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Development and Function of Membrane Systems
in Plant Tissue

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Submitted by

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Abstract

Over the past 11 months we have continued investigation of ion transport mechanisms in corn roots and mitochondria. In mitochondria we find that only citrate and isocitrate are transported by the H^+ /citrate symporter. However, the in vivo function of this carrier remains in doubt because citrate does not appear to be an effective substrate for corn mitochondria. Studies with roots have been directed to why various types of injury or shock all result in temporary blockage of the H^+ -efflux pump in the plasmamembrane. It appears this may be due to an injury-mediated Ca^{2+} influx into the tissue, which by raising free Ca^{2+} in the cytosol activates calmodulin (CaM). In turn, the Ca.CaM complex appears to activate protein kinase, phosphorylating membrane proteins. It is possible that one of these phosphorylated proteins is responsible for inactivation of the H^+ -ATPase.

Future work is planned around the consequences of Ca^{2+} influx into the root cell subsequent to injury, investigating the recovery of the H^+ -ATPase and the initiation of the biosyntheses which lead to augmented ion transport.

Results

1 - Citrate transport in corn mitochondria.

Dr. Paul Birnberg has continued his investigation of citrate transport in corn mitochondria, attempting to determine the biological significance of the H^+ /citrate symport. As discussed last year, corn mitochondria do not require operation of the phosphate and dicarboxylate carriers to transport citrate. Instead, citrate enters with protons in a symport (or cotransport), a mechanism usually associated with sugar or amino acid uptake in plants. However, the physiological significance and importance of this symport are not obvious. As we discuss in a publication on this work (1) it is unlikely that citrate would be a normal substrate for plant mitochondria. Citrate synthase is only found in mitochondria (and the glyoxysomes of fat metabolising seeds), and citrate export to furnish substrate for ATP-citrate lyase is the type of transport expected; citrate import would constitute a futile cycle.

We first checked the specificity of the H^+ /citrate symport mechanisms using the passive swelling assay previously described. A wide variety of organic acids and three amino acids (glycine, proline and glutamic) show some nigerin- and uncoupler-increased swelling, but none except isocitrate was transported as effectively as citrate. By use of labeled compounds in kinetic studies it was determined that isocitrate competes effectively with citrate for the carrier. Malate does not, and the partial inhibition of ^{14}C -citrate uptake due to malate may be due to removal of accumulated citrate by citrate/malate exchange (i.e., tricarboxylate carrier). This exchange is found in corn mitochondria, although it is not very active in influx of citrate (1).

However, it is active in removing accumulated citrate under respiring conditions (Figure 1), supporting our postulate that citrate_{out}/malate_{in}

is the metabolically rational flux.

Thus corn mitochondria have a H^+ /anion symport which is specific for citrate and isocitrate, and which functions optimally at pH 4.5-5.0 with $K_m = 0.1$ mM. At neutral pH (6.8-7.5) uptake rates are slower but could provide for a modest respiration rate with citrate as substrate. We reinvestigated previous work on citrate oxidation (1,2) and confirmed that phosphate or sulfate are required, and that the pH optimum was 6.5-6.8. This seemed curious in view of what we had learned about the properties of the citrate carrier. Further investigation showed that endogenous respiration had the same optimum. A very recent publication by Horn and Mertz (3) reports the same finding; that is, respiration in State 3 or 4 with all substrates is optimal at a slightly acid pH. This makes it unlikely that the optimal pH is set by substrate transport. The requirement for operation of the phosphate transporter [which also handles sulfate (4)] when using slowly transported citrate suggests to us that the common pH optimum for respiration may lie with this transporter. With malate oxidation, Macrae (5) attributed the slightly acid pH optimum to malic enzyme activity, but this would not be expected to apply to citrate oxidation unless the complete Krebs cycle is functioning.

A paper is being written on this second phase of Dr. Birnberg's research.

2 - Regulation of H^+ efflux pumping in corn roots.

We reported last year on the sensitivity of corn roots to injury or shock. Cutting, rubbing, cold shock, osmotic shock, lead within a minute or two to complete cessation of net H^+ efflux pumping (6,7,8). Fusaric acid, which acts to stimulate the H^+ -ATPase of the plasma membrane;

will restore H^+ efflux and partially K^+ influx. Further investigation showed that those ATPase inhibitors known to block the "proton channel" of mitochondrial ATPase (oligomycin, DCCD, ethidium) could also be reversed by fusicoccin. This is equally true for the blockage of net H^+ efflux by protein synthesis inhibitors. Our interpretation of fusicoccin action is that it serves as a bypass or shunt around the point of control on H^+ transfer through the ATPase.

Injuries also result in "leaky" membranes. As observed for many excised (9) or shocked (10) tissues, K^+ initially leaks from segments of corn root, followed in 40-60 minutes by recovery and net gain of K^+ (11,12). Increased membrane permeability is also manifest in loss of adenine nucleotides, followed by net synthesis and recovery in parallel with the recovery of net K^+ transport (12). As reported last year, Dr. Graziano Zocchi found that injury leads to rapid Ca^{2+} influx (13). Thus it is clear that injury is sensed in the cell membrane which suddenly becomes permeable to ions, much like excitable membranes of animals.

We rationalized that the influx of Ca^{2+} might provide the signal for blockage of the H^+ -ATPase. Free Ca^{2+} concentrations in the cytosol of all organisms is very low, on the order of 10^{-7} M (14). If the Ca^{2+} concentration is raised to 1 μ M or higher calmodulin (CaM), known to occur in plants (15), is activated, and in turn activates a number of enzymes, including Ca^{2+} -ATPase and protein kinase. Our principal question became, "Is the influx of Ca^{2+} responsible for the inhibition of the H^+ -ATPase?"

In order to answer this question it was necessary to learn to isolate membrane vesicles active in H^+ transport. As described last year, Dr. Zocchi has succeeded in doing this by pretreating the roots at room temperature for 15 minutes in a grinding medium containing 0.25 M sorbitol,

25 mM Tris-Mes buffer (pH 7.7), 3 mM EGTA, 2.5 mM dithiothreitol, 5 mg/ml BSA and 5 μ M fusicoccin, then grinding the tissue in a mortar at room temperature. Sealed vesicles are isolated by the method of Sze (16). These preparations have ATPase activity which is increased 25-35% by uncoupler, indicating that some fraction of the total activity resides with "sealed" vesicles which are pumping protons. They also possess Ca^{2+} transport activity which is stimulated by calmodulin and fusicoccin, which we attribute to a Ca^{2+} -ATPase and $\text{Ca}^{2+}/\text{H}^{+}$ exchange, respectively (17). The calmodulin-stimulated transport cannot be uncoupled, but is inhibited by chlorpromazine, while the fusicoccin-stimulated transport can be uncoupled indicating that it is driven by the proton motive force.

We have now begun investigation with these vesicles of one possible mechanism by which Ca^{2+} influx might inhibit the H^{+} -ATPase. Dr. A. J. Trewavas (personal communication) told us of his results using $\gamma\text{-}^{32}\text{P}$ -ATP which show that incubation of microsomal vesicles with Ca^{2+} for as little as 60-90 seconds greatly increased phosphorylation of membrane proteins. He also finds evidence indicating the presence of a phosphoprotein phosphatase which acts more slowly to dephosphorylate the proteins. He suggests that protein phosphorylation due to $\text{Ca}\cdot\text{CaM}$ activated protein kinase might be the means whereby increased cytosol Ca^{2+} could inhibit the H^{+} -pumping ATPase. I amplify and illustrate this suggestion by means of a diagram.

Preliminary experiments support this concept (Table I). We find 30 to 45% inhibition of ATPase activity by pretreatment with $\text{Ca}^{2+} + \text{CaM} + \text{ATP}$ compared to omission of ATP. Pretreatment with $\text{Ca}^{2+} + \text{CaM}$ is ineffective. Some fraction of the total ATPase activity is uncoupled by FCCP, demonstrating that it drives a H^+ current. Since this fraction is reduced by pretreatment with ATP, it is possible that the above hypothesis on how injury or shock acts to inhibit H^+ -pumping is sound. Obviously, many more experiments are needed to confirm this;— dependency on Ca^{2+} and calmodulin, demonstration of protein kinase activity, measurements of ΔpH and/or $\Delta\psi$ to support the uncoupling data. But it looks promising.

There is a problem with this hypothesis that cytosol Ca^{2+} is the agent which activates the control responsible for suppressing H^+ -pumping. Calcium has been described as increasing H^+ pumping in coleoptile tissue (18,19). In line with this are reports of Ca^{2+} hyperpolarizing cells (20) and increasing ion transport (21), the so-called "Viets effect" (21). However, as pointed out elsewhere (23), the promotive effect of Ca^{2+} lies at the external surface of the plasma membrane, where it acts to increase hydrophobicity, closing aqueous channels. As a result net H^+ efflux and hyperpolarization could be achieved by simply restricting back flow of H_3O^+ (protons in aqueous media are hydrated).

This explanation needs experimental verification, and Elizabeth Quintero has started investigation by examining the Viets effect in corn roots. Elzam and Hodges (24) reported that the initial effect of adding Ca^{2+} was to inhibit K^+ uptake. After about 15 minutes the inhibition passed into the usually observed promotion. Quintero (25) could verify this with corn root segments washed in H_2O for 3 hours (i.e., recovered from excision injury), although the initial inhibition was smaller than Elzam and Hodges observed (Figure 2). Similarly, addition of 0.1 mM Ca^{2+} caused an initial

inhibition of net H^+ efflux (Figure 3). Preliminary experiments show addition of Ca^{2+} to depolarize the cells by about 30 millivolts, followed by gradual repolarization. Calcium ($^{45}Ca^{2+}$) influx into a non-exchangeable phase was $0.095 \mu \text{ moles/gFW} \cdot 10 \text{ min}$ for 0-10 min after adding Ca^{2+} , and $0.077 \mu \text{ moles/gFW} \cdot 10 \text{ min}$ for the 20-30 min interval. Thus it is likely that initially there may be a flush of extra Ca^{2+} influx into the cytosol which acts to suppress the H^+ -pump, and thereby K^+ influx. However, the cells quickly recover, possibly by Ca^{2+} efflux pumping, and go on to greater K^+ influx. We find a metabolism-dependent efflux of preloaded $^{45}Ca^{2+}$ from the root segments. It is noteworthy in these experiments that the Viets effect is manifested as sustained uptake, compared to the -Ca control. Quintero also finds increased anion uptake in the presence of Ca^{2+} , which may account for the fact that net H^+ efflux is not increased in parallel with K^+ influx (Figures 2 and 3). Fusicochin, which gives an immediate increase in H^+ efflux, prevents the initial depression of K^+ influx due to Ca^{2+} .

Although there is more work to be done here, it is clear that the observations support the view that the reported promotive effects of Ca^{2+} on H^+ efflux and ion transport do not negate our data suggesting that increased concentrations of cytosol Ca^{2+} act in some fashion to give a transitory inhibition of the H^+ -ATPase. In the future we must find means to directly test the hypothesis that the promotive effect of Ca^{2+} lies with binding to the outer surface of the plasma membrane. We believe that only when there is a flush of Ca^{2+} entry into the cytosol is inhibition of H^+ pumping and ion transport to be seen. As Ca^{2+} is pumped out, the promotive effect of external Ca^{2+} becomes apparent.

Future Plans

I have had this contract to investigate the development and function of membrane systems in plant tissue for 23 years, beginning under the Atomic Energy Commission. In recent years the work has expanded to a point where funds were inadequate, and part of it has been supplemented by grants from NSF. However, the contract has been the foundation for all the work since 1960 which includes the training of 30 odd students and postdocs and the publication of about 100 papers.

It was my intention to retire next year (I will be 65 in March), and to terminate the contract when present funds are exhausted. However, I have two students who will not finish until June 1984, and they want to continue working on the project for their theses. In addition, there are a couple of important questions I would like to resolve before retiring. For these reasons a 17 month terminal contract is requested.

I do not plan further work with mitochondria. There are still interesting aspects of transport in plant mitochondria to be investigated, but I want to devote the time remaining to the more practical and interesting questions emerging in root transport. As a final contribution to the mitochondrial work, I have agreed to write a review chapter on transport in plant mitochondria for a volume in the Encyclopedia of Plant Physiology.

There are two primary questions with root transport. First, how does injury (wounding, shock, stress) act to inhibit the H^+ pump of corn roots? Second, what is the mechanism of spontaneous recovery with washing?

We will continue to investigate the possibility that the influx of Ca^{2+} attending injury provides the agent for inhibiting the H^+ -ATPase of the plasma membrane, following up the indication that Ca-CaM activation of protein kinase is responsible (see Table I and the diagram above).

Although uncoupling of ATPase activity with the protonophore FCCP is excellent evidence that some portion of the sealed vesicles have an H^+ -pumping

ATPase, there is a high ATPase background. We will supplement here with measurements of $\Delta\psi$ using $^{35}\text{SCN}^-$ (26) and ΔpH using acridine orange (27), determining the response to preincubation with ATP and/or Ca^{2+} . We must also establish with $\gamma\text{-}^{32}\text{P-ATP}$ that there is a Ca^{2+} -dependent phosphorylation of membrane proteins, Ca^{2+} and calmodulin will be studied as variables and optimal pH determined. Phosphorylated proteins will be separated in SDS polyacrylimide gels and attempts made to determine which possess ATPase activity.

Assuming a correlation between phosphorylation of membrane proteins and H^+ -ATPase inhibition, we will see if dephosphorylation of these proteins leads to recovery. Dr. Trewavas (personal communication) finds protein phosphatase activity associated with microsomal vesicles. We wonder if the alkaline phosphatase described for Ca^{2+} deficient cucumber roots (28,29) might not be involved.

At present we must deal with vesicles which are derived from several cytological sources. It would help to be able to separate out the vesicles with an active H^+ -pumping ATPase. Our present attempts to do this have not been rewarding, but we will keep trying.

We will continue in our efforts to clarify the external and internal roles of Ca^{2+} in regulating the H^+ pump. We find that the cell wall, with its high concentration of ^{2+}Ca , makes quantitative work very difficult, and we plan studies with isolated root protoplasts. Mrs. Quintero has begun work here following the procedures of Lin (30). We hope to be able to measure Ca^{2+} bound the external surface of the plasma membrane as a fraction readily removed by EGTA washing, and thus follow indirectly its penetration into the protoplast upon experimental treatment (and also any loss to the medium). In this way we may be able to correlate quantitatively Ca^{2+} movements and H^+ pumping as well as quantify the effects of superficial

Ca^{2+} on ion uptake.

Protoplasts may help with the problem of why fusicoccin strongly retards Ca^{2+} influx. This has an important bearing on how injury effects the permeability change that permits Ca^{2+} influx. If injury opens aqueous channels for Ca^{2+} influx, it might be argued that fusicoccin acts by "plugging" them. The same argument might be applied to the restoration of net H^+ efflux in injured tissue (i.e.,: injury opens a hole for H_3O^+ backflow, and fusicoccin plugs it). However, this trivial explanation will not account for the specific reversal by fusicoccin of the inhibition produced by H^+ -channel inhibitors (DCCD, oligomycin, ethidium), protein synthesis inhibitors, or mersalyl (7,8). Also, fusicoccin causes a small decline in ATP content of root tissue (31), a result more in line with increased H^+ -ATPase activity.

The alternative is that the increased H^+ -pumping and cell polarization produced by fusicoccin acts in some fashion to preserve semipermeability, reducing Ca^{2+} influx. That is, a membrane with a high electrical potential across it is a membrane resistant to passive ion fluxes, including Ca^{2+} . We suspect this is the case, and that the role of external Ca^{2+} may be stabilize the membrane so that it can maintain a high $\Delta\psi$. There may be a poise between the binding energy of the Ca^{2+} complex and the very high electrochemical potential for influx which is disturbed by the excitation stimulus of injury or shock, releasing Ca^{2+} for influx, depolarizing the membrane, and opening aqueous channels. If we can effectively use protoplasts ways for examining this postulate may appear. Experimentation along these lines appears to be fundamental to understanding how injury or shock is translated into the observed reactions of the cell.

There is an aspect of the recovery from injury to corn roots that we have not yet investigated to any extent, but which appears to be of great importance. The spontaneous recovery with washing can be divided into two intergrading phases, which we have termed "inductive" and "developmental" (11,12). During the inductive phase, which lasts 40-60 minutes after excision of a root segment, there is rapid (no lag) recovery of electrogenic H^+ efflux, K^+ influx and cell potential. At the same time the initial rapid net loss of K^+ and adenine nucleotides gradually slows down and ceases (12). In the developmental phase, which occupies the next 2 to 3 hours, there is net K^+ uptake, biosynthesis of adenine nucleotides, and development of enhanced solute absorption rates, typified by phosphate uptake (11,12). Inhibitors of RNA and protein synthesis block these developments (11) and the developmental phase is obviously a phase of biosynthesis initiated by wounding injury.

Davis and Schuster (32) report that wounding vegetative plant tissues, including corn roots, initiates polysome formation in as little as 15 minutes. The rapidly transmitted wound signal is believed to be based in changes in membrane potential and ion fluxes. These polysomes represent protein synthesis initiated at the expense of existing ribosomes and m-RNA. By one hour after wounding net synthesis of RNA and protein can be detected. Hence, the developmental phase starts very early, and the recovery from injury clearly involves more than simple reversal of the block in the plasma membrane H^+ -ATPase; translation followed by transcription is induced.

There is other evidence that the developmental phase starts early. The net loss of adenine nucleotides (AdN) slows down as early as 30 minutes after excision of root tissue, and AdN recovery is accelerated in rate and

extent by auxin (12). Also, injury to intact roots, where supplies of metabolites and hormones are maintained, gives less decline in AdN and more rapid and extensive recovery (12).

It is possible that the initial formation of polysomes (translation at the expense of preexisting ribosomes and n-RNA) has some rôle in the sustained recovery of H^+ -pumping during washing. Protein synthesis inhibitors which block chain initiation will also block H^+ efflux after periods of 8 to 25 minutes, depending on the inhibitors used (7). K^+ influx is also blocked. Growing segments of oat coleoptile show a similar phenomenon (33). Fusicoccin will largely restore H^+ -pumping in both tissues. We have attributed the effect of protein synthesis inhibitors to an "internal injury" because of the parallelism with responses to external injury, but this analogy wants clarification of what constitutes an internal injury.

The question we have here is two-fold: are some of the induced biosyntheses of the developmental phase essential to sustained recovery of membrane transport (eg. higher levels of AdN), and is the influx of Ca^{2+} responsible for the induction?

It is known from the extensive investigations of protein synthesis initiation in reticulocytes that c-AMP-independent protein kinases have a regulatory role (see reference #34 for recent review articles). There is some disagreement as to how phosphorylation of the eucaryotic initiation factor (eIF-2) affects initiation, but one persuasive view is schematically presented as follows (35):

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Figure 13
eIF-2-Mediated translational regulation in rabbit reticulocyte lysate.

In this terminal work I cannot hope to resolve the above questions satisfactorily, but an important initial step can be made by determining if there are Ca^{2+} -activated protein kinases associated with the rapid polysome formation observed by Davies and Schuster (32). For this purpose I have the assistance of a close colleague, Dr. T.-H. David Ho, whose main interests are in the metabolic biology of hormonally regulated plant development. He is as interested as I am in the potential of cytosol Ca^{2+} serving as a second messenger, and has the equipment and experience needed for work on protein synthesis initiation.

We have not formulated detailed plans, but in general we will begin by examining normal and injured (e.g., cold shocked or excised) roots for polysome levels and AdN content. Assuming that we can confirm Davies and Schuster (32), we will trace the relative development of polysomes and AdN synthesis during the recovery from injury, using auxin to accelerate AdN synthesis (12). With this data on hand, we will attempt to produce

cell extracts comparable to the reticulocyte lysate and use comparable techniques to measure the formation of initiation complex (34). Presumably eIF (initiation factor) will be present, and we should be able to determine by ^{32}P labeling if it is phosphorylated. All of this will be tricky, and we will have to learn as we go, but we will.

The all-over objective becomes one of tracing the induction of increased AdN biosynthesis to see if it fits this pattern

The role of auxin is obscure, but in addition to its reactivation of growth in excised (injured) tissue (36), it has long been known to initiate RNA and protein synthesis (37), and it promotes ATP synthesis (12,38,39). It has been very difficult to establish that protein synthesis is required for the relatively rapid growth response to auxin (40), but the possibility remains that auxin might activate enzymes required to establish growth. No such enzyme has ever been found, but the possibility that auxin is a cofactor in Ca·CaM activation has never been directly investigated. Indirectly, we have found that auxin treatment of the excised growing region of soybean hypocotyl can double the activity of the Ca^{2+} transporting ATPase of a plasmalemma preparation in 1-2 hours (41).

Auxin is known to increase Ca^{2+} efflux from plant tissue (42). Ca^{2+} -ATPase is dependent upon calmodulin (43). Auxin added directly with the assay has no effect. Possibly auxin has some directive influence on the enzymes synthesized, in this case Ca^{2+} -ATPase. Enhanced efflux pumping of Ca^{2+} from the cytosol of excised tissue may be a requisite for recovery of growth with auxin.

Depending on the interests of the postdoc on the project, we may initially approach the problem through auxin/ Ca^{2+} interaction in growth, rather than on ATP synthesis. For this purpose we would probably shift to using growing corn coleoptiles or mesocotyl segments, making initial physiological observations on Ca^{2+} influx and efflux in control and auxin-treated tissue, and focusing on the properties of membrane vesicles in Ca^{2+} transport.

Personnel

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Table I

Reduction in uncoupler-sensitive microsomal ATPase by treatment conducive to protein kinase activity.

Vesicles were isolated as described in the text, and incubated for 5 minutes in 0.25 M sorbitol, 2.5 mM Tris-Mes (pH 7.4) and 1 mM $MgCl_2$ plus 10 μM $CaCl_2$; 10 $\mu g/ml$ bovine brain calmodulin and 1 mM ATP as indicated. The vesicles were recovered by centrifugation and assayed for ATPase activity in 30 mM Tris-Mes (pH 6.5), 3 mM $MgSO_4$, and 3 mM ATP plus 50 mM KCl and 10 μM FCCP as indicated.

<u>Preincubation</u>	<u>ATPase (μ moles Pi/mg protein $\cdot$ hr)</u>				
	<u>-KCl</u>			<u>+KCl</u>	
Control		<u>%ΔFCCP</u>	<u>%ΔATP</u>	<u>%ΔFCCP</u>	<u>Δ%ATP</u>
-FCCP	22.1			26.7	
+FCCP	28.7	(+30)		31.0	(+16)
Ca^{2+} + CaM					
-FCCP	23.0			25.5	
+FCCP	30.1	(+31)		31.7	(+24)
Ca^{2+} + CaM + ATP					
-FCCP	14.0		(-39)	15.6	(-39)
+FCCP	16.4	(+17)	(-46)	17.5	(+12) (-45)

Figure 1

Figure 1. Citrate accumulation from 0.63 mM ^{14}C -citrate by corn mitochondria oxidizing NADH . At 3 minutes Antimycin A and/or malate was added. Blocking respiration with Antimycin A shows the rapid back leakage of citrate in the absence of $\Delta\bar{\mu}_{\text{H}^+}$ (concentrations in the matrix are about 15-fold greater than those in the H^+ medium). Addition of malate shows the removal of citrate by malate/citrate exchange.

Figure 2

Absorption of K^+ ($^{86}\text{Rb}^+$) from solution by corn root segments washed in aerated distilled water for 4 hours at 30°C . Forty 2-cm segments were placed in 20 ml of aerated 0.2 mM K-phosphate, (pH 6.0) labeled with $^{86}\text{Rb}^+$ (10,000 cpm/ml) at room temperature. CaSO_4 was added to give a final concentration of 0.1 mM as indicated, and 0.1 ml aliquots removed for determining radioactivity at the indicated intervals. An equivalent volume of distilled water was added to the control. $\mu\text{-moles } K^+ (^{86}\text{Rb}^+)/\text{g FW}$.

Figure 3

Net H^+ efflux from root segments washed in aerated distilled water for 3 hours at 30°C . Fifty 2-cm segments were placed in 50 ml of aerated 0.1 mM K_2SO_4 , 0.1 mM MES, 0.1 mM TRIS (pH 6.0). $CaSO_4$ was added to give 0.1 mM at 5 minutes. An equivalent volume of distilled water was added to the control. Net H^+ efflux was determined from pH recordings and the acid titer in the presence of the tissue. $\mu\text{moles } H^+/g \text{ FW}$ (Means $\pm$ SD). The standard deviation bars are not shown when they are smaller than the symbols.