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Original Article

Quantitative Evaluation of the Weak Acid Hypothesis as the Mechanism for 2,4-D Absorption by Corn Root Protoplasts*

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The absorption of 2,4-D by protoplasts enzymatically isolated from the cortex of corn (*Zea mays* L. cv Pioneer 3377) root was investigated in relation to their extracellular and intracellular pH. The 2,4-D absorption increased as the extracellular pH (pH^o) decreased. The ratio of the 2,4-D concentration inside to outside the protoplasts (c^i/c^o) reached *ca.* 1.2, 4.0, 10–12 and 30–40 at pH^o 7.5, 6.5, 5.5 and 4.5 respectively, within 75 min incubation. At pH^o 3.5 and 2.8, c^i/c^o ratio reached *ca.* 90 and 150 respectively within 15 min, but the absorbed 2,4-D was rapidly released after 15 min incubation due to the death of the protoplasts. The determination of the overall intracellular pH (pH^i) by the 5,5-dimethyloxazolidine-2,4-dione distribution technique indicated that the change of the pH^i as influenced by the pH^o variation (7.5 to 4.5) was relatively small (6.9 to 6.6). Using the measured pH^i, the theoretical c^i/c^o ratio at each pH^o was estimated based on the weak acid hypothesis. Although the pH^o dependence of the theoretical c^i/c^o roughly resembled the observed tendency, the measured c^i/c^o ratios at pH^o 6.5 and 7.5 were significantly greater than their respective theoretical maxima. These results suggest that the absorption of 2,4-D by protoplasts cannot be accounted for simply by the weak acid hypothesis and that an active transport is involved at least at pH^o 6.5 and 7.5.

INTRODUCTION

It has been well established that a phenoxy herbicide, 2,4-D, has a "symplastic" property in terms of absorption and translocation in plant systems.[1-3] Several lines of experimental evidence have indicated that 2,4-D is capable of being effectively absorbed into cytoplasm and retained in the symplast.[1-5] If the mechanism for such symplastic mobility of 2,4-D could be exactly described, the results would provide important clues to elucidate plant transport systems as well as to help design chemical structures for new symplastic herbicides or phytohormones. Unfortunately, the exact mechanism of 2,4-D absorption is still unclear in spite of extensive investigations carried out.

The weak acid hypothesis (or the ion trap theory) has been used to explain the symplastic mobility of weak acid xenobiotics including 2,4-D.[3,4,6,7] The hypothesis is briefed as follows: The transport of weak acids across plasmalemma occurs mainly by passive diffusion of the undissociated molecules due to their lipophilicity. Once inside the symplast (the cytoplasm or the sieve tube), the molecules dissociate into anions due to the higher pH of the symplast. The hydrophilic anions are unable to diffuse back through the plasmalemma causing the anions to accumulate within the symplast.

There have been, however, two major

* Influence of Intracellular pH on Absorption of 2,4-D by Corn Root Protoplasts (Part 1).

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difficulties in quantitatively evaluating the validity of this hypothesis. First, the presence of the cell wall or free space in plant systems makes it difficult to accurately determine the intracellular solute concentration. Even the extracellular conditions to which the plasmalemma is directly exposed may be different from the bathing buffer if the cell wall is covering the plasmalemma. Second, the intracellular pH (pH^i) of plant cells must be determined during the absorption process. In fact, although the pH^i is a key parameter for the weak acid hypothesis, few investigations have measured the pH^i along with the absorption studies.

In the present study, in order to overcome these difficulties, the absorption of 2,4-D by corn root protoplasts was investigated considering not only the extracellular pH (pH^o) but also the pH^i. The pH^i of the protoplasts was measured by the 5,5-dimethyloxazolidine-2,4-dione (DMO) distribution technique.[8-10] The protoplasts were used to eliminate the uncertainties associated with the cell wall or Donnan phase.[11] Corn roots were selected as the source of protoplasts since experimental evidence had suggested that enzymatically isolated protoplasts from corn roots retain the normal transport properties.[12-14] Using this system, we quantitatively evaluated the validity of the weak acid hypothesis as the mechanism for the absorption of 2,4-D.

MATERIALS AND METHODS

1. *Plant Material*

Corn (*Zea mays* L. cv Pioneer 3377) seeds were surface-sterilized in 1% NaOCl for 10 min and rinsed with deionized water. The seeds were allowed to germinate on germination paper saturated with 0.5 mM $CaSO_4$ at 27±1°C in the dark. The seedlings were collected for protoplast isolation after 60 hr of germination, at which time the primary roots of the seedlings were typically 5 to 8 cm long.

2. *Protoplast Isolation*

Protoplasts were isolated by the procedures described previously,[12-14] with modifications. Chopped cortical segments from the primary roots were digested with a medium containing 0.6 M mannitol, 1 mM $CaCl_2$, 2% (w/v) Cellulysin (Calbiochem), 0.1% (w/v) Pectinase (Cooper Biomedical), 0.05% (w/v) BSA (Sigma, fraction V) and 0.5 mM dithiothreitol (pH 5.6) for 4 hr in the dark at 27±1°C with circular shaking (50 rpm). The mixture was filtrated through an 80-μm nylon filter and the filtrate was centrifuged (180×g, 6 min). The pellet was resuspended in a suspension medium (0.63 M mannitol, 0.1 mM $CaCl_2$, 0.5 mM dithiothreitol, 2.5 mM Tris-MES, pH 6.5) and centrifuged again. The pellet was suspended in 12% (w/v) Ficoll solution containing 0.63 M mannitol, 0.1 mM $CaCl_2$ and 1.25 mM Tris-MES (pH 6.5), and overlaid with similar solutions of 8, 5 and 0% (w/v) Ficoll. The gradient was centrifuged (360×g, 12 min) and the protoplasts were collected between the 0 and 5% Ficoll layers. The protoplasts were diluted in a suspension medium and centrifuged (180×g, 6 min). The final pellet was used for subsequent experiments by suspending it into an absorption medium (0.63 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM KCl, 10 mM Tris-MES, desired pH from 4.5 to 7.5 depending on the pH^o condition). For experiments at $pH^o < 4.5$, the absorption medium contained 10 mM citrate-KOH instead of Tris-MES. The protoplast number was determined with a hemacytometer and the protein content of protoplasts was determined by a modified Lowry method.[15]

3. *Protoplast Volume Determination*

The protoplast volume was determined by the method described previously,[16] with modifications. The protoplast suspension (500 to 2000×10^3 protoplasts) was added to the absorption medium (pH 6.5) containing 3.11 μCi ^3H_2O (ICN Radiochemicals, 100 mCi/ml) and 0.83 μCi ^{14}C-mannitol (ICN Radiochemicals, uniformly labeled, 42 mCi/mmol) to give a final volume of 2.3 ml in a 10- ml Erlenmeyer flask. After 20 min incubation in the dark at 27±1°C with circular shaking (50 rpm), 200 μl of the mixture was placed on top of a 70-μl layer of 1.0421 g/ml silicone oil which was layered on top of 50 μl of an osmoticum layer (0.78 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM KCl, 1 mM Tris-MES, pH 6.5) in a 400-μl microfuge tube. The microfuge tube was centrifuged with a Beckman Microfuge 11 (6800×g, 30

sec) to separate protoplasts from the radioactive medium by pelleting them through the silicone oil layer. Immediately after the centrifugation, the microfuge tube was frozen in dry ice-acetone. The tip of the tube was cut off at the silicone oil layer and placed in 10 ml of scintillation fluid (0.26 g POPOP, 13.16 g PPO, 131.6 g naphthalene, 632 ml absolute ethanol, 1000 ml 1,4-dioxane, 1000 ml toluene), and the radioactivity was determined with a liquid scintillation spectrometer (Beckman, LS-9800).

The protoplast volume and the occuluded volume were determined assuming that ^3H_2O can freely diffuse through membranes of the protoplasts while ^{14}C-mannitol cannot.

4. Procedure for 2,4-D Absorption

The absorption of 2,4-D was initiated by adding the suspension containing 1200 to 1300×10^3 protoplasts to the absorption medium containing 0.22 μCi of 2,4-dichlorophenoxy[2-^{14}C]acetic acid (^{14}C-2,4-D, Amersham, 55 mCi/mmol) to give a final volume of 2.3 ml in a 10-ml Erlenmeyer flask. The concentration of 2,4-D in the absorption medium was adjusted to 2 μM by adding nonradioactive 2,4-D prior to the addition of the protoplast suspension. The absorption mixture was incubated in the dark at 27 ± 1°C with circular shaking (50 rpm). Periodically, an aliquot (200 μl) of the incubation mixture was sampled and the ^{14}C radioactivity absorbed by the protoplasts was determined by the same microfuge-separation technique described in the volume determination procedure.

5. Determination of pH^i

The pH^i of corn root protoplasts was determined by the DMO distribution technique.[8-10] The suspension containing 500 to 600×10^3 protoplasts was added to the absorption medium to give a final volume of 1 ml in a 10-ml Erlenmeyer flask. The suspension mixture was incubated in the dark at 27 ± 1°C with circular shaking (50 rpm). After incubating for 30 min, 0.15 μCi of 5,5-dimethyloxazolidine-2[^{14}C],4-dione (^{14}C-DMO, New England Nuclear, 43.2 mCi/mmol) in 10 μl of absolute ethanol was added to the suspension and the incubation was continued for another 15 min. The net accumulation of DMO by the protoplasts was determined by the same microfuge-separation technique described in the volume determination procedure. The pH^i was calculated from the following equation:[8-10]

$$pH^i = 6.30 + \log\left[\frac{[DMO]^i}{[DMO]^o}\{1+10^{(pH^o-6.30)}\}-1\right] \quad (1)$$

where $[DMO]^i$ and $[DMO]^o$ are the DMO concentrations inside and outside the protoplasts, respectively.

RESULTS

1. Protoplast Characterization

The protoplast yields were 300 to 700×10^3 protoplasts/g fresh weight cortical tissue, resembling those reported previously.[12-14] The isolated protoplasts accumulated neutral red dye in the vacuole and fluorescein diacetate dye in the cytoplasm, indicating their intactness (Fig. 1). The intactness of the protoplasts shown in Fig. 1 was essential for 2,4-D absorption studies as will be described later. Approximately 10^6 protoplasts contained *ca.* 800 μg protein. The protoplast volume and the occluded volume were linearly correlated to the protein content, and were 6.86 μl and 0.78 μl/100 μg protein, respectively (Fig. 2). These values were used for exact calculation of intracellular solute concentration. From these values, the average diameter of the protoplasts is calculated to be 47.16 μm, which is in good agreement with the microscopic observation (Fig. 1A).

2. Absorption of 2,4-D by Protoplasts

The absorption of 2,4-D by the protoplasts was measured under wide pH variations. The pH^o markedly affected 2,4-D absorption (Figs. 3 and 4). The absorption increased as the pH^o decreased. The ratio of the 2,4-D concentration inside to outside the protoplasts (c^i/c^o) reached *ca.* 1.2, 4.0, 10–12 and 30–40 at pH^o 7.5, 6.5, 5.5 and 4.5 respectively within 75 min incubation (Fig. 3). At pH^o 3.5 and 2.8, the c^i/c^o ratio reached *ca.* 90 and 150 respectively within 15 min, but the once absorbed 2,4-D was rapidly released from the

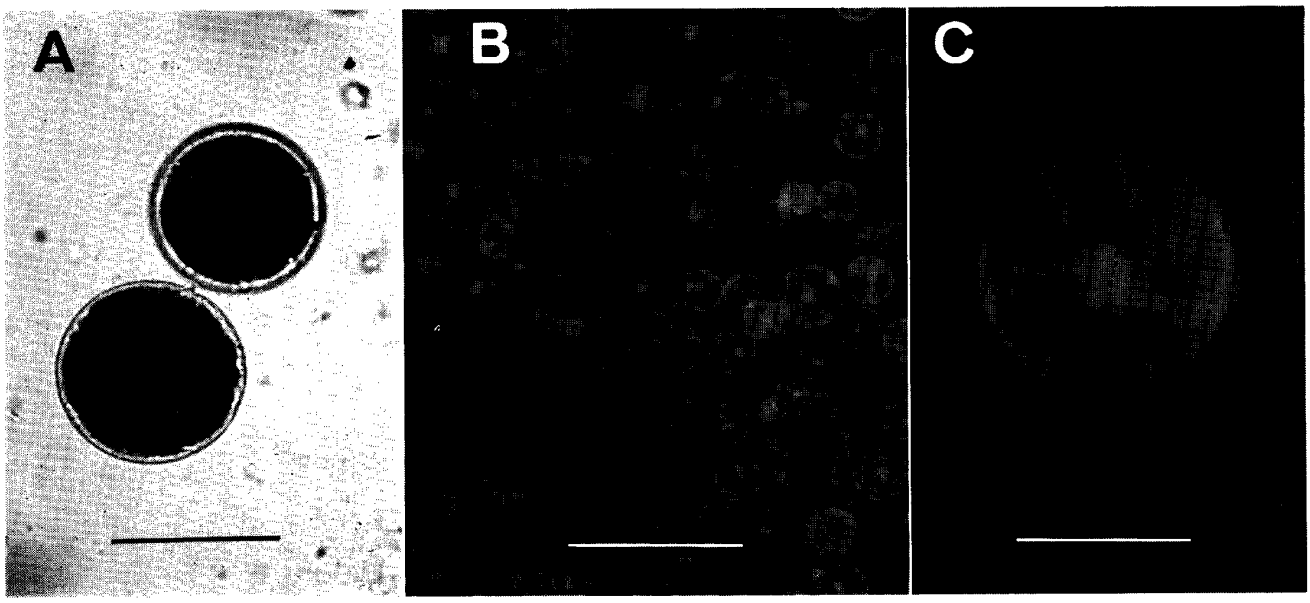

Fig. 1 Light micrographs of corn root protoplasts.

(A) Bright field observation with the treatment of neutral red dye. Bar represents 50 μm. The diameters of typical protoplasts are approximately 50 μm, and neutral red dye is accumulated in the large central vacuole. (B) Reflected fluorescence observation with the treatment of fluorescein diacetate. Bar represents 200 μm. Most of the population of the protoplasts accumulate fluorescein, indicating their intactness. (C) Same as (B) except for magnification. Bar represents 50 μm. The thin layer of cytoplasm accumulates fluorescein higher than large central vacuole, and transvacuolar strands and nucleus (a bright spot at the center of the protoplast) can be seen.

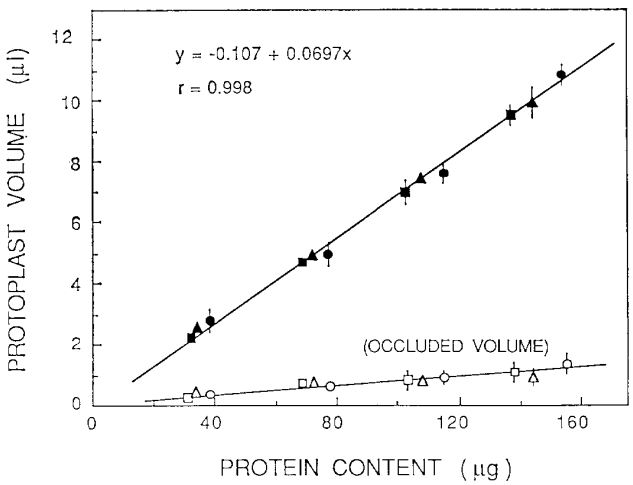

Fig. 2 Relationship between protein content and protoplast volume.

Closed and open symbols represent protoplast volume and occluded volume, respectively. Different shapes of symbols represent different isolation dates. In this and the subsequent figures, data points and error bars represent the mean $\pm$ SD, and points without error bars had the SD smaller than symbols used.

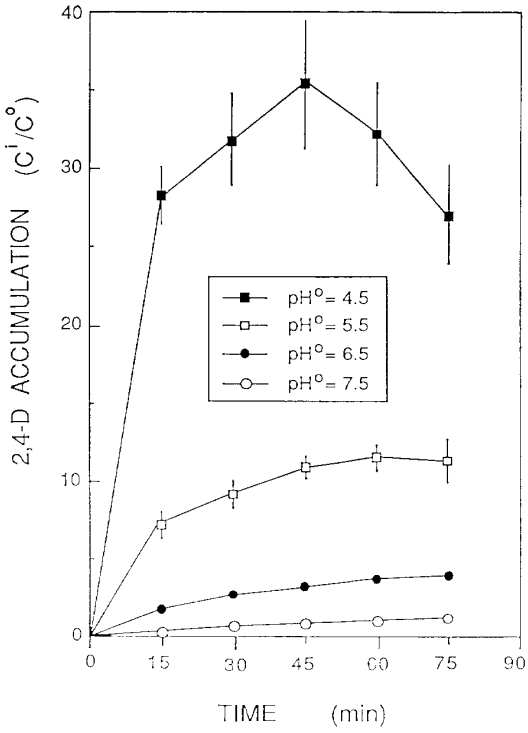

Fig. 3 Effect of pH^o (7.5 to 4.5) on 2,4-D accumulation by corn root protoplasts.

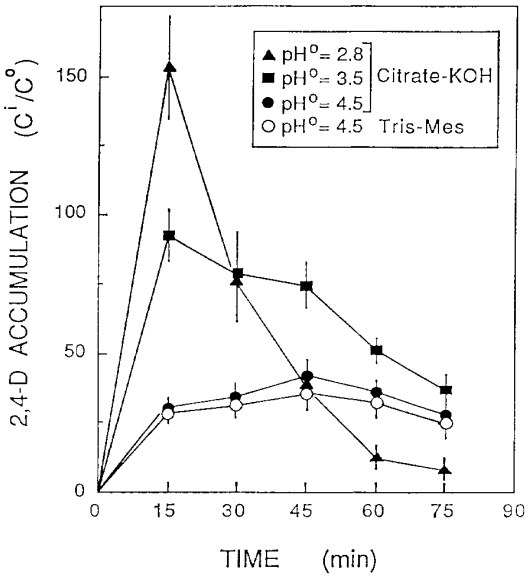

Fig. 4 Effect of low pH° (4.5 to 2.8) on 2,4-D accumulation by corn root protoplasts.

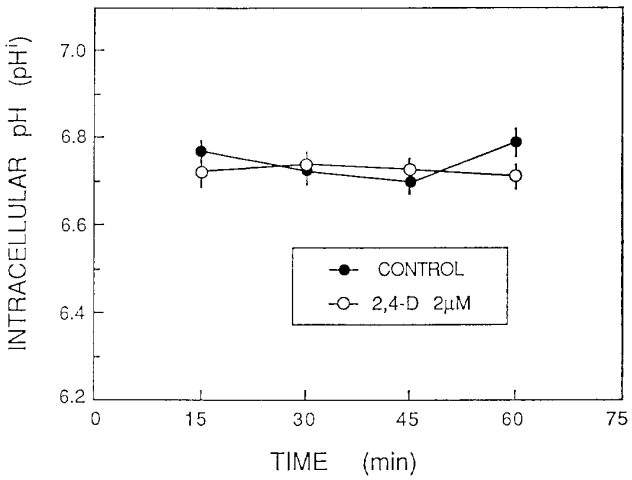

Fig. 5 The pH^i of corn root protoplasts during incubation time at pH° 6.5 with and without 2,4-D treatment.

The incubation conditions were identical to that for the 2,4-D absorption studies. For the 2,4-D treatment, 2 μM of the non-radiolabeled 2,4-D was added prior to the incubation.

protoplasts after 15-min incubation (Fig. 4). The microscopic observation using fluorescein diacetate dye indicated that the release of the once absorbed 2,4-D shown in Fig. 4 was a function of the death of the protoplasts, suggesting that the 2,4-D absorption process is not a purely physical process but at least requires cell intactness.

3. *pH^i of Protoplasts*

The pH^i of the protoplasts was stable at approximately 6.7 to 6.8 during the 60-min incubation at pH° 6.5, and the treatment with 2 μM 2,4-D did not affect the pH^i throughout the incubation time (Fig. 5). The change of the pH^i as influenced by the pH° variation (7.5 to 4.5) was relatively small (6.9 to 6.6), indicating that the cells are able to maintain their pH^i within a small range even under wide pH° variations (Fig. 6). The presence of 2 μM 2,4-D did not affect the pH^i and pH° relationship as shown in Fig. 6.

The pH^i determined by the DMO distribution technique is for "overall intracellular pH" since the technique has no spatial resolution of subcellular compartments.[8-10] However, although one cannot determine cytoplasmic pH (pH^{cyt}) and vacuolar pH (pH^{vac}) from the pH^i, one can estimate the pH^{cyt} and pH^{vac} which give the observed pH^i from the following relationship.[8,9]

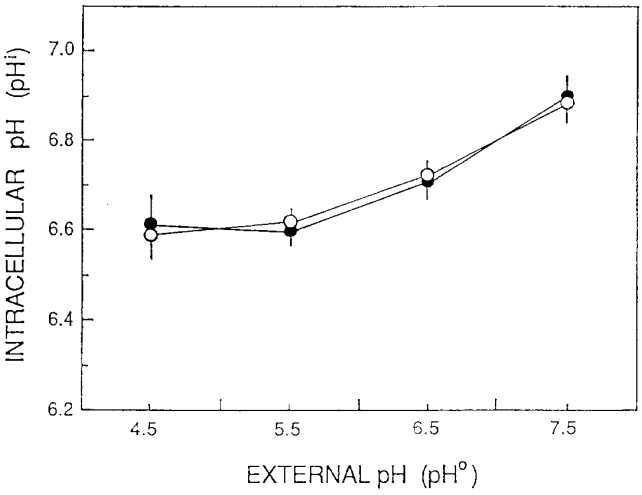

Fig. 6 Effect of pH° on the pH^i of corn root protoplasts.

Closed and open symbols represent the data measured in the absence and the presence, respectively, of 2 μM of nonradioactive 2,4-D. The incubation conditions were identical to that for the 2,4-D absorption studies.

$$10^{pH^i} = V^{cyt}10^{pH^{cyt}} + V^{vac}10^{pH^{vac}} \qquad (2)$$
$$(V^{cyt} + V^{vac} = 1)$$

where V^{cyt} and V^{vac} are the relative volumes of cytoplasm and vacuole respectively. It is important to note that Eq. (2) is not a general principle but a valid equation when pH^i is

measured by the DMO method.[8,9] Assuming that cytoplasm and vacuole occupy 10% and 90% respectively of the cell volume,[4,8] substitution of these volumes and the measured pH^i (*e.g.* 6.70) into Eq. (2) results in

$$10^{6.70}=0.1\times10^{pH^{cyt}}+0.9\times10^{pH^{vac}} \quad (3)$$

By using Eq. (3), if a typical pH^{vac} is assumed to be 5.5,[17] pH^{cyt} is calculated to be 7.67. These values of pH^{cyt} (7.67) and pH^{vac} (5.5) are similar to those of other plant cells reported previously.[8,9,18,19]

DISCUSSION

According to the quantitative expression of the weak acid hypothesis,[4,7] the ratio of cytoplasmic to outside concentration (c^{cyt}/c^o) at the diffusion equilibrium can be described as:

$$\frac{c^{cyt}}{c^o}=\frac{(1+k^{cyt})\left\{\frac{P_m}{P_a}-\frac{\phi k^o}{1-\exp(\phi)}\right\}}{(1+k^o)\left\{\frac{P_m}{P_a}-\frac{\phi k^{cyt}\exp(\phi)}{1-\exp(\phi)}\right\}} \quad (4)$$

$[k^{cyt}=10^{(pH^{cyt}-pKa)},\ k^o=10^{(pH^o-pKa)},\ \phi=zFE/RT]$

where P_m and P_a are the permeability coefficients of the molecule (the undissociated form) and the anion (the dissociated form) of the weak acid respectively, pKa is the dissociation constant of the weak acid, and z, F, E, R and T are the charge number of the ion, the Faraday constant, the plasmalemma potential, the gas constant and the absolute temperature, respectively. Compared with the plasmalemma potential (E), the tonoplast potential is generally very low and has been assumed to be negligible.[4,7] Therefore, adopting the principle of Eq. (4) to the ratio of vacuolar to cytoplasmic concentration (c^{vac}/c^{cyt}) gives

$$\frac{c^{vac}}{c^{cyt}}=\frac{(1+k^{vac})(P_m/P_a+k^{cyt})}{(1+k^{cyt})(P_m/P_a+k^{vac})} \quad (5)$$

$$[k^{vac}=10^{(pH^{vac}-pKa)}]$$

$$(\because \lim_{\phi\to0}\phi/\{1-\exp(\phi)\}=-1)$$

where P_m/P_a is assumed to be common to plasmalemma and tonoplast. Considering the relative volumes of cytoplasm (V^{cyt}) and vacuole (V^{vac}), the accumulation ratio inside to outside the cell (c^i/c^o) can be calculated by

$$c^i/c^o=V^{cyt}(c^{cyt}/c^o)+V^{vac}(c^{cyt}/c^o)(c^{vac}/c^{cyt}) \quad (6)$$

By Eqs. (4), (5) and (6), we calculated the theoretical accumulations of 2,4-D by the protoplasts at pH^o 4.5, 5.5, 6.5 and 7.5 (Table 1) assuming a wide range of P_m/P_a ratios from 1×10^1 to infinity (∞), because the P_m/P_a ratio of 2,4-D has not been determined precisely and has been only indirectly estimated to be 1.1×10^3 or 5.4×10^4.[4,20] The V^{cyt} and V^{vac} were taken to be 0.1 and 0.9 respectively,[4,8] and the pH^{cyt} was calculated by substituting the measured pH^i (Fig. 6) into Eq. (2) assuming $pH^{vac}=5.5$.[17] The plasmalemma potential (E) was taken to be -120 mV.[4,7] The absolute temperature was 300 K (27°C). Other constants used for the calulations were $R=8.3143$ J/mol/K,[21] $F=9.649\times10^4$ J/mol/V,[21] and pKa$=2.96$.[20]

In Table 1, the measured values of the 2,4-D accumulation by the protoplasts at each pH^o condition are shown together to be compared with the theoretical values. The comparison

Table 1 Theoretical 2,4-D accumulations estimated by weak acid hypothesis in comparison with measured values.

P_m/P_a ratio[a] assumed	Theoretical 2,4-D accumulations (c^i/c^o)[c]			
	pH^o conditions			
	4.5	5.5	6.5	7.5
	pH^i:[b]			
	6.61	6.60	6.71	6.90
1×10^1	0.07	0.02	0.01	0.01
1×10^2	0.49	0.06	0.01	0.01
1×10^3	2.01	0.21	0.02	0.01
1×10^4	7.82	0.80	0.08	0.01
1×10^5	43.8	4.46	0.48	0.05
1×10^6	105.3	10.6	1.31	0.18
1×10^7	123.4	12.4	1.59	0.24
1×10^8	124.9	12.6	1.62	0.25
∞	125.1	12.6	1.62	0.25
Measured values (c^i/c^o)[d]	30–40	10–12	4.0	1.2

a) P_m and P_a represent the permeability coefficients of undissociated 2,4-D molecule and dissociated 2,4-D anion, respectively.
b) The pH^i data were taken from Fig. 6.
c) The theoretical 2,4-D accumulations (c^i/c^o) were calculated by Eqs. (4), (5) and (6).
d) The measured values (c^i/c^o) were taken from Fig. 3.

of the values presents two important indications.

First, in terms of a qualitative comparison, the observed effect of pH° on 2,4-D accumulation is similar to the theoretically expected accumulation from the weak acid hypothesis, where a lower pH° gives a higher c^i/c^o. When the P_m/P_a ratio is to be 1×10^5 or larger, some of the theoretical values are similar to the observed ones at pH° 4.5 and 5.5. This suggests that the mechanism of the weak acid hypothesis may be partly involved in 2,4-D absorption, provided that the P_m/P_a ratio of 2,4-D is at least 1×10^5.

Second, in terms of a strictly quantitative comparison, the theoretically expected values did not completely agree with the observed data. In Table 1, the c^i/c^o ratio theoretically becomes maximal when P_m/P_a is assumed to be infinite (∞) at either pH° condition. Thus, the 2,4-D accumulation (c^i/c^o) cannot exceed 1.62 and 0.25 at pH° 6.5 and 7.5 respectively, as long as only the weak acid hypothesis operates 2,4-D absorption. The observed 2,4-D accumulations at pH° 6.5 and 7.5, however, were 4.0 and 1.2 respectively, which are significantly greater than their respective theoretical maxima. It is clear that the weak acid hypothesis alone does not explain the observed absorption of 2,4-D by protoplasts, and some additional mechanism(s) must be involved at least at pH° 6.5 and 7.5. This mechanism should be an active transport process because the observed absorption of 2,4-D exceeded the passive diffusion equilibrium expected from the weak acid hypothesis. The active component might be a carrier-mediated transport by the proposed auxin carrier.[22] In the following paper, we will report on further study on the active component.

In conclusion, the present results suggested that the mechanism of the weak acid hypothesis may be partly involved but it alone cannot explain 2,4-D absorption by protoplasts and that an active process must be involved at least at pH° 6.5 and 7.5.

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要　　約

トウモロコシ根プロトプラストの 2,4-D 吸収機構における弱酸仮説の定量的評価*

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トウモロコシ根皮層より酵素的に単離されたプロトプラストの 2,4-D 吸収を細胞内外の pH と関連して検討した．2,4-D 吸収は細胞外 pH(pH^o) の低下とともに増大した．プロトプラスト外部に対する内部の 2,4-D 濃度比 (c^i/c^o) は pH^o 7.5，6.5，5.5，4.5 において 75 分以内の培養でおのおの約 1.2，4.0，10～12，30～40 に達した．pH^o 3.5，2.8 においては 15 分以内に c^i/c^o 比がおのおの約 90，150 に達したが，吸収された 2,4-D はその後プロトプラストの死により速やかに放出された．5，5-ジメチルオキサゾリジン-2，4-ジオン分配法による細胞内 pH(pH^i) 測定の結果，pH^o 変化 (7.5～4.5) による pH^i 変化は比較的小さかった(6.9～6.6)．これらの pH^i 測定値を用いて各 pH^o における理論的 c^i/c^o 比を弱酸仮説に基づいて推定した．pH^o 変化に対する理論値の傾向は大まかには実測値の傾向を反映したが，pH^o 6.5 および 7.5 における c^i/c^o 比実測値はおのおのの理論的最大値を顕著に上回っていた．これらの結果は，プロトプラストによる 2,4-D 吸収が単に弱酸仮説のみでは説明されえず，少なくとも pH^o 6.5 および 7.5 においては能動的な輸送の関与していることを示唆する．

* トウモロコシ根プロトプラストの 2,4-D 吸収に及ぼす細胞内 pH の影響（第1報）