DOE/ER/13337--4

DE93 007961

FINAL TECHNICAL REPORT

MEMBRANE VESICLES -- A SIMPLIFIED SYSTEM FOR STUDYING AUXIN TRANSPORT

Introduction

Indoleacetic acid (IAA), the auxin responsible for regulation of growth, is transported polarly in plants. IAA stimulates a rapid increase in the rate of electrogenic proton secretion by the plasma membrane. This not only acidifies the cell wall leading to its loosening and growth but contributes to the pH and electrical gradients that provide the driving force for polar auxin transport and the uptake of inorganic ions, sugars, and amino acids.

Several different models have been suggested to account for IAA transport by cells and its accumulation by membrane vesicles.

(1) *Diffusion of IAA driven by a pH gradient.* The anion of a lipophilic weak acid like IAA or butyrate accumulates in an alkaline compartment in accord with the size of the pH gradient. The accumulation of IAA may be diminished by the permeability of its lipophilic anion. This anion leak may be blocked by NPA. With anion efflux blocked, a gradient of two pH units would support an IAA accumulation of less than 50-fold at equilibrium.

(2) *Diffusion of IAA, in parallel with a saturable symport ($IAA^- + nH^+$), driven by both the pH gradient and membrane voltage (Hertel, 1985).* Such a symport should be highly accumulative, however, with a lipophilic weak acid such as IAA, net diffusive efflux of IAAH whenever $IAAH_i > IAAH_o$ would constitute a leak.

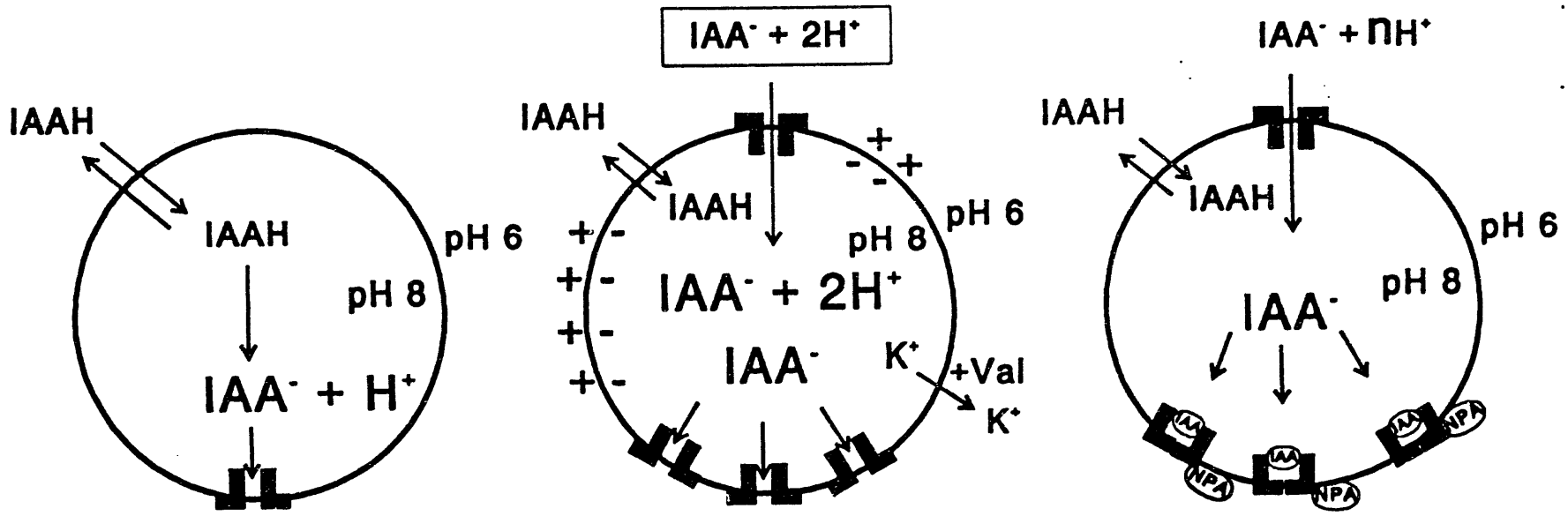
(3) Δ *pH-driven IAA uptake and saturable symport enhanced by internal binding sites.* Following pH gradient-driven accumulation of IAA, the anion may bind to an intravesicular site, permitting further uptake of IAA. NPA, by blocking anion efflux, enhances this binding.

We have reported that membrane vesicles isolated from actively growing plant tissues are a good system for studying the mechanisms involved in the transport and accumulation of auxin. In response to a pH gradient ($pH_o < pH_i$), ^{3}H-IAA accumulates several times more than expected from the magnitude of the pH gradient (Fig. 1). The herbicide naphthylphthalamic acid (NPA) further enhances IAA uptake. To determine whether the membrane potential enhances IAA accumulation (model 2), we imposed a K^+ and added the K ionophore, valinomycin. If a negative membrane potential contributes to IAA accumulation by the vesicles, we would expect that:

(1) IAA uptake would be enhanced even in the absence of any pH gradient because according to chemiosmotic theory, pH and membrane voltage are interchangeable driving forces for transport.

(2) electrogenic IAA transport would decrease the membrane potential, and

Models for IAA Uptake in Membrane Vesicles



Model 1.
ΔpH-driven uptake

Model 2.
ΔpH and saturable ΔV_m -driven uptake

Model 3.
ΔpH-driven, NPA-stimulated uptake with binding sites

(3) IAA accumulation would be in accord with the magnitude of the pH gradient when the membrane potential has been dissipated.

Although a negative membrane potential transiently increases IAA uptake in the presence of a pH gradient (Fig. 2), we did not find evidence to support the above points. Intravesicular binding sites provide an alternative explanation for the enhanced accumulation of IAA.

Experimental Conditions

Microsomal vesicles were isolated by differential centrifugation from hypocotyls of dark-grown zucchini (Cucurbita pepo L. var. Black Beauty). The homogenization medium was buffered at pH 8 and contained 100 mM K^+ (sulfate or gluconate). Unless otherwise noted, all media were 350 mOsM. To establish transmembrane pH and K^+ gradients, vesicles were diluted into pH 6 buffer with 0 - 100 mM K-salt, ionically balanced with Na-salt. The pH gradient was dissipated with nigericin (a H^+/K^+ exchanger) and valinomycin was added to enhance K^+ permeability and generate a membrane potential. Uptake of ^{3}H-IAA was measured at 10°C simultaneously with either ^{14}C-Bta (as pH probe) or $^{86}Rb^+$ (membrane voltage probe). Millapore filtration was used to recover the vesicles. Since their pK_as are similar, IAA and BtA should accumulate to similar extents if a pH gradient alone drives accumulation. The component of IAA and BtA accumulation that is ΔpH-dependent (that is sensitive to CCCP plus Val) has been expressed as C_i/C_o, the internal concentration of IAA or BtA relative to that outside. The membrane potential generated by the K^+ - diffusion potential was calculated according to Nernst from the distribution of Rb^+.

Results

Vesicles prepared in 50 mM K_2SO_4 at (pH 8) were diluted 100-fold into 50 mM Na_2SO_4 (pH 6) containing ^{3}H-IAA and ^{14}C-BtA (Fig. 1). Since the accumulation of IAA in the presence of 5 μM NPA was greater than that of BtA (the pH probe), it can not be attributed to the pH gradient alone. The jump in the accumulation of $^{86}Rb^+$ on addition of valinomycin (Fig. 2 top) indicates an enhanced membrane negativity and correlates with a transient increase in IAA accumulation (Fig. 2 bottom). The increase in IAA uptake does not occur in the presence of 5 μM IAA. Valinomycin increases the permeability of the vesicles to K^+, presumably increasing the K^+ diffusion potential and making the membrane voltage more negative. BtA uptake (not shown) was not influenced by valinomycin addition indicating that its anion is impermeable and distributes independently of the membrane voltage.

Vesicles prepared in 100 mM K-gluconate were diluted 100-fold into a solution ionically balanced with Na^+-gluconate containing $^{86}Rb^+$ and 1 to 100 mM K^+-gluconate. Addition of valinomycin induced a redistribution of Rb^+ that was proportional to the K^+ diffusion gradient (Fig. 3). The observed membrane voltage (calculated from the Nernst equation based on the accumulation of Rb^+) was linearly related to the expected E_K

calculated from the imposed K^+ gradient); however, the slope was less than 56 mV indicating that the vesicles are permeable to other ions besides K^+.

After the pH gradient had been dissipated with nigericin, a negative membrane potential could still be generated by valinomycin, but it did not enhance IAA accumulation (Fig 4).

Even when $[K^+]_i$ and $[K^+]_o$ were equal (50 mM); that is, no K^+ diffusion potential to contribute to V_m, IAA accumulation still exceeded that expected from the magnitude of the pH gradient as measured by BtA uptake (Fig. 5), suggesting that enhanced saturable accumulation of IAA in NPA is not explicable by postulating membrane voltage as a driving force.

The possibility of K^+-diffusion potential was eliminated by preparing the vesicles and diluting them into solution of the same concentration, 50 mM K_2SO_4. Under these conditions, increasing the external osmoticum from 200 to 850 mOsM caused osmotic shrinkage of the vesicles. Although virtually all the BtA uptake was eliminated by shrinking the vesicles as was the ΔpH-dependent, saturable IAA uptake, the excess NPA-stimulated IAA was not affected (Fig. 6). This suggests that much of the IAA is not freely soluble in the intravesicular volume.

Conclusions and Observations

(1) At IAA concentrations below saturation, ^{3}H-IAA accumulates more than expected from the magnitude of the pH gradient (Figs. 1,2,4,5,6).

(2) In the presence of a pH gradient, specific IAA accumulation is transiently enhanced by a negative membrane potential (Figs 2,3,5).

(3) In the absence of a pH gradient, a negative membrane potential is without effect on IAA accumulation (Fig. 4).

(4) Even in the absence of a negative membrane potential, IAA accumulation still exceeds that predicted fronm the size of the pH gradient (Fig.5).

(5) ΔpH-dependent uptake followed by a saturable binding could account for the excess accumulation of IAA (Fig. 6). The kinetics of IAA uptake are slower than the other probes and may reflect the binding step (see Figs 1,2,5). One possibility is that both NPA and the membrane potential may change the conformation of IAA carrier sites in such a way as to block efflux and enhance binding.

INVESTIGATIONS OF AN INWARDLY RECTIFYING CURRENT IN OAT MESOPHYLL PROTOPLASTS ATTRIBUTED TO K^+ CHANNELS

During the extension of this grant without additional funds, we also carried out a preliminary investigation of the whole-cell currents of the plasma membrane of mesophyll protoplasts of young oat leaves using patch clamping techniques. This investigation was undertaken after the PI participated in the course "Single Channel Methods" at Cold Spring Harbor during the summer of 1985. The purpose was to master these techniques in order to be able to investigate the transport of K^+, other cations, and anions during growth and cell expansion. We have reported the properties of an inward rectifying current carried by K^+ (Kourie and Goldsmith, 1992). Both the whole-cell and single-channel currents are activated when the cell is hyperpolarized. The current is highly selective for K^+ and blocked by Na^+ and Cs^+. The channels have long open periods without inactivating that would be suitable for uptake of K^+.

Growing plant cells must acquire potassium ions in order to maintain relatively stable ionic and osmotic potentials during cell expansion. Our calculations indicate that these channels could contribute significantly to K^+ influx when $[K^+]_o$ is in the millimolar range and the plasma membrane voltage is more negative than E_K. Our lab is continuing to characterize single channels in mesophyll cell membranes. We are also using Arabidopsis and have recently reported on activation of K^+ channels by light and their regulation by ATP. The present work is a direct outgrowth of the earlier investigation that was supported in part by DOE.

INVENTION STATEMENT

This research did not lead to any inventions or patents.

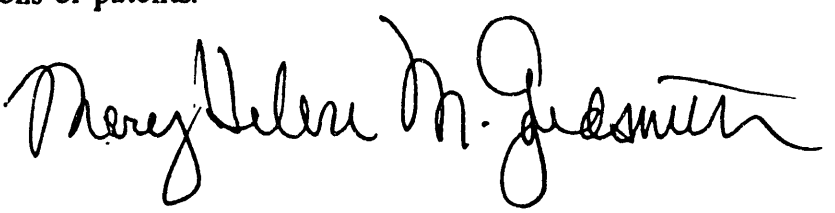


Figure Legends

Figure 1. In the presence of NPA, the accumulation of IAA exceeds that expected from the pH gradient measured with ^{14}C-butyric acid.

Figure 2. Addition of valinomycin transiently enhances the uptake of $^{86}Rb^+$ and IAA. The presence of NPA and saturating IAA concentrations do not affect $^{86}Rb^+$ accumulation.

Figure 3. Generation of a negative membrane voltage on addition of valinomycin to vesicles with an imposed transmembrane K^+ gradient ($[K^+]_i > [K^+]_o$). Inset: The E_K observed (based on Rb^+ accumulation) as a function of the expected E_K (based on the K^+ gradient).

Figure 4. In the absence of a pH gradient, a negative membrane potential appears to be ineffective in IAA accumulation.

Figure 5. In the absence of any membrane potential, accumulation of IAA was still several fold greater than could be accounted for by the existing pH gradient. This eliminates model 2 as a viable explanation for the enhanced IAA accumulation.

Figure 6. Only a portion of the specifically accumulated IAA varies with vesicle volume; therefore, binding of IAA to a saturable site inside the vesicles provides a plausible explanation for much of the ΔpH-driven uptake of 3H-IAA. The data points were fit by a linear regression.

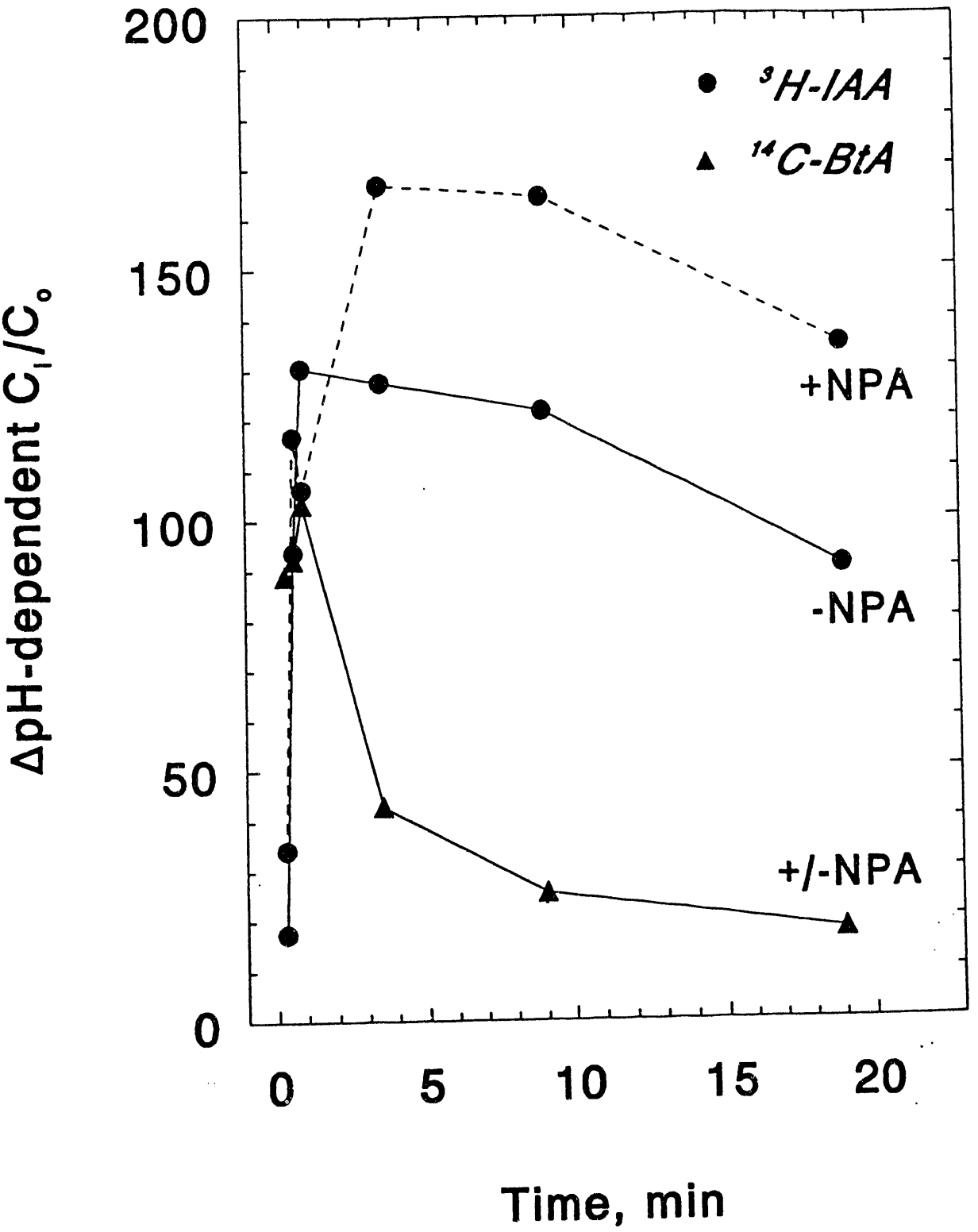

200
150
100
50
0
0
5
10
15
20
ΔpH-dependent C_i/C_o
Time, min
^{3}H-IAA
^{14}C-BtA
+NPA
-NPA
+/-NPA

Fig. 1

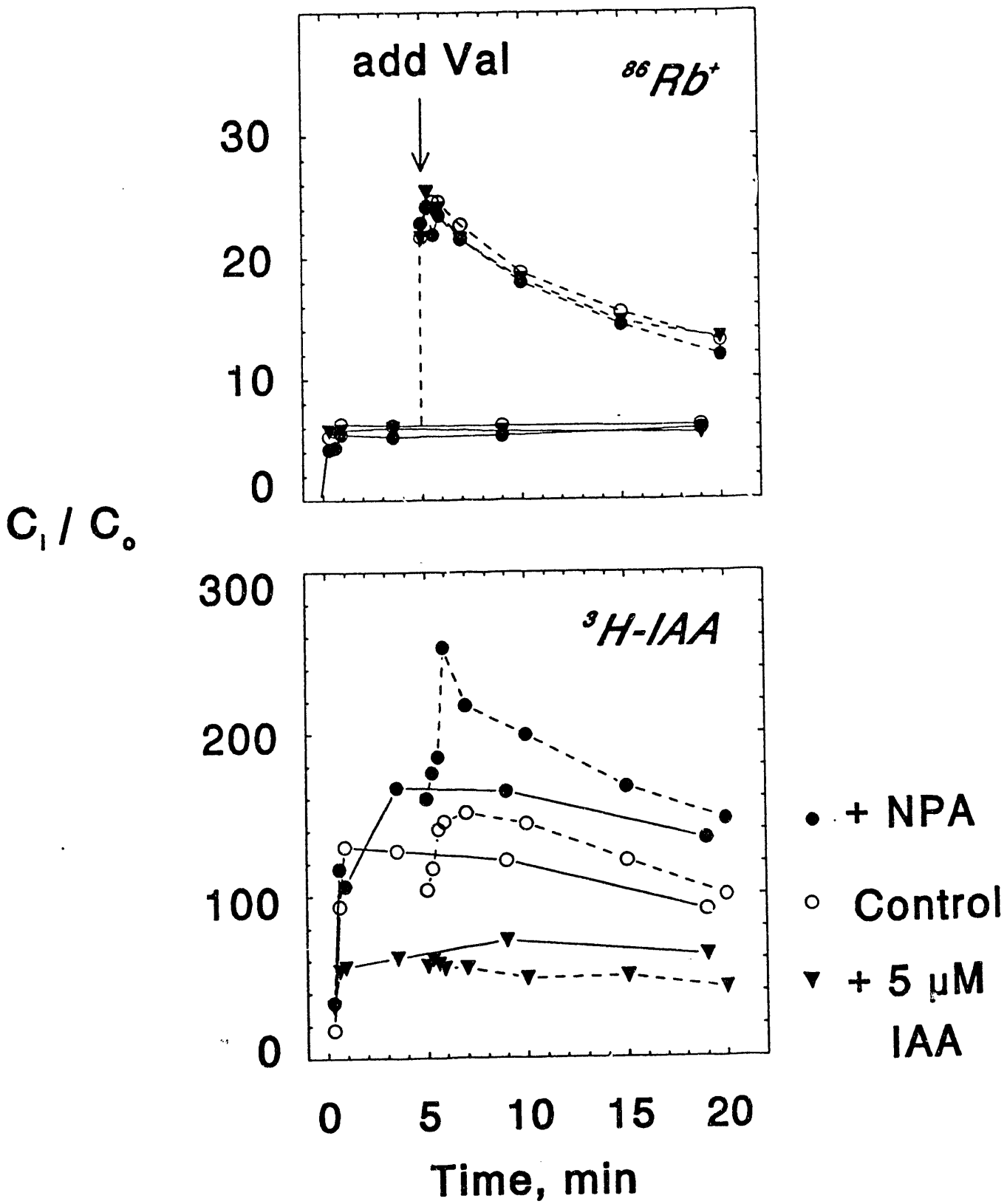

add Val
$^{86}Rb^{+}$
30
20
10
0
C_i / C_o
300
200
100
0
^{3}H-IAA
+ NPA
Control
+ 5 µM IAA
0
5
10
15
20
Time, min

Fig. 2

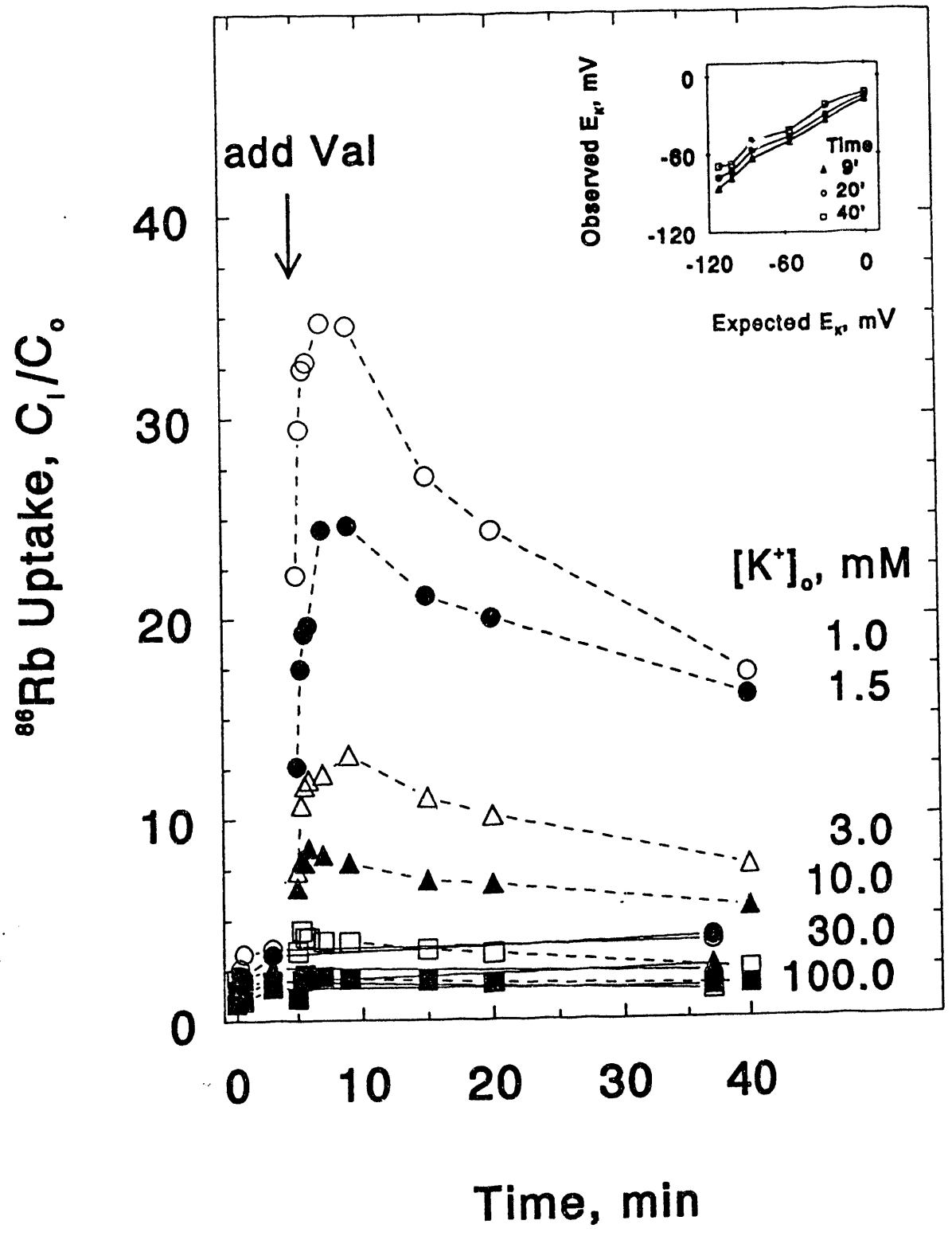

add Val
^{86}Rb Uptake, C_i/C_o
Time, min
0
10
20
30
40
$[K^+]_o$, mM
1.0
1.5
3.0
10.0
30.0
100.0
Observed E_K, mV
Expected E_K, mV
Time
9'
20'
40'
-120
-60
0

Fig. 3

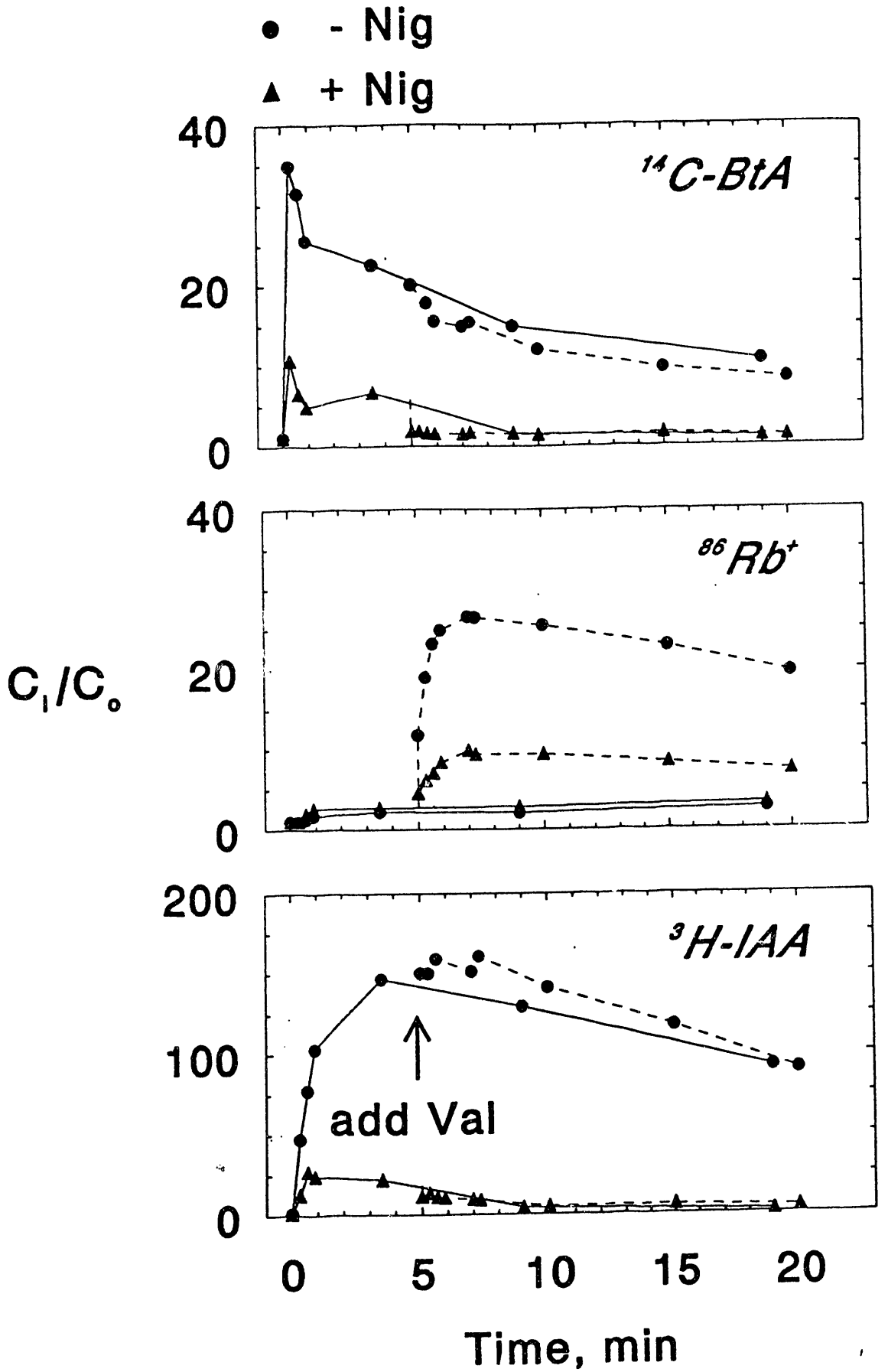

● - Nig
▲ + Nig
40
20
0
^{14}C-BtA
40
20
0
$^{86}Rb^{+}$
200
100
0
^{3}H-IAA
add Val
0
5
10
15
20
Time, min
C_i/C_o

Fig. 4

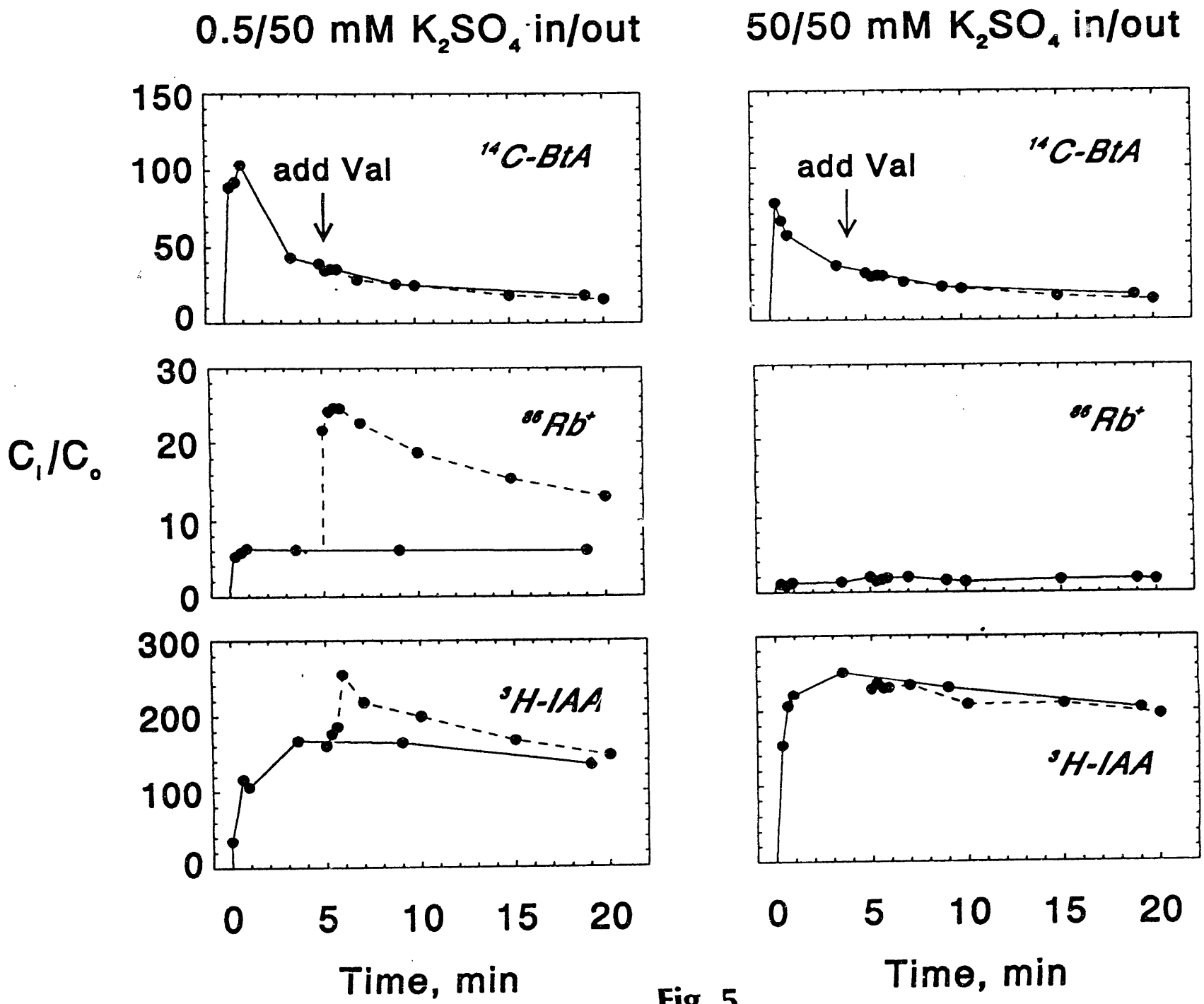

0.5/50 mM K_2SO_4 in/out
50/50 mM K_2SO_4 in/out
C_i/C_o
150
100
50
0
add Val
^{14}C-BtA
30
20
10
0
$^{86}Rb^+$
300
200
100
0
^{3}H-IAA
0
5
10
15
20
Time, min

Fig. 5

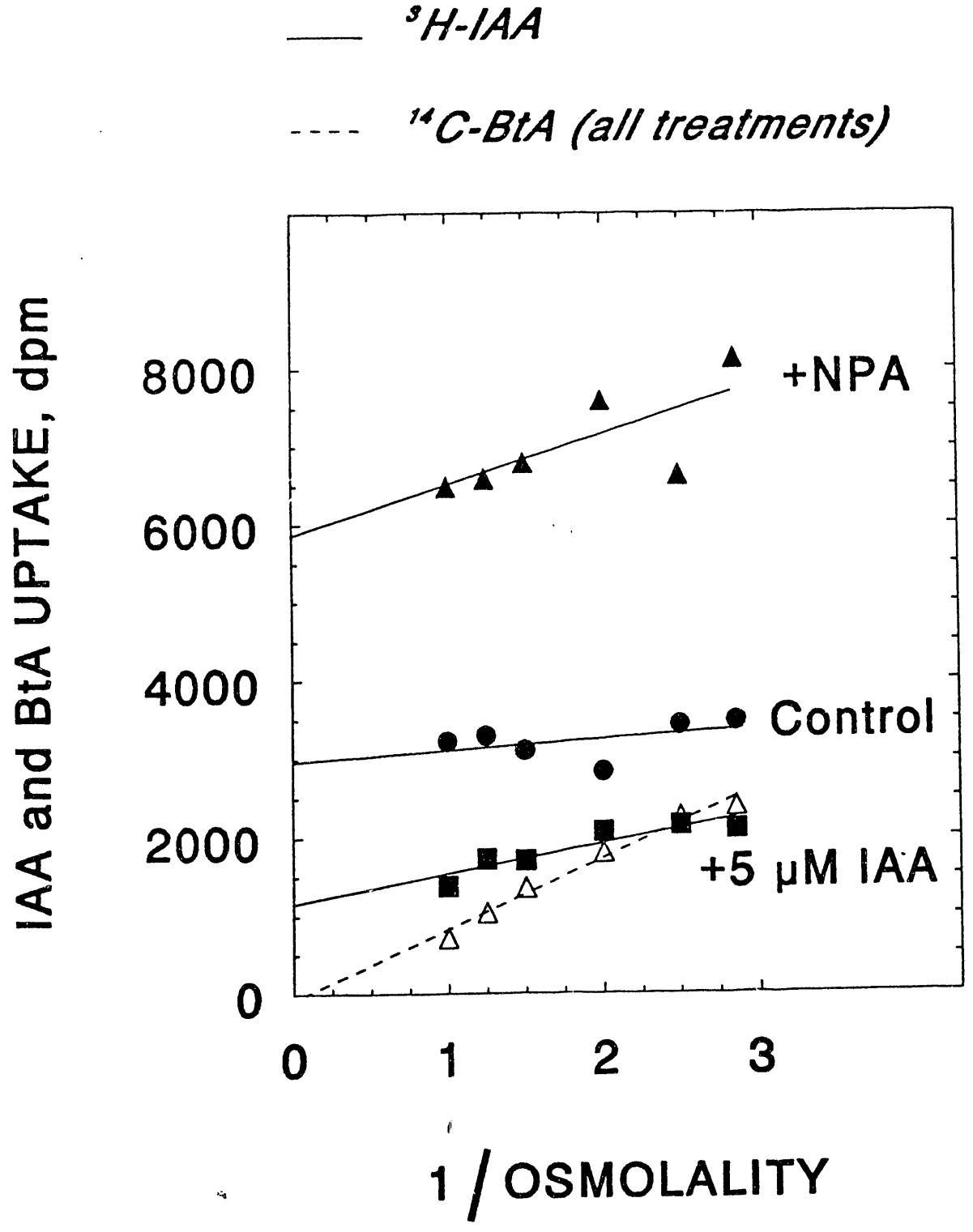

^{3}H-IAA
^{14}C-BtA (all treatments)
IAA and BtA UPTAKE, dpm
8000
6000
4000
2000
0
+NPA
Control
+5 µM IAA
0
1
2
3
1 / OSMOLALITY

Fig. 6

PERSONNEL

"USE OF MEMBRANE VESICLES AS A SIMPLIFIED SYSTEM FOR STUDYING TRANSPORT OF AUXIN"

Dr. Kathleen Clark, Postdoctoral Associate 2/15/85-8/31/87

Project: "pH-dependent accumulation of indoleacetic acid by membrane vesicles from zucchini hypocotyls"

Assistant Professor, Department of Plant Biology, Southern Illinois University Carbondale, IL. 9/1/87-5/30/92.

Present situation: Graduate student in Library Science Program, University of Illinois, Champaign, IL.

Dr. Joseph Kourie, Postdoctoral Associate 6/88-6/89

Project: Characterization of an inwardly-rectifying K^+ channel in the plasma membrane of mesophyll protoplasts from oat leaves. Pilot study of whole cell currents and single channel activity by patch clamping.

Present Position: Muscle Research Group, Division of Neuroscience, John Curtin School of Medical Research Australian National University, Canberra, ACT 2061 Australia

PUBLICATIONS

Clark, K. and M. H. M. Goldsmith. 1986. Roles of transport and binding in the specific ΔpH-dependent accumulation of auxin by zucchini membrane vesicles. In: **Plant Growth Substances 1985** Ed. M. Bopp. Springer. 203-208.

Clark, K.A., M.H.M Goldsmith. 1987. Effect of surface and membrane potentials on IAA uptake and binding by zucchini membrane vesicles. In: **Plant Hormone Receptors** Ed. D. Klambt. NATO ASI series vol. H10 Springer. 99-112.

Martin, M.H., M.H.M. Goldsmith, T.H. Goldsmith. 1990. On polar auxin transport in plant cells. J. Math. Biol. 28:197-223.

Kourie, J., & M.H.M. Goldsmith. 1992. K^+ channels are responsible for an inwardly-rectifying current in the plasma membrane of mesophyll protoplasts of Avena sativa. Plant Physiol. 98:1087-1097.

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