COO-790-65

TECHNICAL PROGRESS REPORT

ERDA Project 790

Annual Progress Report

15 July 1974 - 14 July 1975

Title: Development and Function of Membrane Systems in Plant Tissue

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15 July 1975

DISCLAIMER

Annual Progress Report 15 July 1974 - 15 July 1975

I. Research accomplishments

Research on mitochondrial ion transport has been stopped pending the return of Professor Hanson. Research on the development of enhanced ion transport in corn root tissue due to "washing" has continued.

A. Mr. Willy Lin has continued the study of enhanced cell potentials developed with washing by investigating whether there are corresponding changes in cell resistance. (The terms "cell potential" and "cell resistance" are used since there parameters are measured between the vacuole and the external medium, and thus refer to measurements across both the vacuolar and cell membranes.) Washing proves to be without effect on resistance, which falls in the range of 11-13 megaohms in both fresh and 4 hour washed root tissue. There is thus no evidence to support the hypothesis that the increase in electrical potential is due to increased permeability (P_K). Rather, the increased PD probably arises from energy-linked electrogenic transport, as previously deduced from the collapse of the increased PD with the uncoupler, FCCP (Plant Physiol. 54: 799, 1974).

If enhanced electrogenic transport is responsible for the increase in PD with washing, the next question becomes, "what ion is being transported?". Mitchell's chemiosmotic hypothesis proposes that proton efflux pumping is responsible for salt transport; that is, an electrochemical gradient of protons established by respiratory "loops" or ATP hydrolysis provides the potential for ion transport. Energy-linked proton efflux could then account for the electrogenic transport component of PD.

However, we observed that net proton efflux was high in fresh tissue (about 2 μmoles/g/hr) and essentially nil in washed tissue (Plant Physiol. 54: 799, 1974). This is the opposite of what would be expected. In probing this perplexing matter we learned that sulfhyryl binding reagents such as mersalyl would increase net proton efflux, and conversely that sulfhydryl protecting agents would institute rapid net proton influx. Mr. Lin has spent most of his time exploring the action of these agents in causing proton influx with the following results.

1. Dithioerythritol (DTE) and several other SH-protecting reagents are equally effective in causing rapid proton influx. Maximum effectiveness of DTE is at 0.5 mM.

2. The net proton efflux is highly pH dependent, and so is the effect of DTE in causing net proton influx.

net proton fluxes in fresh tissue
μmoles H^+/g/hr

pH	4.0	5.0	6.0	6.5	7.0
Control	-0.86	-1.42	-2.11	-3.11	-4.55
DTE	0.90	-0.76	-0.31	-1.36	-1.75
ΔH^+	0.04	0.66	1.80	2.30	2.80

A negative sign indicates efflux

It appears that the respiring tissue might lose more protons to an alkaline medium simply because of a reduced gradient of chemical potential. Work by Poole (Can. J. Botany 52: 1023, 1974) with washed beet root slices shows that increasing H^+ efflux with increasing pH is paralleled by increasing K^+ influx and increasing PD. Our confirmation here is not yet complete but it does appear that the amount of H^+ extruded is related to the amount of K^+ taken up, and thus we are dealing with an energy-linked H^+/K^+ exchange mechanism which somehow enhances PD. The action of DTE is in opposition to this system, but it cannot be simply a permeability change because at low pH (high in/out H^+ gradient) it has little effect.

3. DTE increases the net uptake of anions (P_i^-, Cl^-, $SO_4^=$) and decreases the net uptake of K^+ and Na^+. The changes are small (20-40%). In these experiments, as in all others not otherwise specified, the pH was 6.0.

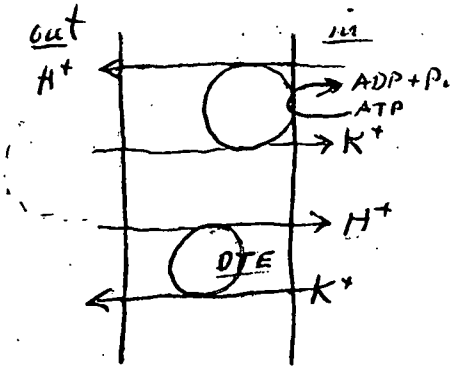
4. By labeling the cellular K^+ with $^{86}Rb^+$ during a pretreatment period, and then following the efflux of label with and without DTE, it was learned that the extra H^+ influx is almost balanced by K^+ efflux.

Ion fluxes in washed tissue
μmoles H^+/g/hr

pH	4.0	5.0	6.0
control	0.40	0.25	0.18
+DTE	0.45	1.17	1.59
ΔH^+	0.05	0.92	1.41
	μmoles K^+/g/hr		
control	-5.20	-4.35	-2.71
DTE	-5.26	-5.48	-4.11
ΔK^+	-0.06	-1.13	-1.40

A negative sign indicates efflux; positive, influx

These experiments show K^+ efflux to decrease with decreasing H^+ concentration externally, suggesting a H^+/K^+ exchange dependent on H^+ gradient and running counter to the energy-linked H^+/K^+ exchange discussed in #2 above. The effect of DTE appears to be in augmenting this passive H^+/K^+ exchange. Schematically, our tentative view of these systems can be depicted thus:



← Energy-linked (ATPase?) H^+/K^+ exchange, probably not electroneutral and increased at high pH externally.

← Passive exchange driven by electrochemical gradients of H^+ and K^+, the exchange operating maximally when -SH groups are reduced.

At pH 4, there is little net uptake of K^+ or net loss of H^+, especially in fresh tissue. DET has little effect at low pH, and the high K^+ efflux which is observed suggests the passive exchanger is operating maximally without DTE. The energy-linked H^+/K^+ exchange may be operating efficiently but, if it is, the back exchange via the passive "antiporter" is off-setting its effect. According to Poole, the electrogenic PD component is small at low pH. We deduce the energy-linked H^+/K^+ exchange must be close to stoichiometric (i.e.: equal H^+ out, K^+ in).

With washing there is not much change at pH 4.0. However, at pH 6.0 there is an increase in electrogenic PD and an increase in rates of K^+ (and other ion) uptake. The net proton extrusion of fresh tissue has disappeared, and DTE now has a pronounced effect in causing net H^+ uptake and K^+ efflux. This can be explained by higher efficiency and non-stoichiometric operation of the energy-linked H^+/K^+ exchanger, plus inefficiency of the passive H^+/K^+ exchanger until DTE is added. Under this circumstance, the extra K^+ accumulated during washing could contribute significantly to the electrochemical potential of protons by driving passive H^+/K^+ exchange.

5. DTE increases the PD of both fresh and washed tissue by about 20 mV. This increase cannot be collapsed by FCCP, and hence is not electrogenic.

6. DTE decreases the resistance in both fresh and washed tissue from about 12-13 megaohms to 7-8 megaohms.

Both of these results might be explained by DTE-enhanced operation of the passive H^+/K^+ exchanger as speculated above, but only if the coefficients for K^+ exit were greater than for H^+ entry, suggesting some degree of independence in the respective fluxes.

7. There is no evidence that DTE affects the energy relations of the tissue.

	ATP content nmoles/g.		Microsomal ATPase µmoles P_i/g. prot./hr		Respiration µmoles O_2/g/hr	
	control	DTE	control	DTE	control	DTE
fresh	58	60	16.4	16.5	23.6	23.6
4 hr washed	73	75	20.4	19.6	23.3	23.3

Tissue was incubated for 30 minutes in buffer with and without 0.5 mM DTE. prior to making determinations. The rapid decline in ATP content of fresh tissue over the first 30-60 minutes of submersion followed by recovery at 4 hours was reported last year (Plant Physiol. 54: 250, 1974) and forms a special study (see Section B). The small increase in microsomal ATPase with washing was reported in our initial study (Plant Physiol. 49: 436, 1972).

8. Kinetic studies on K^+ and Pi^- uptake show that DIE has only a small affect on Km or V max.

K^+ uptake (0.05 - 0.25 mM)

	Km		V max	
	Control	DIE	Control	DIE
Fresh	0.028	0.038	0.85	0.86
4 hr wash	0.022	0.040	3.23	3.04

Pi^- uptake (0.05 - 0.25 mM)

	Km		V max	
	Control	DIE	Control	DIE
Fresh	0.017	0.014	0.25	0.26
4 hr wash	0.025	0.019	0.87	0.79

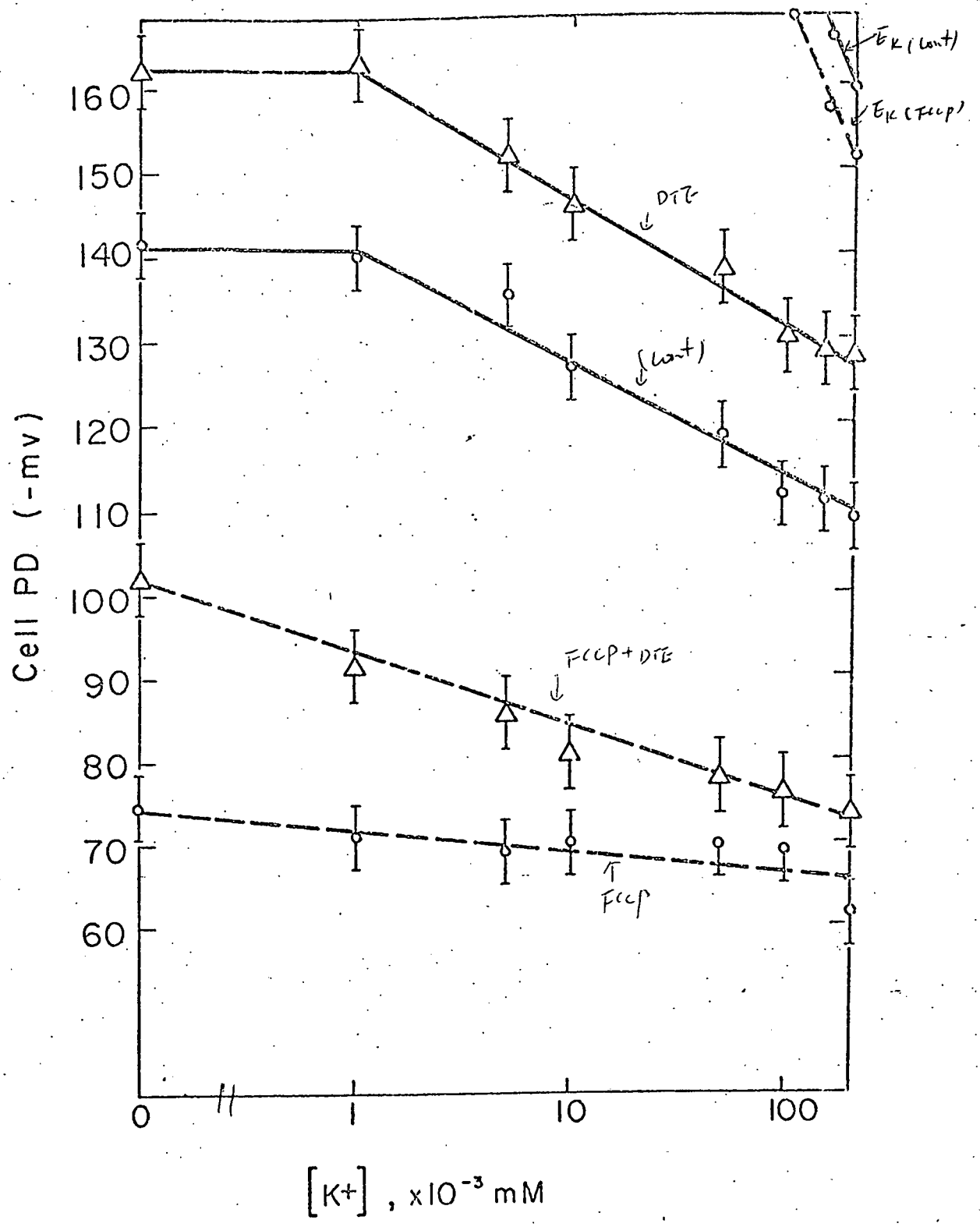
9. Cell potential was measured as a function of salt concentration and DIE. For the present the data are more perplexing than enlightening. Using the basic 0.2 mM $CaCl_2$ solution and varying KPO_4 buffer (pH 6.0) from 0.05 mM to 1.0 mM had no effect on PD of fresh or washed tissue, nor was the hyperpolarization. Sample data are given below for washed tissue.

	PD (-mV)			
PO_4 (mM)	K^+		Na^+	
	Control	DIE	Control	DIE
0.05	115	138	116	131
0.2	113	135	138	162
1.0	110	129	152	163

The hyperpolarization produced by washing is also more evident with Na^+ than with K^+ (e.g.: from -75 to -113 mV at 0.2 mM K PO_4, and from -85 to -138 mV at 0.2 mM $NaPO_4$). A partial explanation of these results is that P_{Na} is very low compared to P_k, and that lowering external K concentrations (by substituting Na) increased the diffusion potential. The only significant response to DIE was in the sharper increase in PD between 0.05 and 0.2 mM $NaPO_4$. This would occur if DIE increased P_k.

Fig 1

Slide 6


E_K (cont)
E_K (FCCP)
DTE
(cont)
FCCP + DTE
FCCP
Cell PD (-mv)
160
150
140
130
120
110
100
90
80
70
60
0
1
10
100
[K+], x10⁻³ mM

When variable concentrations of KCl were added to washed tissue in the basic medium, the result obtained in Figure 1 was obtained.

The decline in PD was far short of the 58 mV per 10-fold increase in K^+ concentration as would be expected from the Nernst equation. The usual explanation for this is that electrogenic ion transport maintains the PD over a range of low external concentrations. Since E_K (the potential difference required for electrochemical equilibration of the K^+ concentrations in the tissue with the external medium) is higher than the observed PD, there is evidence that K^+ is being pumped inward. However, use of the uncoupler FCCP did not introduce a Nernst response in PD; indeed, the decline in PD was lowered. We have no explanation as yet for this result.

DIE increased the PD without changing the response to variable KCl concentration (Figure 1). Where FCCP was used to collapse the proton gradient, DIE increased the slope as well (Figure 1). Again, we know too little about the transport processes to properly interpret this result.

B. Mr. John Gronewald has been investigating the inductive phase which precedes the enhanced ion uptake in corn root tissue.

Figure 2 gives additional data on the initial drop in ATP levels induced by cutting and/or washing. It can be seen that there is also a drop in ADP and AMP concentrations, thus giving a drop in the level of total adenine nucleotides (AdN). The reasons for this rapid decline in AdN are not known, but will be investigated in the coming year (see Proposed Research).

A further attempt was made to distinguish whether cutting or submersion is responsible for the induction of enhanced ion uptake rates, and for the decline in AdN. In our initial work we found that washing the roots of intact seedlings (no cuts), followed by excision and assay of the standard 0.5-2.5 cm section, gave about the same enhancement of ion transport as did washing cut secitons. This indicated washing as the primary physical inducer. However, it was also learned that roots raised in liquid culture responded to cutting and further washing. This means cutting has an effect as well. Previous work by us and others has shown that washing does not produce its effect by simple leacking of ions.

As shown in Figure 3, the standard treatment (curve C) produced only slightly greater augmentation than that secured by holding the sections on wet filter paper (curve B). Reducing the number of cuts (curve A) reduces the response, and increasing the number of cuts increases the response (curve D). Hence, one would deduce that wounding is a primary factor.

However, we repeated the initial experiment comparing the results obtained with washing of sections with that from washing intact roots (Figure 4). The previous results are confirmed; wounding is not essential to induction. We conclude that either form of stress -- cutting or submersion (partial anerobiosis) -- is adequate to trigger the inductive response in the tissue.

Figure 2

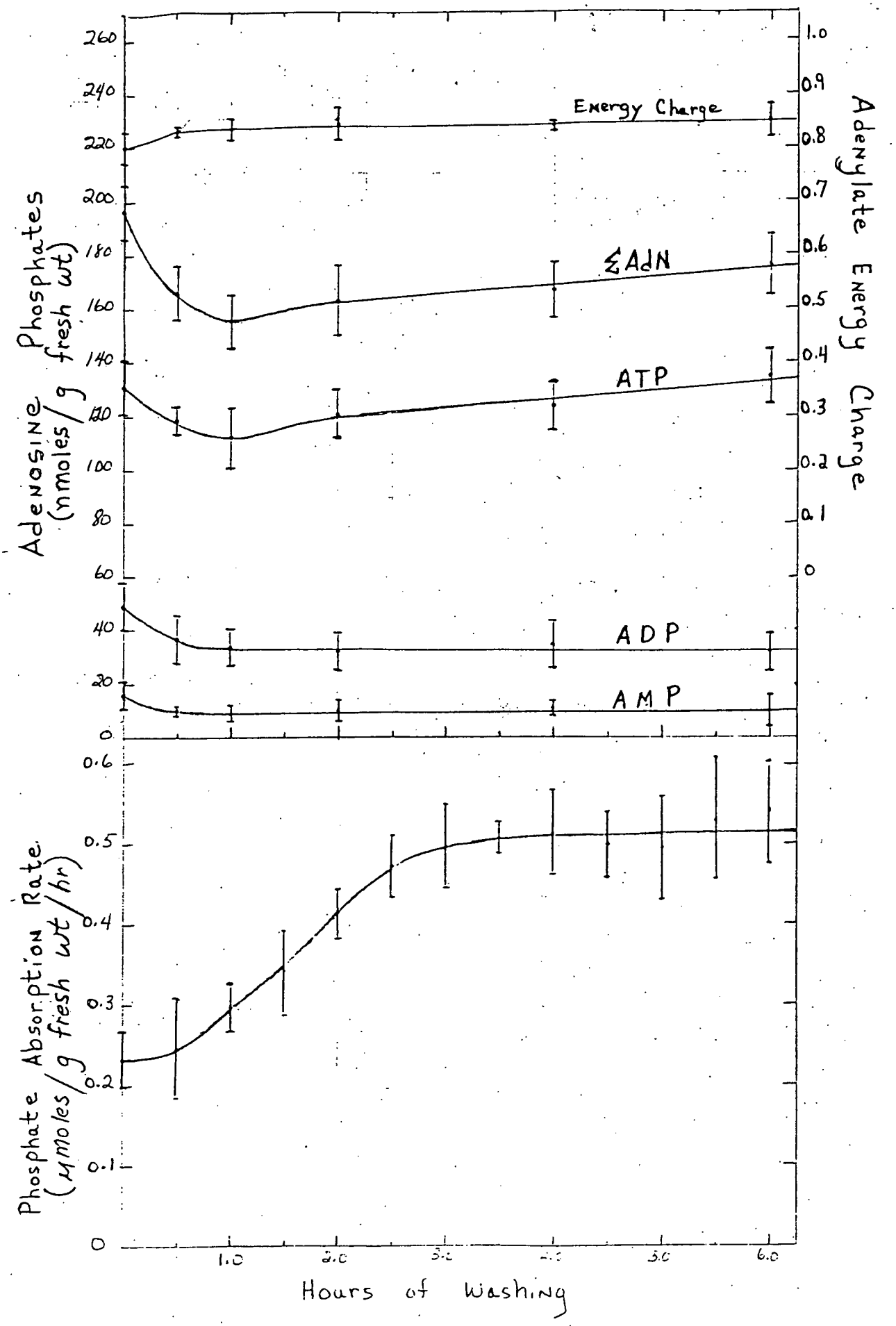

Energy Charge
ΣAdN
ATP
ADP
AMP
Adenosine Phosphates (nmoles/g fresh wt)
Adenylate Energy Charge
Phosphate Absorption Rate (μmoles/g fresh wt/hr)
Hours of Washing
260
240
220
200
180
160
140
120
100
80
60
40
20
0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.6
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
5.0
6.0

Figure 3

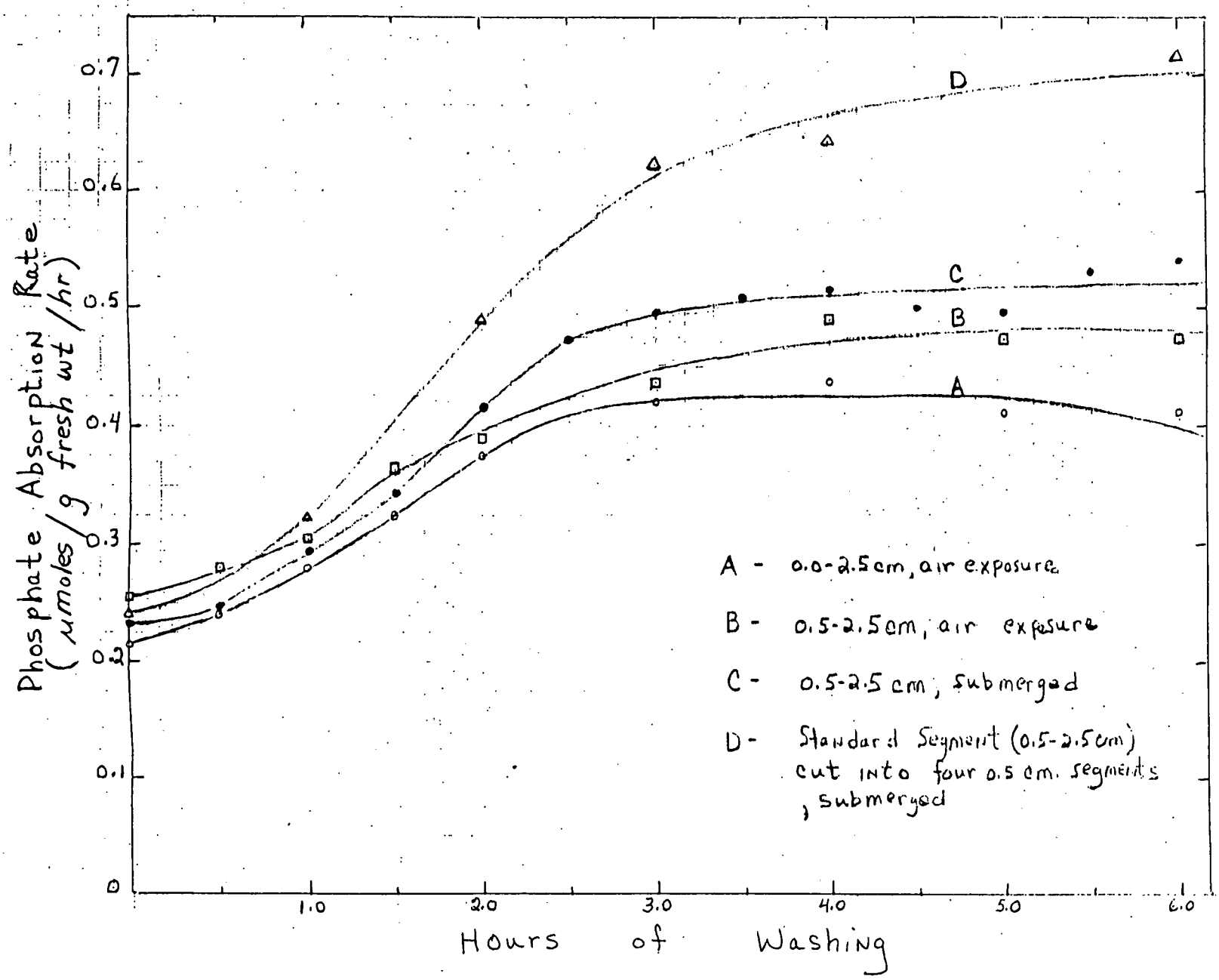

Phosphate Absorption Rate
(μmoles / g fresh wt / hr)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1.0
2.0
3.0
4.0
5.0
6.0
Hours of Washing
A
B
C
D
A - 0.0-2.5 cm, air exposure
B - 0.5-2.5 cm, air exposure
C - 0.5-2.5 cm, submerged
D - Standard Segment (0.5-2.5 cm) cut into four 0.5 cm. segments, submerged

Figure 4

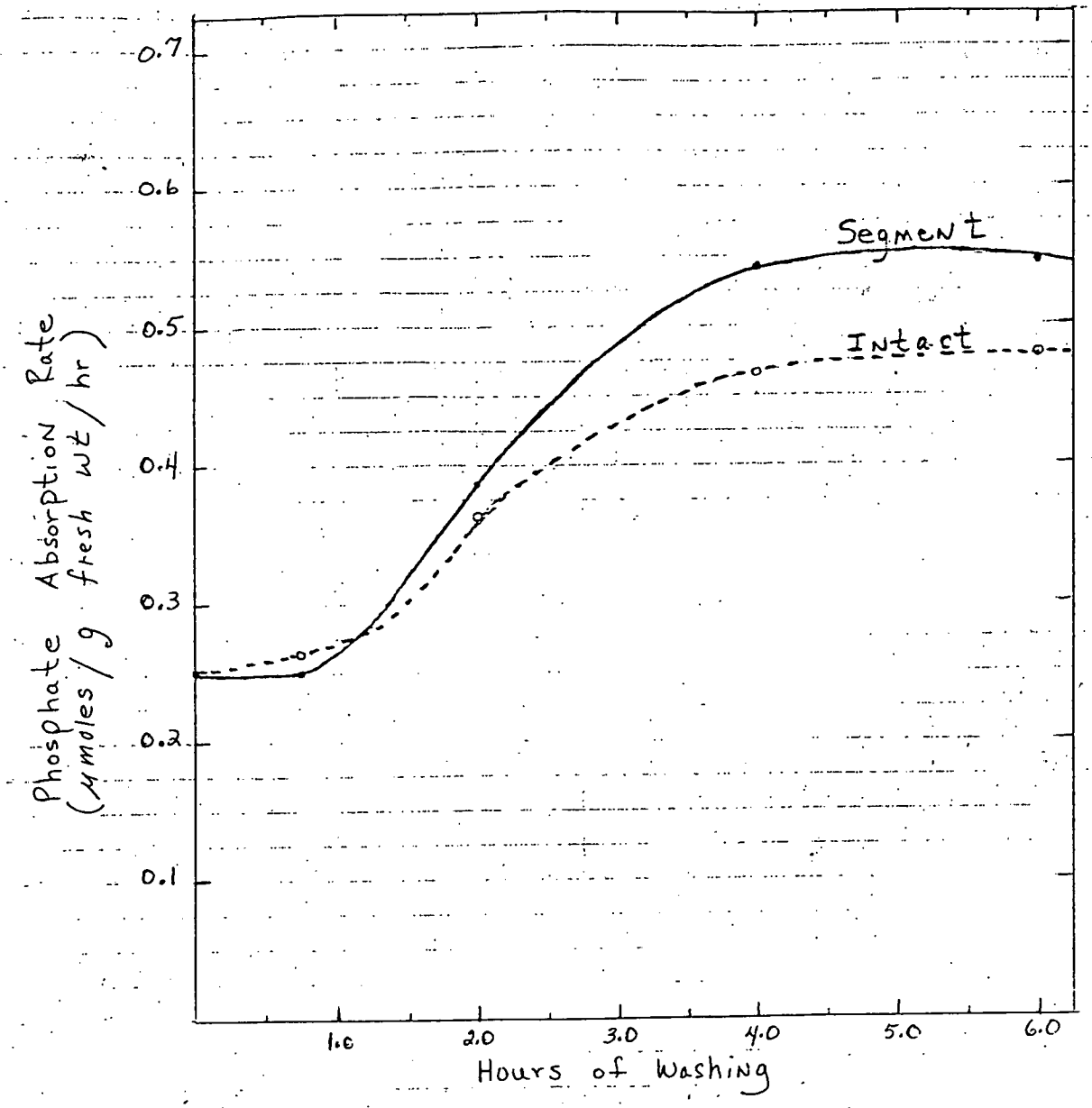

Phosphate Absorption Rate
(μmoles / g fresh wt / hr)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Segment
Intact
1.0
2.0
3.0
4.0
5.0
6.0
Hours of Washing

Table I

Hours of Washing	Standard Segment (0.5 – 2.5 cm)			Intact Root (0.5-2.5cm segment excised after washing)			Standard Segment washed with 10^{-4} M 2,4D			Standard Segment cut into four 0.5cm Segments.		
	nmoles/g f wt			nmoles/g f wt			nmoles/g f wt			nmoles/g f wt		
	ATP	ΣAdN	Energy Charge	ATP	ΣAdN	Energy Charge	ATP	ΣAdN	Energy Charge	ATP	ΣAdN	Energy Charge
0	131 ± 10	196 ± 10	.80 ± .03	131 ± 10	196 ± 10	.80 ± .03	131 ± 10	196 ± 10	.80 ± .03	95 ± 8	167 ± 8	.73 ± .02
1	112 ± 11	155 ± 10	.83 ± .02	117 ± 4	169 ± 3	.81 ± .03	121 ± 11	168 ± 15	.83 ± .01	99 ± 11	141 ± 13	.80 ± .03
2	119 ± 8	162 ± 13	.84 ± .03	124 ± 6	184 ± 4	.78 ± .01	128 ± 5	177 ± 5	.83 ± .03	116 ± 14	161 ± 12	.83 ± .03
4	121 ± 9	167 ± 10	.84 ± .01	141 ± 5	203 ± 5	.81 ± .02	138 ± 11	189 ± 9	.83 ± .01	130 ± 9	167 ± 7	.86 ± .04
6	135 ± 10	175 ± 12	.86 ± .03	178 ± 9	223 ± 5	.88 ± .02	161 ± 10	206 ± 6	.87 ± .04	133 ± 7	174 ± 9	.86 ± .03

Table I shows the change in AdN with various washing treatments. Previous work has shown that washing in 2,4-D inhibits the development of enhanced ion uptake rates. Washing of standard segments and of intact roots give comparable results, while cutting into 0.5 cm segments increases the response (see above).

The initial drop in AdN is not significantly different in standard segments with and without 2,4-D and the intact root. However, the recovery in AdN is greater with intact roots and 2,4-D. Wounding by making extra cuts seems to cause an initial sharp depression of AdN, but this is recovered with washing to the level of the standard segment. Hence, there appears to be no correlation between ATP or AdN concentrations and the enhanced ion uptake rates.

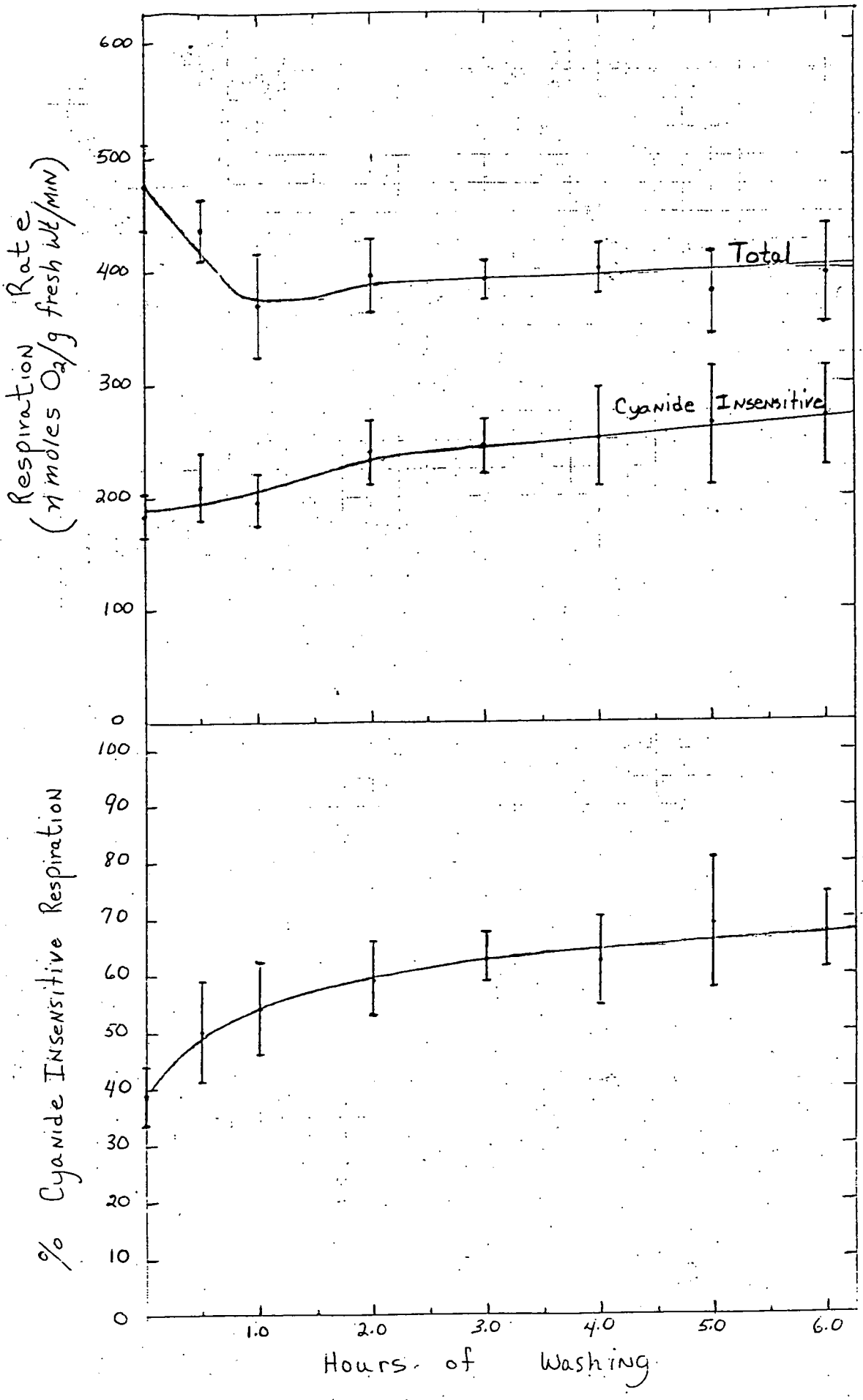
Past research has shown no statistically significant change in respiration rate during washing. However, we did not closely follow changes in the inductive lag over the first hour. Investigation showed that there was a small decline in respiration rate followed by a rise to normal in parallel with the changes in AdN. Figure 5 is the same experiment but, with the standard section cut into two 1 cm pieces to augment the loss of AdN. There is about a 20% decline in respiration rate over the first hour, with partial recovery subsequently. It is possible that the decline in respiration is due to loss of adenylate acceptor for oxidative phosphorylation.

It has often been observed in storage tissue that washing is associated with an increase in cyanide-insensitive respiration. Out of curiosity we checked the sensitivity of corn root segments to 10^{-3} M KCN. The increase is from about 40% to about 70% CN-insensitive respiration over 5-6 hours. The percentage increase curve parallels that of the increase in PD (Plant Physiology 54: 799, 1974). However, this is deceptive. The absolute rate of CN-insensitive respiration shows a lag period about like that for ion transport, and the percentage curve really reflects the drop in CN-sensitive respiration (Figure 5).

II. Proposed Research, 15 July 1975 - 15 July 1976

We will continue with investigations of the electrogenic component of ion transport in root cells. Like most others in this field our thinking has been highly conditioned by Mitchell's chemiosmotic hypothesis. (Higinbotham, Ann. Rev. Plant Physiol. 24: 25, 1973; Spanswick, B. B. Acta 228: 73, 1972; Smith, New Phytol. 69: 903, 1970; Hodges, Adv. Agronomy 25: 163, 1973). It is becoming evident, however, that simple proton pumping at the expense of an ATPase followed by ion transport via exchange carriers driven by the proton gradient is not adequate to explain all of the experimental results. For example, we have difficulty accounting for the observed effects of sulfhydryl protecting agents, such as dithioerythritol, on causing H^+ influx/K^+ efflux, increasing PD, decreasing membrane resistance, and increasing anion transport (see above). This mutiplicity of effects may have a simple explanation in the activity of several enzymes, but what are the enzymes? A way must be found to get at these one by one.

Figure 5


Respiration Rate
(n moles O_2/g fresh wt/min)
600
500
400
300
200
100
0
Total
Cyanide Insensitive
% Cyanide Insensitive Respiration
100
90
80
70
60
50
40
30
20
10
0
1.0
2.0
3.0
4.0
5.0
6.0
Hours of Washing

Last year we proposed to use "cold osmotic shock" (Amar and Reinhold, Plant Physiol. 51: 620, 1973) to see if we could release proteins essential to ion transport from the membranes, and then study the properties of these proteins. However, we were unable to establish that cold osmotic shock affected *~~cell transport or~~ the ATPase activity of membranes. It still remains a desireable goal to find techniques of removing and replacing membrane components critical to transport. We will continue to explore methods of doing this.

We will also continue with efforts to understand the parameters that govern proton uptake and extrusion from the roots. Most perplexing is the decline in H^+ efflux which accompanies washing and which parallels most closely the increase in electrogenic potential (membrane resistance and diffusion potential do not change). That is, there is no lag phase to the decline in rate of H^+ efflux, which is quite unlike ion absorption rates. The fact that H^+ efflux is very pH sensitive in fresh tissue and much less sensitive in washed tissue (report above) suggests that the original mechanism for H^+ extrusion has been superseded by another mechanism of creating electrogenic potentials. Perhaps in washed tissue a K^+ efflux pump has been developed. We will attempt to determine this by following short-term active efflux of pre-accumulated ^{42}K or ^{86}Rb, looking to see if K^+ efflux increases as H^+ efflux decreases with washing.

We want to determine if the proton fluxes associated with transport are altered if a non-electrolyte is substituted for salts. Washing was found to promote glucose uptake (Plant Physiol. 49: 430, 1972), but we have not yet determined if this enhanced uptake is linked to H^+ cotransport, as in the case of fungi (Slayman and Slayman, PNAS 71: 1935, 1974).

Work on the inductive phase will proceed along present lines. First, we must clear up where the adenine nucleotides are going. We will try to make a balance sheet on degradation (AMP → IMP → inosine?), leaking to solution, and incorporation into RNA and poly-A. The other nucleotides will also be followed. In this way we will have data indicating whether the decline in AdN is causally related to the RNA and protein synthesis occuring during the lag phase.

An attempt will also be made to understand the significance, if any, of the increase in cyanide-insensitive respiration. There has always been some dispute as to whether ATP is the energy source for ion transport, or whether some direct linkage to respiration is involved (Polya and Atkinson, Aust. J. Biol. Sci. 22: 573, 1969). Our exploration of this question suggested that ATP could be the energy source, but only if membrane ATPase possessed certain properties, including sensitivity to oligomycin and uncouplers (Plant Physiol. 54: 250, 1974). Such sensitivity is not found in isolated membrane preparations, and thus leaves open the Atkinson-Polya hypothesis of some more direct linkage to respiration. There is a possibility that the cyanide-insensitive respiration might serve as the energy source; the correlation between increased ion absorption rates and increased CN-insensitive respiration is reasonably good. However, there is still a lot that is unknown about CN-insensitive respiration (Bonner, Phytochemistry, Vol. III, L. Miller, ed., 1974). It is sensitive to substituted hydroxamic acids and can be found in association with

* correction: cell transport could be lowered in some cases,

the mitochondria, but the terminal oxidase is unknown, other than that it is not cytochrome oxidase (the oxidase has a loss 0_2 affinity). There is no suggestion that CN-insensitive respiration can be located in the cell membranes, but no one has eliminated this possibility, either.

We will attack this problem by first determining if we can entirely account for the rise in CN-insensitive respiration by that associated with the mitochondria. Also, we will determine any changes in other known oxidases (ascorbic acid axidase, polyphenol oxidase, catalase, peroxidase). The sensitivity of ion transport to the inhibiting hydroxamic acids will be ascertained. Depending on what we learn here, we may proceed to isolating cell membranes and examine them for the ability to oxidize potential substrates (NADH, malate?) in association with cytoplasmic oxidases. This becomes very speculative research and difficulty to describe, but in some fashion we need to determine whether the CN-insensitive respiration of these corn root sections can be coupled to ion transport. No clear pathway to answering this question is evident, but by probing we may find one. Mr. John Gronewald is interested in doing this research for a thesis problem.

A new post-doctoral associate, Dr. David Day, who has had considerable experience with plant mitochondria, will join the laboratory this fall. We plan to continute the investigations of the mitochondrial ATPase started by D. W. Jung (Arch. Biochem. Biophys. 158: 139, 1973; Biochem. Biophys. Acta 325: 189, 1973; Arch. Biochem. Biophys. 168: 358, 1975). The principal problem is to establish whether or not there is a direct exchange transfer of ATP/ADP between the AdN translocator and the F_i-ATPase as we proposed on the basis of arsenate uncoupling studies (Plant Physiol. 52: 431, 1975). We also need to determine the H^+ efflux/ATP hydrolyzed ratio involved in the concerted transport-hydrolysis reaction. Our hypothesis is that the concerted reaction produces a H^+/ATP ratio of unity, plus the membrane potential associated with ATP^{4-}/ADP^{3-} exchange. (in Membrane Transport in Plants, Zimmerman and Dainty, eds., Springer-Berlag, p. 317, 1974). We believe the membrane potential may be translated into additional proton transport in the presence of uncouplers and ionophores, giving the $2H^+$/ATP ratio which Mitchell observes (Eur. J. Biochem. 4: 530, 1968). We will aslo investigate the atractyloside-insensitive accumulation of AdN which we have discovered (Arch. Biochem. Biophys. 168: 358, 1975).

III. Personnel, 15 July 1974 - 15 July 1975

C. J. Arntzen, principal investigator
J. T. Gronewald, research associate (100%)
W. Lin, research assistant (50%; partially paid by NSF grant)

IV. Publications, 15 July 1974 - 15 July 1975

COO-790-59. Lin, W. and J. B. Hanson, 1974. Phosphate absorption rates and ATP concentrations in corn root tissue. Plant Physiol. 54: 250-256.

COO-790-60. Lin, W. and J. B. Hanson. 1974. Increase in electrogenic membrane potential with washing of corn root tissue. Plant Physiol. 54: 799-801.

COO-790-61. Hanson, J. B. 1974. On the reversible ATPase of plant mitochondria. In Membrane Transport in Plants, ed. U. Zimmermann and J. Dainty. Springer-Verlag, Berlin, pp. 317-320.

COO-790-62. Ramakrishnan, C. V. and J. B. Hanson. 1974. Oxidation of exogenous NADH by corn mitochondria. Indian J. Biochem. Biophys. 11: 134-137.

COO-790-63. Jung, D. W. and J. B. Hanson. 1975. Activation of 2,4-dinitrophenol-stimulated ATPase activity in cauliflower and corn mitochondria. Arch. Biochem. Biophys. 168: 358-368.

COO-790-64. Hensley, J. R. and J. B. Hanson. 1975. The action of valinomycin in uncoupling corn mitochondria. Plant Physiol. 56: 13-17.

In press:

Hanson, J. B. and D. E. Koeppe. 1975. Ion transport in plant mitochondria. In: Ion Transport in Plants, ed. D. E. Baker and J. R. Hall, Springer-Verlag, Berlin.