DOE/EV/00790-1

Development and Function of Membrane Systems in Plant Tissue

Annual Technical Progress Report

15 July 1979 - 15 June 1980

Submitted by

J. B. Hanson
Botany Department
University of Illinois
Urbana, IL 61801

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DISCLAIMER

Abstract

Over the past 11 months we have continued to investigate ion transport mechanisms in corn roots and mitochondria. With mitochondria we find the H^+/K^+ antiporter is active in passive influx of KCl, and that the Cl^- gradient drives the swelling. Citrate uptake by corn mitochondria requires phosphate or sulfate as an activating cofactor, but malate does not appear to be required. In the root investigations, we find fusicoccin to relieve the block in H^+ efflux pumping caused by protein synthesis inhibitors. Fusicoccin also repolarizes the cells. Inhibition of H^+ efflux by the H^+ channel inhibitors, oligomycin and DCCD, can also be relieved or bypassed by fusicoccin. Nigericin, a H^+/K^+ exchanging antibiotic will give partial release of the H^+ efflux block caused by protein synthesis inhibitors, repolarizing the cells, but in no other respect does it resemble fusicoccin. It will not restore the K^+ influx or growth inhibitions resulting from treatment with protein synthesis inhibitors.

RESULTS

Due to the late refunding of this project we were delayed 5 months in some of the work, especially with mitochondrial transport. However, with student help some progress was made, selecting some of the simpler problems. None of these investigations has been completed yet, but should be by September or October.

1. Citrate transport in corn mitochondria.

We confirmed our previous finding that citrate uptake is much greater at pH 6.0 than at pH 7.5, and that phosphate or sulfate are needed to activate extensive uptake (1). Phosphate or sulfate do promote citrate uptake at pH 7.5, a point that had been in question. Addition of malate at either pH slightly stimulated citrate swelling, provided phosphate or sulfate were present, having greater effect at pH 7.5. However, the slight stimulation of uptake might be attributed to the 50% increase in respiration rate upon addition of malate. Present evidence is inadequate to demonstrate that malate/citrate exchange is a factor of consequence in citrate uptake, and especially so at low pH.

Mg^{++} promotes the phosphate or sulfate activated citrate uptake, especially at low pH, but without significant change in respiration. Subsequent addition of malate increases the respiration more if Mg^{++} is present. Since Mg^{++} promotes phosphate and sulfate uptake (2) the promotive action on citrate uptake might be indirect. This proves to be true, for Mg^{+} does not promote citrate uptake in the absence of phosphate or sulfate.

Our present work, then, indicates that citrate uptake is critically dependent on phosphate or sulfate, is promoted by Mg^{++} if the anions are present, and is probably proton-compensated (hence, promoted by low pH).

In all respects except the proton-compensation the net uptake of citrate resemble the net uptake of ADP, and we have already reported that citrate competitively inhibits ADP uptake (3). To finish the work off we must determine the role of phosphate or sulfate in activating citrate uptake. As described in last year's comprehensive report, we know that the requirement is for exogenous phosphate or sulfate.

2. H^+/K^+ exchange transport in corn mitochondria.

It is now widely accepted that plant mitochondria have a H^+/K^+ exchanging mechanism or antiport in their inner membrane (4,5,6,7,8) presumably a transport enzyme although firm evidence on this point is lacking. We have proposed that the active shrinkage of corn mitochondria when the P_i transporter is blocked with mersalyl is due to activation of the H^+/K^+ antiporter (4), particularly when valinomycin is added to lower the resistance of K^+ efflux on the enzyme. This concept of a high degree of resistance in K^+ penetration to the enzyme was also called upon to explain the active shrinkage upon addition of valinomycin to corn mitochondria which had accumulated K_2SO_4 (9). Likewise, the active contraction or shrinkage of mitochondria which have passively absorbed KCl is attributed to efflux pumpong of salt in which the H^+/K^+ antiporter participates (7). The antiporter might best be described as a $H^+/cation^+$ exchange transporter which in plant mitochondria operates more effectively with K^+ than Na^+. The opposite is true for animal mitochondria (10).

There is some evidence that the H^+/K^+ exchange is subject to control. Pomeroy (11) found that mitochondria from cold-hardened wheat lose the ability to drive KCl efflux, although they would still swell in KCl. This signifies loss of H^+/K^+ antiporter activity. Huber and Moreland (6) attributed

the rapid KCl swelling of mung bean mitochondria treated with tripropyl tin, which carries out Cl^-/OH^- exchange, to participation of the H^+/K^+ antiporter. They also suggest that the antiporter can be activated by respiration. Jung and Brierley (8) report, however, that the H^+/K^+ antiporter of potato mitochondria only participates in salt efflux (ie: H^+ in, K^+ out).

This last report troubles us for we reported some years ago that uncouplers would accelerate passive swelling of corn mitochondria suspended in 100 mM KCl (12). Reinvestigation shows the results given in Figure 1. Analysis shows these corn mitochondria to contain about 140 mM K^+ and 13 mM Cl^-, which means that when they are suspended in 100 mM KCl there is little or no gradient in K^+ activity, but a large gradient in Cl^- activity. Addition of valinomycin (Val) produces very rapid KCl penetration, demonstrating that Cl^- permeability is high, producing a negative membrane potential down which K^+ fluxes when resistance is lowered by valinomycin. However, addition of the uncoupler FCCP, which effectively lowers resistance to H^+ penetration, also produces additional rapid swelling. In simple chemiosmotic terms this can only be explained by the operation of an H^+/K^+ antiport, which in this case is effective in driving K^+ influx (see diagram, Fig. 1). A check on the relative efficiency of the native antiporter can be made by adding nigericin, which is an H^+/K^+ exchanging ionophore. Saturating the H^+/K^+ exchange capacity of the membrane increases the rate of response to FCCP, but the data show that the endogenous antiporter can operate at about half the maximum rate. Thus we have shown that the H^+/K^+ antiporter of corn mitochondria can function in influx (Fig. 1) or efflux (4).

It has frequently been shown that an added impermeable solute such as sucrose or mannitol will decrease the rate of passive salt influx (Fig. 1).

Our explanation of this is that osmotic shrinkage of the matrix lowers the Cl^- gradient, and thus the driving force for swelling.

We have also studied the effect of pH on swelling. In agreement with earlier work (13), swelling rates are minimal at about pH 6.5 and rise sharply above pH 7.5. Azzi and Azzone (14) attribute this to increasing Cl^- permeability with increasing pH in liver mitochondria; they found Cl^- exchange to increase with pH but not K^+ exchange. Very likely anion permeability in general increases with pH.

It is possible that the high State 4 respiration rates typical of plant mitochondria (compared to animal mitochondria) might arise from the salt permeability of the membrane. That is, recycling of K^+ and/or P_i consumes the proton gradient, allowing for a high respiration rate. As a check on this we studied State 4 respiration rates as a function of pH, using NADH as substrate to avoid the problem of organic acid transport, which can become limiting to respiration (15). There is a sharp increase in State 3 and State 4 respiration between pH 6 and pH 8. (State 3 from 165 to 275 μmoles O_2/hr/mg protein, and State 4 from 70 to 148 μmoles O_2/hr/mg protein).

Summarizing, there is a very active H^+/K^+ antiport in corn mitochondria which manifests itself in several ways, including both influx and efflux pumping of salt, and in the high acceptorless and State 4 respiration. We believe this antiport also participates in transcellular transport to the root xylem (16), and it appears that as an H^+/Na^+ exchanging enzyme it may act in excluding Na^+ from plant cells (17,18).

We have some preliminary evidence which suggests that divalent cations, particularly Ca^{++}, may serve to activate the H^+/K^+ antiport during active salt efflux. We plan to investigate this possibility. We suspect the

activation of H^+/K^+ exchange due to respiration (6) may be a consequence of increasing Δp, and thus the electrochemical gradient driving H^+ influx.

3. Regulation of H^+ efflux pumping in corn roots.

In last year's comprehensive report we described the effects of various types of injury on the H^+ efflux, K^+ influx and cell potential of corn roots. In less than one minute there is a rapid collapse of up to 50% in these processes, followed by a slower recovery (1-2 hours) during washing. H^+ efflux recovers very rapidly compared to K^+ influx. We believe that injury signals a partial collapse of the H^+/K^+ exchanging ATPase, and washing restores full activity in a process which resembles that induced by fusicoccin. These results are now published (19).

More recent work has been to investigate in another fashion the energy-linked H^+ efflux from washed corn root tissue. It has been repeatedly demonstrated in a wide variety of plant tissues that inhibitors of protein synthesis, such as cycloheximide, will also inhibit energy-linked ion absorption, although no specific protein(s) has been identified as responsible. A related phenomenon lies with the inhibition of auxin-induced growth by protein synthesis inhibitors, and it has recently been shown that this inhibition is accompanied by inhibition of H^+ efflux (20,21). Fusicoccin-induced growth shows much less inhibition of H^+ efflux. We thought it worthwhile to investigate the situation with corn roots, for it would be useful to know if the plasmalemma ATPase were inhibited by protein synthesis inhibitors.

Our results show that four different protein synthesis inhibitors will completely block H^+ efflux from washed corn root tissue within 8 to 20 minutes (Fig. 2). However, addition of *10* µM FC will give 80-90% release of the

inhibition. Several different ionophores and uncouplers ("protonophores") were also tested, and one of these, nigericin, also gave partial release of the block in H^+ efflux (Fig. 2). Nigericin, which will carry out H^+/K^+ exchange across membranes, was less effective than FC, restoring about 50% of the original H^+ efflux.

We checked the effect of protein synthesis inhibition on cell potentials. In parallel with the rapid decline in H^+ efflux there is a decline in ψ_p of about 15 mvolts, and addition of FC or nigericin restored the potential.

As expected, protein synthesis inhibition also blocked K^+ influx (from 4.77 µmoles K^+/g fr wt·hr to 0.91 with MDMP inhibition). Fusicoccin was not so effective in restoring K^+ influx as it was with H^+ efflux (from 0.91 to 2.02 µmoles K^+/g fr wt·hr with 10 µM FC). However, nigericin did <u>not</u> restore K^+ influx, and was inhibitory to K^+ influx in control tissue. Similarly, growth of corn root tips was strongly inhibited by MDMP (from 2.0 to 0.2 mm/3hr) and FC gave partial restoration (from 0.2 to 1.1 mm/3 hr), but nigericin had an inhibitory effect. Thus nigericin resembles FC in only one respect; it will partially restore H^+ efflux and cell potential.

We are continuing work on the mechanism of action of nigericin but have at present little to report. The restoration of H^+ efflux does not seem to depend on the concentration of K^+ in the medium, but appears to be sensitive to H^+ concentration, working better at pH 6.0 than 5.5. We are now starting work with isolated plasmalemma membrane ATPase to determine if preincubation of the roots in protein synthesis inhibitors will lower K^+-stimulated ATPase activity.

PLANNED RESEARCH

We wish to finish up the work on citrate transport in corn mitochondria, with particular attention as to why phosphate or sulfate are required. As mentioned in last year's comprehensive report, there is evidence that the phosphate transporter becomes a dicarboxylate carrier after it has bound P_i or octylphosphate (22), and we wonder if it might also serve to transport citrate or ADP.

We want to complete the investigation of Cl^- permeability of plant mitochondrial membranes as responsible for passive swelling in KCl. For this purpose we will use inhibitors of Cl^- transport in red blood cell membranes —— DIDS, SITS & phloretin (23).

The action of Mg^{++} in promoting active phosphate and sulfate transport also needs to be cleared up. One ancillary problem here is that very little is known about conditions under which Mg^{++} is actively transported in any type of mitochondria. Is it the uptake of Mg^{++} which promotes phosphate and sulfate uptake? We have some preliminary data on Mg acetate uptake which suggests this salt may be more useful in determining parameters of Mg^{++} transport.

As mentioned above, we also have preliminary data which suggests that Ca^{++} may activate the H^+/K^+ antiporter, and if time permits we want to investigate this. In this case we wonder of Ca^{++} may act to increase Δp by "tightening" the membranes. Techniques for determining Δp have been applied to plant mitochondrial (24).

Our studies of the root H^+/K^+ ATPase will continue. We have started a program of attempting to get plasmalemma preparations which will respond to uncouplers and ionophores on the basis that if the membranes are "tight",

the rate of ATP hydrolysis will be controlled. Sze and Fox (25) have reported such preparations from tobacco callus, and Lin (private communication) from corn root protoplasts. We have had some success with corn root preparations stimulated by nigericin, and are encouraged to continue. We will also look to the supernatant for a regulatory protein which we speculate may be the causal factor in the blockage of H^+ efflux caused by protein synthesis inhibitors. A new postdoctoral associate, Dr. Graziano Zocchi from the Milan group will join us for this work.

Personnel

J. B. Hanson, Professor of Plant Physiology

John M. Cheeseman, Postdoctoral Associate (to Sept 1979)

Chris Chastain, Graduate Assistant

M. Jane Fluegel, Graduate Assistant

Peter R. LaFayette, Graduate Assistant

Gail Johnson, Undergraduate Assistant

PUBLICATIONS

15 July 1979 - 15 June 1980

Abou-Khalil, S. and J. B. Hanson (1979) Energy-linked adenosine diphosphate accumulation by corn mitochondria. I. General characteristics and effect of inhibitors. Plant Physiol. 64: 276-280.

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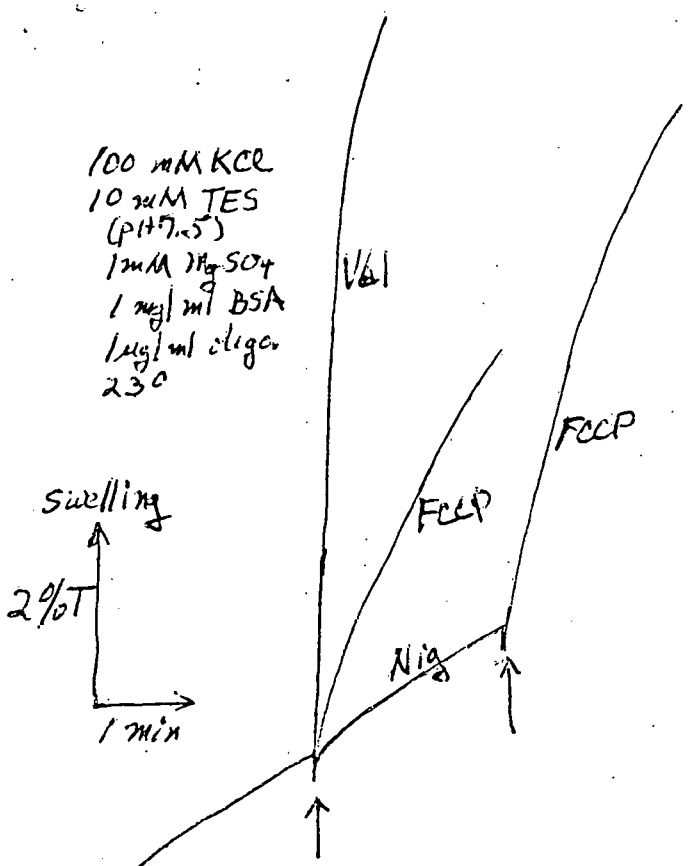
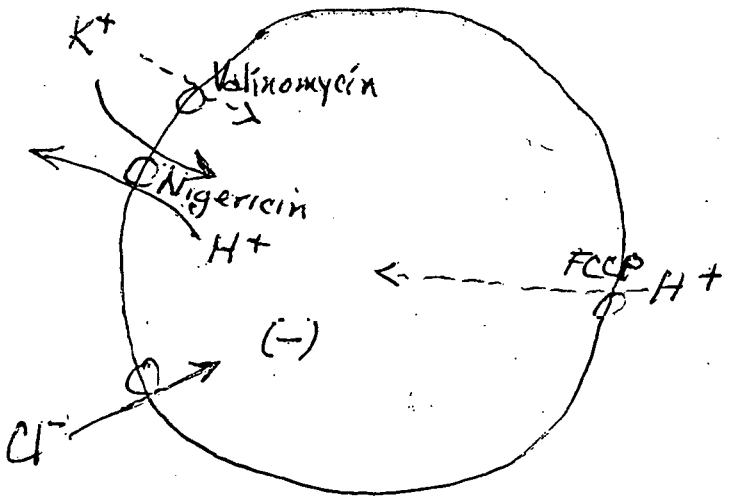
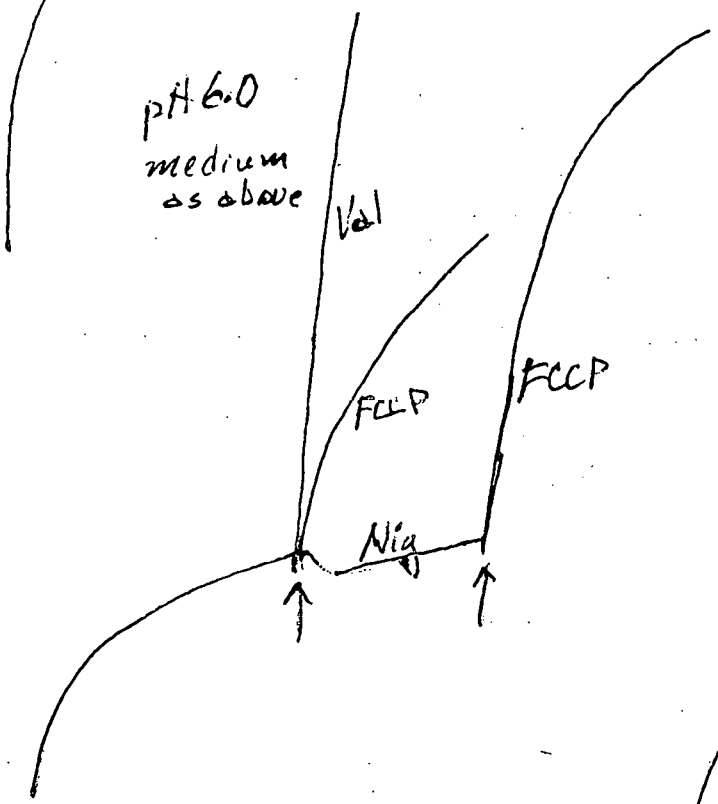
Fig. 1


100 mM KCl
10 mM TES
(pH 7.5)
1 mM $MgSO_4$
1 mg/ml BSA
1 μg/ml oligo.
23°
Val
FCCP
FCCP
Nig
Swelling
2% T
1 min


K^+
Valinomycin
Nigericin
H^+
FCCP
H^+
(−)
Cl^-


pH 6.0
medium
as above
Val
FCCP
FCCP
Nig

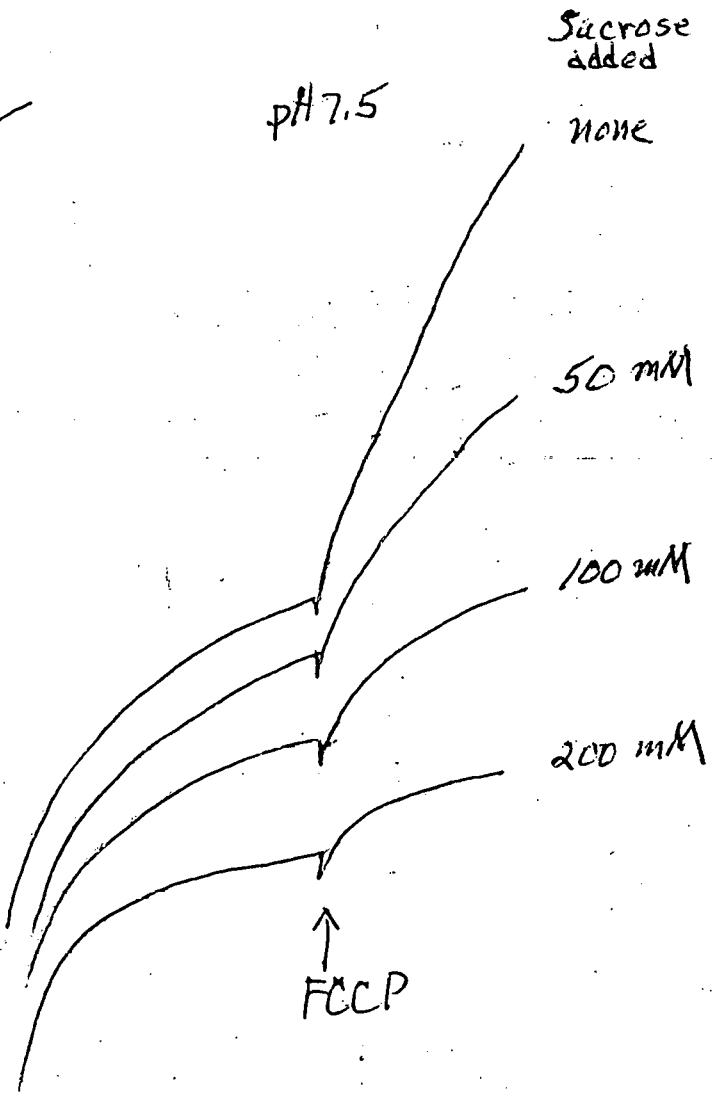
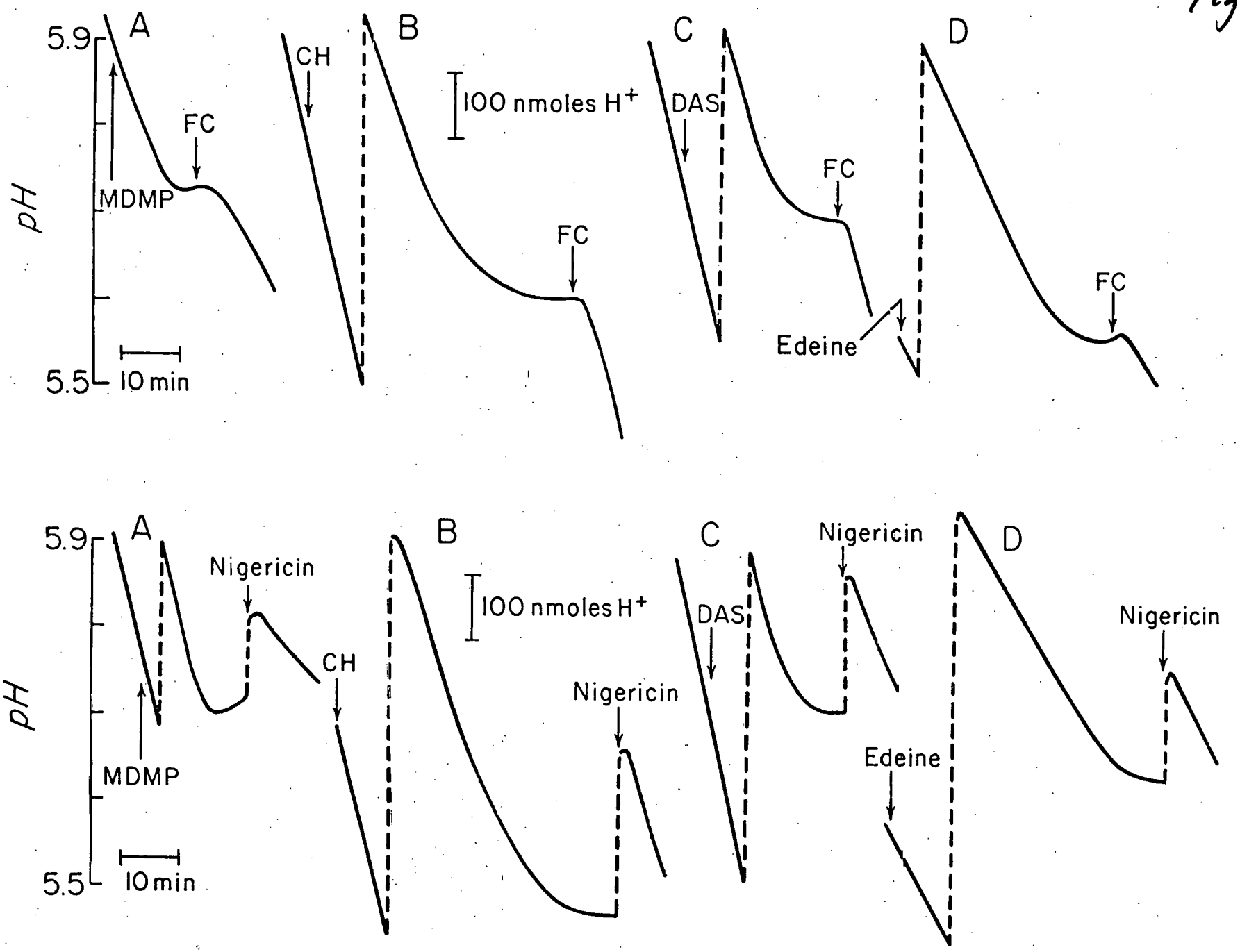

pH 7.5
Sucrose added
none
50 mM
100 mM
200 mM
FCCP

Fig 2


5.9
5.5
pH
A
MDMP
FC
10 min
CH
B
100 nmoles H+
FC
C
DAS
FC
Edeine
D
FC
Nigericin
CH
Nigericin
Nigericin
Edeine
Nigericin

Request for Continued Support

Contract DE-AC02-76EV00790.A005

U. S. Department of Energy

from

Department of Botany

University of Illinois at Urbana-Champaign

Urbana, Illinois 61801

Technical Scope

Technical Program

Proposed Budget

Financial Statement

Statement of Other Support
and Applications

Title: Development and function of membrane systems in plant tissue

Principal Investigator: J. B. Hanson, Professor of Plant Physiology

Proposed Technical Scope

Investigation of ion transport mechanisms in plant membranes, with special attention to mitochondrial ion transport and the development of membrane transport mechanisms in corn roots.

Proposed Technical Program

The details of the program are given in the Annual Technical Report. There is no change in the basic program. We will be investigating citrate transport in corn mitochondria, with special attention to the requirement for phosphate or sulfate. The promotive action of Mg^{++} will be studied, including investigation of parameters governing Mg^{++} transport. In corn roots we will study the mechanism which drives active H^{+} efflux and k^{+} influx; this will require techniques for isolating plasmalemma vesicles competent in transporting H^{+} and K^{+}.

Proposed Budget

DOE Contract DE-AC02-76EV00790.A005

1 November 1980 - 31 October 1981

A - II(a) Reimbursable or Cost Sharing Portion

1.	Salaries and Wages		
	Scientific Discipline Personnel		
	Professor J. B. Hanson, Principal Investigator 50%, 2 months summer salary		$4,780
	Postdoctoral Research Associate 100%, 12 months		14,000
	Support Personnel		
	Hourly student labor		2,500
		Sub Total	$21,280
2.	Indirect Costs		
	Approved rate (DHEW, 20 June 1979) 68% of Salaries and Wages		$14,470
3.	Supplies and Materials		4,000
4.	Equipment		none
5.	Publications		500
6.	Travel		
	Am. Soc. Plant Physiologists meeting		500
7.	Other		
	Fringe Benefits		1,270
		Total	$42,020

It is requested that the Department of Energy provide 100% of the A-II(a) portion of $42,020.

A - II(b) Items Excluded from Cost-Sharing to be Furnished by the University

none

A - II(c) University Contribution of Principal Investigator J. B. Hanson, 10% of academic year (9 months)

Financial Statement

DOE Contract DE-AC02-76EV00790.A005

1 November 1979 - 31 October 1980

1.	Estimated total project costs for current period	$ 34,779
2.	Total amount chargeable to DOE (100% of #1)	34,779
3.	Cumulative Support Costs (10/31/78)	323,131
4.	Estimated Total Cumulative Support Costs	357,910
5.	Cumulative Support Ceiling	357,910
6.	Anticipated difference between the Estimated Total Cumulative Support Costs and the Cumulative Support Ceiling	none

Statement of other Government agency support of a similar or identical undertaking.

NSF PCM-79-13406 for $47,941, 15 January 1980 to 31 July 1981, "Increased ion absorption rates induced by washing of root tissue".

This project complements that supported by DOE on Contract DE-AC02-76EV00790.A005, but does not support the same undertaking.

Statement of whether a similar or an identical proposal has been submitted to another Government agency for evaluation for award.

This proposal is not being submitted to any other agency.

APPROVALS

J. B. Hanson — 20 June 1980
J. B. Hanson
Principal Investigator
Professor of Plant Physiology
Department of Botany

Paul F. Mortensen — date
Assistant Director
Business Affairs
School of Life Sciences

J. J. Kamerer — date
Assistant Director
Business Affairs
University of Illinois

Linda S. Wilson — date
Secretary
Campus Research Board
University of Illinois