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# The Uptake of (+)-*S*- and (−)-*R*-Abscisic Acid by Suspension Culture Cells of Hopbush (*Dodonaea viscosa*)[1]

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## ABSTRACT

**The uptake of (+)-*S*- and (−)-*R*-abscisic acid (ABA) by suspension culture cells of hopbush (*Dodonaea viscosa* L. Jacqu.) was followed over a range of temperatures, pH values, and time intervals. The natural (+)-*S*-ABA was taken up about five times faster than the unnatural (−)-*R*-ABA. Each 10°C rise in temperature from 1 to 31°C increased the rate of uptake ($Q_{10}$) of (+)-*S*-ABA about 2.2-fold, whereas that of the (−)-*R* increased with a $Q_{10}$ of 1.4. (+)-ABA was taken into the cells by a saturable carrier, but (−)-ABA and both enantiomers of 2-*trans*-ABA were not; they appeared to enter by passive diffusion. The uptake of (+)-ABA was linear over the first 8 hours but concentrations within the cells decreased after 2 hours to remain constant after 4 hours as rapid metabolism was induced. Electron microscopy of thin sections of the cells, combined with a stereological analysis of their shape, showed that the vacuoles comprised 80% of the cell volume and the cytoplasm plus nucleus comprised 20%. There were no photosynthetically active plastids in the cells. Concentrations of the endogenous ABA in the cytoplasm (pH 7.32) and vacuoles (pH 5.88) were calculated by applying the Henderson-Hasselbalch equation (ABA $pK_a$ 4.7) so that, provided no active metabolic redistribution occurred, the concentration in the cytoplasm was 7.9 micromolar and that in the vacuole was 0.3 micromolar. *In vivo* pH was measured by $^{31}P$ nuclear magnetic resonance spectroscopy.**

Rates of uptake and metabolism of ABA have been measured for a variety of cells, but the bulk of many tissues used introduces complications of distribution. We have measured several of the factors affecting uptake of ABA by a cell suspension culture from hopbush (*Dodonaea viscosa* L. Jacqu.). The disadvantages of using such cells are that they are undifferentiated and have no exact counterpart in the intact plant. They most resemble wound callus or cambial cells. However, the single cell type, reproducible physiology, and the ease with which all cells can be exposed uniformly to the bathing solution allow measurements of uptake rates per unit area to be made with an accuracy that cannot be matched in experiments with pieces of tissue or organs.

An important feature of the investigation was that we used labeled (+)-*S*-ABA, the naturally occurring enantiomer, and (−)-*R*-ABA, the unnatural enantiomer, that had been resolved by HPLC. Although this allows us to define uptake of the enantiomers separately and precisely, it also means that the results reported here are not strictly comparable with almost all of the previous work in which racemic ABA [(±)-*R,S*-ABA] was used. The magnitude of the differences between the rates of uptake of the (+) and (−) enantiomers and their metabolism (32) underlines the necessity of using (+)-*S*-ABA for experiments such as those described here.

[1] This work was supported by the Australian Research Committee.

## MATERIALS AND METHODS

### Initiation and Maintenance of Cell Suspension Cultures

Seeds of hopbush (*Dodonaea viscosa* L. Jacqu.) were sterilized by immersion for 2 min in 70% ethanol (v/v in water) followed by 15 min in sodium hypochlorite (1% available chlorine) and three washes in sterile water. The seeds were germinated on UNE#B medium (7) and tissue from seedlings innoculated onto Schenk and Hildebrant medium with agar (1%), napthalene-1-acetic acid ($\alpha$) (10 $\mu$M), and kinetin (10 $\mu$M). Soft callus was obtained after two subcultures (each 28 d at 25°C under continuous fluorescent light) and 5 g was inoculated into liquid medium (50 mL, pH 5.8) in 250-mL conical flasks. Suspension cultures formed after 3 d of shaking at 110 cycles/min. The cultures (10 mL) were inoculated into fresh medium (50 mL) every 12 to 14 d.

### Chemicals

HPLC-grade organic solvents were purchased from Mallinckrodt Australia Pty Ltd (S. Clayton, Victoria). Laboratory-grade ethanol was refluxed over solid NaOH and then distilled. Water was purified by a Milli-Q reagent water system from Millipore-Waters (Millford, MA). All HPLC solvents were filtered and degassed before use. When water-free solvents were required, they were allowed to stand over a desiccated molecular sieve (Union Carbide, Sydney, NSW).

(±)-*R,S*-[2-$^{14}$C]ABA (35.2 $\mu$Ci/mmol) and (±)-*R,S*-[G-$^{3}$H]-ABA[2] (50 mCi/$\mu$mol) were purchased from the Radiochemical Centre, Amersham (Bucks, UK), and unlabeled (±)-*R,S*-ABA from Sigma, as were *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide, naphthalene-1-acetic acid, kinetin, and Mes buffer. Triethyl phosphate was from Aldrich Chemical Co. (Milwaukee, WI) and pentaflourobenzylbromide was obtained from Fluka, A.G. (Buchs, Switzerland).

[2] Abbreviations: [G-$^{3}$H]ABA, ABA labeled generally, but not uniformly, with tritium; $Q_{10}$, change in rate of uptake caused by a 10°C rise in temperature; ABAH, unionized conjugate base.

### EM

Cells were fixed in glutaraldehyde (2.5%, w/v) in phosphate buffer (50 mM, pH 6.8, 4 h, 25°C) then washed in buffer three times and held in it for 16 h. Subsamples were fixed in osmium tetroxide (0.2%) in buffer (2 h) and then washed again. The cells were dehydrated in ethanol:water (1:10, v/v) and in 10% increments every 10 min to 100% ethanol for 1 h. The sample was transferred to fresh 100% ethanol for another 1 h and then infiltrated in Spurr's resin (30). The sample was embedded in fresh resin, which was polymerized at 70°C over 24 h. The preparations were sectioned (0.6 μm) and stained in lead acetate followed by uranium acetate (5).

### Preparation of ABA for Experiments

(±)-*R,S*-[2-$^{14}$C]ABA, (±)-*R,S*-[G-$^{3}$H]ABA, and unlabeled (±)-*R,S*-ABA were methylated with ethereal diazomethane and resolved by HPLC in a column with a chiral stationary phase (26). The (+)-*S*- and (−)-*R*-ABA fractions were completely separated and either isomerized under bright incandescent lights and then separated into methyl ABA and methyl 2-*trans*-ABA by HPLC or hydrolyzed directly in ethanol:60% aqueous KOH (2:1, v/v) at 37°C for 2 h. The mixture was acidified to pH 3.0 with 0.1 N $H_2SO_4$ and extracted twice with diethyl ether. The combined ether extracts were evaporated and the ABA purified by reversed-phase HPLC.

### Uptake Experiments

The cells were pelleted at 310*g* for 60 s. Packed cell volumes of 20 to 30 mL were obtained from 50 mL of culture medium. The cells were resuspended to 150% of the original volume in fresh medium. ABA in 100 μL of ethanol was added to 100 mL of cell suspensions, in 250-mL Ehrlenmeyer flasks, to 3 μM and 0.22 μCi $^{14}$C.

Incubations were carried out in dull light in a reciprocating shaker bath (150 oscillations/min, 25°C, pH 5.8) and aliquots (8 mL) were removed at intervals and the cells were separated by suction filtration through celite- coated filter paper discs (50 mm diameter). The cells were washed with fresh medium (5 mL) then weighed and assayed for [2-$^{14}$C]ABA.

### Extraction of ABA and Metabolites

Cell pellets were ground with celite and extracted overnight with approximately 10 volumes of acetone:acetic acid (99:1, v/v) containing 2,6-di-*tert*-butyl-4-methylphenol (20 mg/L). The mixture was filtered, dried, dissolved in ethanol (0.1 mL), diluted with 0.2% aqueous acetic acid (10 mL), and passed through a C-18 Sep-Pak cartridge (Millipore-Waters). The cartridge was washed with 0.2% aqueous acetic acid (10 mL) and then the ABA fraction was eluted in ethanol:0.2% aqueous acetic acid (10 mL, 7:13, v/v).

Suspension culture filtrates were acidified to pH 3 with acetic acid and then passed through Sep-Pak cartridges, washed, and eluted with ethanol-0.2% aqueous acetic acid as before.

### GC

Methylated samples were chromatographed in a Hewlett-Packard gas chromatograph (Waldbron, Germany) interfaced to a Hewlett-Packard 9000 series 300 workstation. The column used was a 15 m × 0.53 mm (i.d.) SE-54 with a lining film thickness of 1.2 μm (Alltech Associates, Deerfield, IL) at 80°C initially for 1 min then programmed to rise 50°C/min to 180°C. This temperature was maintained for 1 min, then was raised at 10°C/min to 200°C for 5 min. The electron capture detector was held at 240°C.

### HPLC

Resolution of racemic methyl ABA was carried out in a 250 × 4.6 mm (i.d.) Chiralcel O.D. coated silica gel column (Daicel, Los Angeles, CA). Aliquots (0.5 mg) of methyl ABA were resolved using propan-2-ol:hexane (1:9, v/v) at 1 mL/min. Retention times were (+)-*S*-methyl ABA, 8 min; (−)-*R*-methyl ABA, 13 min. The free acids were liberated by saponification in 3 N KOH in ethanol:water (3:1, v/v) and repurified by normal-phase HPLC.

The metabolites of ABA were separated by reversed-phase HPLC conducted in an analytical C18 column (250 × 4.6 mm i.d.) (Phenomenex, Rancho Palos Verdes, CA) with ethanol:0.2% aqueous acetic acid (15:85, v/v) for 15 min then a linear gradient to ethanol:0.2% aqueous acetic acid (35:65, v/v) over 15 min, all at 1 mL/min. Fractions were collected over 2-min intervals.

Normal-phase HPLC was carried out on an A.S.I. (Santa Clara, CA) semipreparative column (250 × 8 mm i.d.) with propan-2-ol:hexane (3:97, v/v) at 3 mL/min.

The loading areas of silica gel thin-layer plates were deactivated with methanol before the samples were applied. The solvent (toluene:ethyl acetate:acetic acid [50:30:4, v/v]) also contained 2,6-di-*tert*-butyl-4-methylphenol (20 mg/L). Multiple development of the plates was used to narrow the zones.

### Liquid Scintillation Counting

The zones of the TLC plates were eluted and counted in toluene:triton (2:1, v/v) containing 0.5% 2,5-diphenyloxazole (United Technologies). The filtered cells, together with the celite-impregnated discs, were placed in the scintillant solution, shaken vigorously, and allowed to extract overnight.

### $^{31}$P NMR Spectroscopy

The pH of the cytosol and vacuoles of living cells that had just reached stationary phase (12–14 d after inoculation) was determined by $^{31}$P NMR spectroscopy of the Pi in these compartments.

Cells (approximately 10 mL pelleted volume) were resuspended in Mes buffer (10 mM, 20 mL) made up in $^{2}H_2O$ (20 atom %). The pH of the medium, as measured with a conventional pH meter, was adjusted to 6.5. The $^{31}$P spectra were recorded in a Bruker CXP-300 FT NMR Spectrometer operating at 121 MHz, spectral width 10,000 Hz, 8 K data points, 60°C pulse, with a recycle time of 2 s and heteronuclear broadband decoupling. Spectra were zero filled to 16 K, exponentially multiplied, with line broadening to 10 Hz

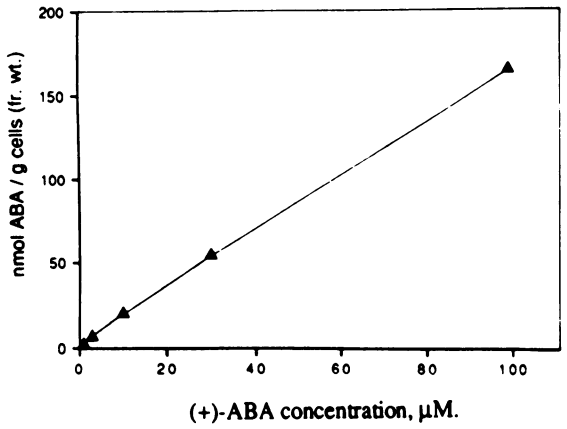


**Figure 1.** Uptake of (+)-*S*-ABA by *D. viscosa* suspension culture cells in relation to the concentration in the medium. These data are taken from the experiment plotted as Figure 7, where it can be seen that proportionally less (+)-*S*-[2-$^{14}$C]ABA is taken up by the carrier as the concentration in the medium increases. The ABA present in the cells comprises the labeled *S*-ABA (5.5 μCi/μmol, 100 mL, 0.733 μM with increasing amounts of unlabeled S-ABA added to give the concentrations shown). The samples were taken after 2 h incubation at 22°C, 30 min. The cells were separated on celite-covered filter paper, washed with blank medium, and $^{14}$C was measured by scintillation counting so free (+)-*S*-ABA and metabolites were included. Data recalculated from Figure 7.

before Fourier transformation. The pH of the cellular compartments was obtained by interpolation from a calibration curve made by plotting the observed chemical shifts of the phosphate signals in cell sap adjusted to a range of pH values. A cell pellet was homogenized and the solids removed by centifugation. The pH of the cell sap was then adjusted sequentially at 0.5 pH unit intervals from pH 3 to 8 and chemical shifts were obtained for each. The chemical shifts were referenced internally to the triethyl phosphate resonance at -0.0727 ppm relative to 85% phosphoric acid at 0.00 ppm. The triethyl phosphate signal was found to be unaffected by variations in pH, $^2H_2O$ was added to 20%, pH was adjusted with N HCl and N NaOH to give a range of pH values, and the chemical shifts of the phosphate signal of each was measured by $^{31}$P NMR. This was done to compensate as realistically as possible for any chemical shifts induced by paramagnetic ions in the cell sap and the departure from the true pH caused by $^2H_2O$. The measurements of the living cells can be determined with an accuracy of about 0.05 pH unit.

## RESULTS AND DISCUSSION

### Environmental Factors

The amount of ABA sequestered into an "apparent free space" during the first few seconds of the cells' exposure to a 3-μM solution of labeled ABA was negligibly small in relation to the amounts taken up during the course of the experiment. Probably because of the small size of the cells and their thin cell walls, almost all of the material that penetrated into the cell walls passed out again during the brief washing procedure. The amounts were similar for (+)-*S*-ABA, (−)-*R*-ABA, and (±)-*R,S*-2-*trans*-ABA.

The surface areas of the plasmalemma and of the tonoplast and the volumes of the cytoplasm and vacuoles were calculated from a stereological analysis of electron micrographs of typical cells. Although the analysis was cursory, and the parameters calculated may be inexact, any error is constant over all measurements.

The quantitative measurements of uptake, leakage, and metabolism of (+)-*S*-ABA show that many of the data in earlier publications may be attributed to the operation of several complicating factors, namely the use of racemic labeled material and the rapid metabolism of the labeled ABA absorbed, the rapid induction of metabolism of ABA by ABA, and the faster leakage of (−)-ABA compared with (+)-ABA.

### Effects of Concentration on Uptake

The uptake of (+)-ABA increased almost linearly as the concentration increased (Fig. 1), although the proportion taken up by the saturable carrier decreased. Because the experiment lasted 2 h, relatively little ABA would have been metabolized (Fig. 2).

Uptake of (+)-[2-$^{14}$C]ABA was virtually linear over the first 5 h (Fig. 2), and during this time about half of the labeled (+)-ABA was absorbed. After 1.5 h, the concentration of labeled ABA within the cells started to fall from 2 nmol/g (i.e. about five times the normal endogenous content) and by 7 h it had reached the normal endogenous level and thereafter remained constant for 30 h. This occurred in spite of continuing uptake. It is concluded that the high concentrations of ABA induced the activation and/or synthesis of catabolic enzymes (Fig. 3).

The pattern of uptake of (−)-[2-$^{14}$C]ABA was similar al-

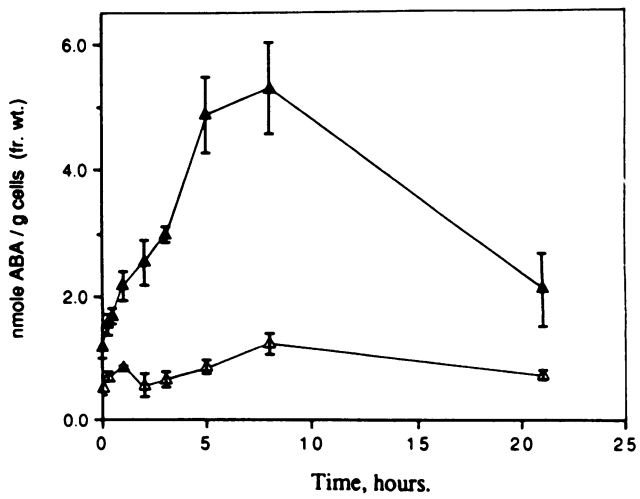


**Figure 2.** Uptake of (+)-*S*-[2-$^{14}$C]ABA and (−)-*R*-[2-$^{14}$C]ABA. Both natural, (+)-ABA and unnatural, (−)-ABA were fed, separately, to cell suspension cultures of *D. viscosa* (3 μM, 0.733 μCi/μmol, 100 mL, 22°C). Samples were taken at intervals and cells were removed from the medium by suction filtration. Uptake of ABA was assayed by liquid scintillation counting. Data are the means of three experiments; bars = SD. (+)-*S*-ABA, ▲; (−)-*R*-ABA, △.

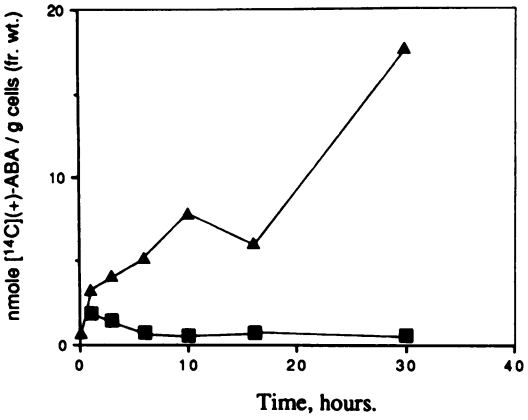


**Figure 3.** Uptake and metabolism of (+)-*S*-[2-$^{14}$C]ABA (3 μM, 0.733 μCi/μmol, 100 mL, 22°C). The 10-mL sub-samples were harvested and the cells extracted. An aliquot of the extract was taken and the total $^{14}$C measured. The ABA was isolated using Sep-Paks and HPLC, and the $^{14}$C content determined. Total (+)-*S*-[2-$^{14}$C]ABA in the cells, ▲; (+)-[$^{14}$C]ABA remaining as ABA in the cells, ■.

though the rates of uptake and metabolism were considerably smaller (Fig. 2). After an initial rise to 1 nmol/g from 1.5 to 2.5 h, the internal concentration fell to about 0.5 nmol/g by 10 h and remained constant until 30 h (Fig. 4). As with (+)-ABA, the majority of the labeled material absorbed was present as metabolites after about 2 h.

### Effects of pH

A progressive decrease in the pH of the medium from 7.5 increased the uptake of (+)-ABA to reach a maximum at pH 4.5. Uptake of (−)-ABA also increased with increasing acidity, but less entered the cells at any pH and the rate of penetration increased progressively to pH 3.5 (Fig. 5). This is in accord with earlier work for weak organic acids and for ABA (1, 15).

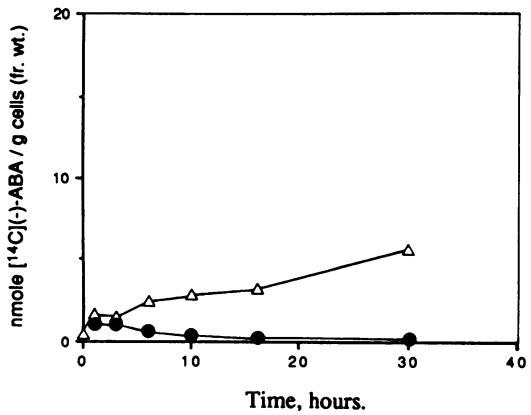


**Figure 4.** Uptake and metabolism of (−)-*R*-[2-$^{14}$C]ABA (3 μM, 0.733 μCi/μmol, 100 mL, 22°C). The samples were harvested and treated as for (+)-ABA, described in Figure 3. Total (−)-*R*-[2-$^{14}$C]ABA in the cells, △; (−)-*R*-[2-$^{14}$C]ABA remaining as ABA in the cells, ●. Approximately one-third as much (−)-ABA as (+)-ABA was absorbed (a similar result can be seen in Fig. 2).

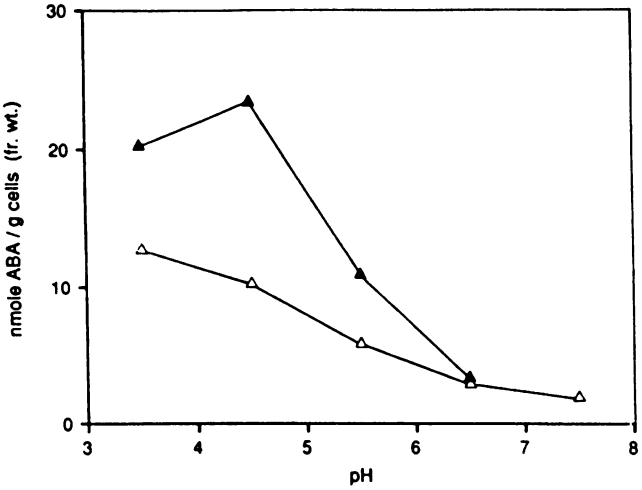


**Figure 5.** Effect of pH on uptake of (+)-*S*- and (−)-*R*-ABA. Suspension cultures were adjusted to various pH values by the addition of citric acid (0.1 mM) and Na phosphate buffers (0.2 mM) prior to the addition of the [2-$^{14}$C]ABA. Samples were taken after 3 h of incubation with 3 μM ABA (1.15 μCi/μmol (+)-*S*-ABA; 0.86 μCi/μmol (−)-*R*-ABA, 100 mL) and uptake of the hormone was determined by liquid scintillation counting. (+)-ABA ▲; (−)-ABA △.

These authors agree that this indicates that the ABA anion cannot enter the cells by penetration through the plasmalemma, and Astle and Rubery (1) suggested that the uptake by the carrier increases as the pH is lowered. The data in Figure 5 support this. In all subsequent experiments, the medium was adjusted to pH 5.8.

### Effects of Temperature

The $Q_{10}$ of 2.2 for uptake of (+)-ABA and the $Q_{10}$ of 1.4 for the (−) enantiomer suggests that an energy-requiring process is involved in the uptake of the former (Fig. 6). Daie and Wyse (6) also found that temperatures close to 0°C almost abolished the uptake of (±)-ABA by sugar beet root discs. All subsequent experiments were carried out at 25°C.

A marked increase in the amount of 2-*trans*-ABA absorbed was observed at the highest concentration of unlabeled (+)-ABA present (90 μM), in comparison with the amounts taken up at 5 to 10 μM (Fig. 7). In contrast with this, the amount of labeled (+)-[$^{14}$C]2-*trans*-ABA absorbed was unaffected by the presence of 90 μM unlabeled (+)-2-*trans*-ABA (data not shown). A possible interpretation of these phenomena is that (+)-ABA at 90 μM causes the membranes to become more permeable than they are at 3 μM. No mechanism for this can be suggested.

### Intracellular pH

Extraction and measurement of the free ABA from centrifuged cell pellets gave a mean value of 96 ng/g (364 nM, SD = 40, $n$ = 6), but if this is expressed on the cell volume within the plasmalemma, determined by EM, then the overall concentration is 1.82 μM.

The pH of the cytosol of *D. viscosa* cells was measured by

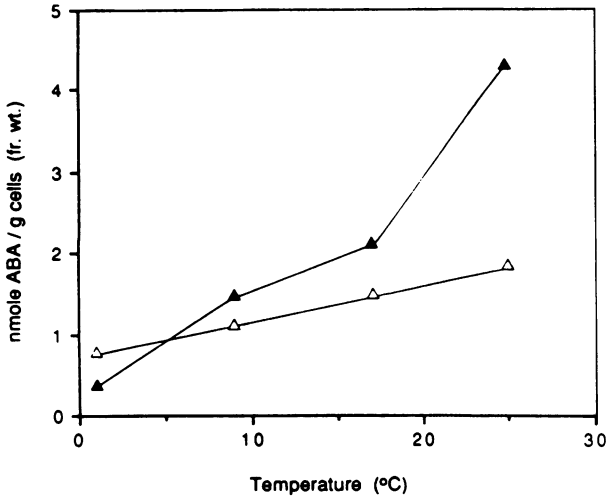


**Figure 6.** Effect of temperature on uptake of (+)-*S*- and (−)-*R*-ABA. Cell suspensions were incubated with (+)-*S*- or (−)-*R*-[2-$^{14}$C]ABA (0.22 μCi/μmol, 3 μM, 100 mL) at varying temperatures. Uptake of (+)-ABA showed a greater dependence on temperature ($Q_{10}$ of 2.2) than uptake of (−)-*R*-ABA, which had a $Q_{10}$ of 1.4. Symbols as for Figure 2.

$^{31}$P NMR as 7.2 and that of the vacuole as 5.88. If it is assumed that the distribution of ABA between these two compartments is at an equilibrium dictated by Henderson-Hasselbalch kinetics (13, 17), then the concentration in the cytosol is 7.9 μM and that in the vacuole is 0.3 μM. If labeled ABA in the medium has free access through the cell wall to the plasmalemma and the apoplastic pH is close to that of the medium (5.8), then it can be concluded that the uptake of (−)-ABA and both of the mirror-image antipodes of the 2-*trans* isomer can occur by passive uptake, not by a carrier, and not against a concentration gradient of the conjugate base, ABAH. The concentration of un-ionized ABAH within the cytosol is extremely low because, from the Henderson-Hasselbalch equation, a weak acid with a $pK_a$ of 4.7 would be almost totally ionized at pH 7.3. Consequently, un-ionized ABAH would enter the cell by passive diffusion through the plasmalemma down a concentration gradient. On the other hand, if, as has been suggested by Rubery and Astle (28), the uptake carrier imports the $ABA^-$ anion, then the $ABA^-$ in the medium (2.7 μM, i.e. approximately 90% ionized) has to enter the cells against a gradient of $ABA^-$ (7.9 μM) across the plasmalemma.

Some (+)-ABA, of course, would also enter passively as ABAH, presumably at the same rate as the 2-*trans*-ABA enantiomers and (−)-ABA, since they have similar $pK_a$ values.

## Saturable Uptake Carrier

The action of a saturable uptake carrier for (+)-ABA in the cells was demonstrated by the sharp decrease in rate of uptake of (+)-[$^{14}$C]ABA (2.56 nM) caused by unlabeled material (Fig. 7). (−)-ABA may be capable of acting as a poor substrate for the uptake carrier, because in several experiments there was a slight reduction in the amount of labeled (−)-ABA absorbed as the concentration of unlabeled (+)-ABA increased. However, the uptake of (−)-ABA by the carrier was negligible in comparison with the effect of (+)-ABA on the uptake of (+)-[2-$^{14}$C]ABA. The (+)-2-*trans*-ABA was taken up at the same rate as the (−)-2-*trans*-ABA, and the rate was slower than for (+)-ABA. It was linear for 8 h (Fig. 8).

In an earlier paper, Milborrow and Rubery (24) found that root tip segments of runner bean (*Phaseolus multiflorus*) possessed saturable carrier activity for (+)-ABA, but at least half of the radioactive material entering the tissues did so by a noncarrier path. The presence of the (−)-ABA in the labeled racemate used would account for part of this noncarrier uptake. About 50% of the labeled (+)-ABA is taken up by the carrier in *D. viscosa* cells at pH 5.8 (Fig. 7).

Astle and Rubery (1) calculated the $K_m$ of the carrier as 2.6 μM for (±)-ABA, whereas Milborrow and Rubery obtained a figure of 3.6 μM for (±)-ABA and assumed that the value for the (+)-ABA would be half of this *i.e.* 1.8 μM. This correction may not be valid because the presence of the (−)-ABA is already taken into account in the Dixon plot. The $K_m$ of the *D. viscosa* carrier for (+)-ABA was 4.5 μM, which is close to the 4.9 μM value we calculate for (+)- and (−)-ABA for *Amaranthus tricolor* cells. After this paper was submitted, Bianco-Colomas *et al.* (4) reported that cells of a suspension culture of *Amaranthus tricolor* have a saturable uptake carrier that accepts (+)- and (−)-ABA equally. We cannot establish at present whether this is a species difference or the chance selection of a cell type that expresses just one of a variety of carriers that occur in different tissues. The difference in stereoselectivity between the carriers for ABA is reminiscent of the uptake of IAA by *Parthenocissus tricuspidata* crown gall culture cells (27), where one carrier accepted IAA and its synthetic analog 2,4-D, and another was specific for IAA.

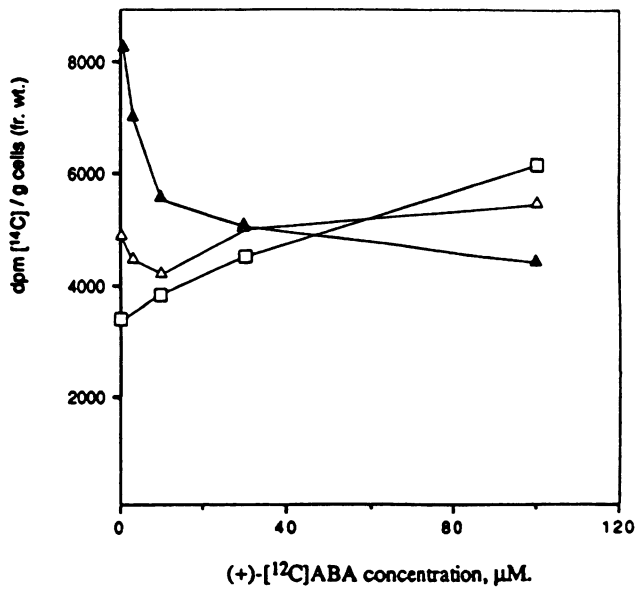


**Figure 7.** Effect of different concentrations of unlabeled (+)-*S*-ABA on uptake of $^{14}$C labeled (+)-*S*-, (−)-*R*-, and (±)-*R,S*-2-*trans*-ABA. Each solution comprised 5.5 μCi/μmol, 340 pM, 100 mL total, 12 mL samples. Uptake of (+)-ABA was saturable. (+)-*S*-ABA, ▲; (−)-*R*-ABA, △; (±)-*R,S*-2-*trans*-ABA, □.

The nonsaturable uptake of (−)-ABA, and of both enantiomers of 2-*trans*-ABA, is not driven directly by metabolic energy. The $Q_{10}$ of 2.2 for (+)-*S*-ABA and 1.4 for (−)-*R*-ABA requires active uptake for the former (Fig. 6). Similarly, the saturability of the import of the (+) enantiomer while the (−)-ABA and both (+)- and (−)- forms of the 2-*trans* isomer show no marked decrease in uptake at high concentration suggests that their import is by passive, diffusive penetration of the membrane in un-ionized form.

The progressively faster penetration of (−)-ABA as the pH is lowered (Fig. 5) is also in accord with passive penetration. The rate of uptake of (+)-ABA increases until it reaches a maximum at about pH 4.5 and then falls.

A saturable uptake carrier has been sought in several mature tissues of bean plants but, other than in root tips, none has been found. Hartung and Dierich (12) failed to find evidence for an uptake carrier even in root tips, but they used racemic ABA and, if the uptake carrier were only weakly active, its effect could have been swamped by passive, diffusive uptake of most of the (+)-ABA and an equal, passive uptake of the (−)-ABA. Faster uptake of (+)-ABA than (−)-ABA has been observed in other tissues (29), which may indicate the presence of uptake carrier activity. HPLC methods to resolve racemic ABA (26, 32) has made labeled (+)-ABA available, so investigations of the saturability of uptake can now be readily undertaken and may show that carrier activity is more widespread than was hitherto believed.

Baier and Hartung (2) reported results that they claimed showed the presence of faster uptake through the tonoplast of guard cells of *Vicia faba* and *Valerianella locusta* than could be accounted for by passive diffusion. Part of the analysis depended on the application of Collander's linear equation, and his results have been recalculated (25) to show a significant quadratic term in the partition coefficient, so there may not be exact equivalence between the calculated measurements and the true values. Furthermore, they measured total radioactivity, not labeled ABA, and if the cells metabolized ABA at a similar rate to *D. viscosa* cultures then the majority of the labeled material absorbed would have been converted into metabolites over the 16 h of pretreatment.

### Stimulation of Metabolism

One of the difficulties that beset experiments with ABA is that the hormone supplied may alter the permeability of the cellular membranes, even to itself. Glinka and Reinhold (8) reported changes in the permability of carrot discs to $^3H_2O$ induced by (±)-*RS*-ABA, and Stillwell and Hester (31) found that (±)-*RS*-ABA increased the permeability of lecethin vesicles. Altered permeability of cell membranes by the presence of the high concentrations of (+)-ABA may account for the differences in uptake of 2-*trans*-ABA (Fig. 7). The faster metabolism of ABA after 1.5 h of exposure to ABA in the medium is another manifestation of altered metabolism caused by ABA.

### No Leakage of Metabolites

The *D. viscosa* cells behaved like tomato petiolar segments supplied with labeled ABA from agar blocks (20) in that the metabolites formed from the ABA stayed within the tissues and only ABA leaked out into the medium or into receiver agar blocks. No labeled metabolites of (+)-[2-$^{14}$C]ABA could be detected in the cell suspension culture medium, and similar results have been found by Dr. S.R. Abrams using duckweed (*Lemna gibba*) (unpublished).

There is as yet no report of the presence of an equivalent of the P-glycoproteins in plant cells, other than yeast, to facilitate the leakage of xenobiotics. A sample of protoplast membranes prepared from a culture of *D. viscosa* cells was tested by Dr. R.A. Davey for its ability to react with a monoclonal antibody (C219) to human P-glycoprotein using the methods of Haber et al. (9). No reaction whatsoever was detected, so this highly conserved protein is not present in these cells. The result does not exclude the existance of proteins having a similar function because other, related proteins have failed to react with C219 antibody.

### Metabolism

It has been tacitly assumed that the receptors for the plant hormones that affect "slow" responses involving protein synthesis (21) are within the cells, analogous to the estrogen receptors within the nuclei of uterine cells in animals (18). Hooley and colleagues (16) have produced compelling evidence that the receptor for $GA_1$ is in the outer surface of the barley aleurone protoplast. Thus the effect, at least in this cell type, is analogous to that of the peptide hormones, such as glucagon in animals, which do not enter cells to exert their action. Hartung (11) has convincingly demonstrated that the receptor for ABA that causes closure of stomata is located in the outer face of the plasmalemma of guard cells, but the

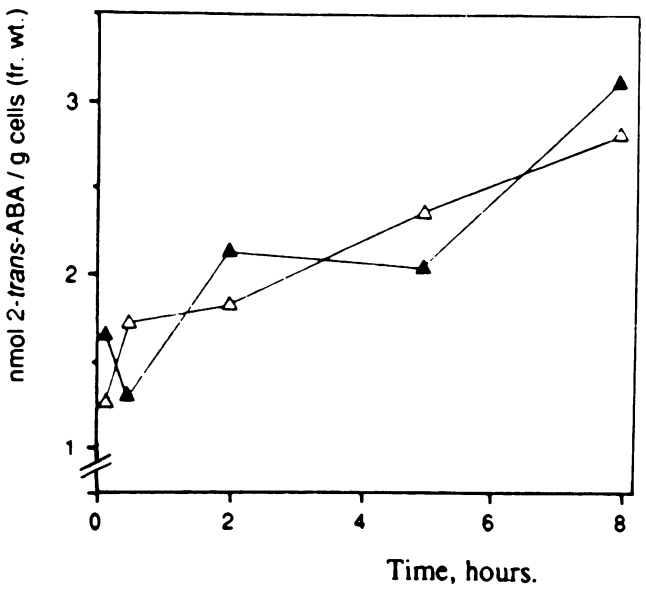


**Figure 8.** Uptake of (+)-*S*- and (−)-*R*-[2-$^{14}$C]2-*trans*-ABA (3 μM, 0.73 μCi/μmol, 100 mL). Experimental conditions were the same as those used to study uptake of (+)- and (−)-ABA (cf. Fig. 2). The natural and unnatural enantiomers of the biologically inactive 2-*trans*-ABA were taken up by the cells at equal rates. (+)-*S*-2-*trans*-ABA, ▲; (−)-*R*-2-*trans*-ABA, △.

closing reaction is dependent on ion pumping and is a "fast" reaction and so may not be directly comparable with "slow" ones. Nevertheless, the point must be made that internal cellular concentrations of ABA may not be the ones that affect growth processes in unstressed cells.

In short-term experiments, lasting for 10 to 20 min, the amount of the labeled ABA metabolized was negligible. After about 2 h, the majority of the labeled (+)-ABA was converted into metabolites (Fig. 3). The same is true for (−)-ABA, but uptake and metabolism occur at slower rates (Fig. 4). Most of the $^{14}C$ or $^{3}H$ within the cells, therefore, does not represent ABA, but ABA that had been absorbed and metabolized.

A similar pattern of metabolites was formed from (+)-ABA by *D. viscosa* as is found in tomato shoots, but the rate and pattern of metabolism may be quite unlike that occurring endogenously in the intact plant, since the cell suspension cells were exposed to the high concentrations of kinetin and naphthalene-1-acetic acid necessary to sustain growth. These compounds could have influenced the rate of catabolism.

### Stereological Analysis

The volumes and surface areas were calculated by assuming that the individual cells were symmetrical ellipsoids, because this form most closely resembled their appearance under the light microscope. Major "a" and minor "b" radii were measured from a number of cells (22) after the cell pellet had been fixed, sectioned, and examined by EM.

The mean surface areas of the plasmalemma and tonoplast and mean volumes of vacuoles, cytoplasm (including the nuclei and excluding the vacuoles), and total protoplasm were calculated for an ellipsoid radially symmetrical about the long axis (i.e. an average sized cell):

$$\text{surface area} = 2\pi ab^2 + [2\pi a^2 b/(a^2 - b^2)^{0.5}]\cos^{-1}(b/a)$$
$$= 4.11 \times 10^{-9}\ \text{m}^2$$
$$\text{volume} = 4/3\pi ab^2$$
$$= 2.20 \times 10^{-14}\ \text{m}^3$$

Therefore, the volume of cytosol = $0.44 \times 10^{-14}$ m$^3$, or 20% of the volume of the total protoplasm.

### Measurements of Uptake Rates and Intracellular Concentrations

The cells contained an average of 96 ng (SD = 40, $n = 6$) ABA/g fresh weight (364 p mol/g) at the end of the growth phase, which started even days after inoculation with a 25-mL volume of packed cells and ended about day 14 with 45 mL. One gram fresh weight contained $9.1 \times 10^6$ cells.

The vacuolar pH was determined by $^{31}P$ NMR as 5.88 and that of the cytosol as 7.32.

By rearranging the Henderson-Hasselbalch equation and using $pK_a = 4.7$

$$\frac{\text{vacuolar ABA}}{\text{cytosolic ABA}} = \frac{1 + \text{antilog}_{10}(\text{pH vacuole} - pK_a)}{1 + \text{antilog}_{10}(\text{pH cytosol} - pK_a)}.$$

The calculated ABA contents at equilibrium were: cytoplasm, 7.9 μM; vacuole, 0.3 μM. The penetration rate of (+)-ABA was calculated as defined by Baier and Hartung (2) at $7.4 \times 10^{-12}$ mol s$^{-1}$ m$^{-2}$.

The permeability coefficient for a solute was defined by Baier and Hartung (2) as:

$$Ps = Js/Co,$$

where *Ps* values of the plasma membrane were calculated from the initial fluxes (*J*) of the solute (*S*). The initial concentration of the radioactively labeled solute in the medium (*Co*) took into account the pH of the medium and the $pK_a$ value of the solute. The situation for (+)-ABA is more complex because it enters the cells in two ways: by a saturable uptake carrier and by passive diffusion. (−)-ABA shows virtually no effect on the uptake carrier and its rate of penetration (4000 dpm/g cells at 3 μM in Fig. 7) can be taken as the same value as that of noncarrier-mediated uptake of (+)-ABA, which is approached asymptotically as the concentrations of unlabeled material increase.

The pH of the medium was 5.8, one pH unit above the $pK_a$ for ABA, consequently the concentration of the permeant species (ABAH) was one-twentieth of the nominal, initial concentration. Fitting these values to the equation above, we obtain a permeability coefficient of $4.9 \times 10^{-8}$ m s$^{-1}$ for passive penetration of (+)- and (−)-ABA. This is comparable to the figure of $2.5 \times 10^{-7}$ m s$^{-1}$ obtained for racemic ABA through the plasmalemma and $1.3 \times 10^{-8}$ m s$^{-1}$ through the tonoplast of guard cells by Baier and Hartung (2).

There is an apparent incongruity between the mean concentration of endogenous ABA: (96 ng/g [0.364 μM] of packed, washed cell volume) and the concentrations of 7.9 μM in the cytoplasm (20% of protoplasmic volume) and 0.3 μM in the vacuole (80% of volume), which gave an overall concentration of 1.58 μM. Earlier work by Behl and Hartung (3) has given comparable data for stomatal guard cells. This value of 1.58 μM is five times higher than the 0.364 μM described above, and the difference arises because the volume of the protoplasts in a fixed preparation was one-fifth of the total. The remaining four-fifths comprise cell walls, intercellular spaces, and cell fragments.

Hartung et al. (14) measured the concentration of ABA in xylem sap forced out of cotton leaves by overpressure and obtained values of between 0.1 to 0.5 μM in the earlier fractions (0–60 μL), rising to 1.3 μM (70 μL), and then stabilizing at approximately 0.5 μM (in the 200- to 250-μL fractions). These values may be lower than the cytosolic concentrations of the leaf cells because of dilution by the apoplastic solution, and the plasmalemma may act to some extent as an ultrafiltration membrane, retaining molecules of ABA and allowing the water to pass through. Nevertheless, the data do show that the concentration in the exuded sap is close to the expected concentration in the cytoplasm. The overall content of ABA in the leaf was 0.12 μM.

Lahr and Raschke (19) reported concentrations of ABA in guard cell protoplasts of *Vicia faba* of 36 to 230 μM, and of 2.7 to 3.3 μM in mesophyll protoplasts. These are high values, since vacuoles are included, and may have been caused by the hypertonicity of the mannitol solution augmented by oligosaccharides formed by cellulase and pectinase from hydrolysis of cell wall materials. There is a critical water potential deficit of approximately 90 MPa, above which a rapid

increase in ABA concentrations occurs (22). Additions of betaine to an osmotic potential of 180 MPa failed to elicit any further increase in ABA concentrations in the guard cell protoplasts, so they were probably already stressed.

Harris and Outlaw (10) found that isolated *V. faba* guard cells increased their contents of ABA from 0.5 μM (on a dry weight basis) to 5 μM within 0.5 h, over which time the leaflets lost 10% of their fresh weight.

Behl and Hartung (3) calculated the concentrations of ABA in guard cells (1.28 μM in cytoplasm; 0.57 μM in vacuole) of *Valerianella*, and Harris and Outlaw (10) compared the amounts in guard cells with those in other leaf cells and found that they were similar on a dry weight basis but that the amounts of ABA in guard cells increased before those of other leaf cells in response to stress. Behl and Hartung also found that the guard cells lost a considerable proportion of their cytoplasmic ABA on wilting. Hartung (11) has produced strong evidence that the receptor for ABA that causes stomatal closure is on the outside of the plasmalemma, so the release of ABA from the cells would allow it to exert its effect. Milborrow (23) analyzed coleoptile growth responses to obtain an estimate of the $K_m$ for the ABA growth inhibition receptor and arrived at a figure of 0.6 μM on a whole tissue basis. The concentrations were obtained with an external bathing solution giving 50% maximum effect at 0.5 μM.

The rise and the subsequent fall in concentration of labeled (+)-ABA taken up by the *D. viscosa* cells (Fig. 3) indicates that activation and/or synthesis of degradative enzymes occurs over a period of about 2 h. Thereafter, the constant rate of uptake was balanced by catabolism and the internal concentration of labeled ABA remained constant.

## CONCLUSION

The rates of uptake and the factors that affect them have been defined for suspension culture cells using (+)- and (−)-ABA. This has provided a yardstick for comparison with other tissues, and rates of penetration and internal concentrations are reasonably similar. One of the most striking features is the magnitude of the differences between the enantiomers. The cells used are somewhat unnatural but the uptake phenomena measured were uncomplicated by limitations of transport into bulky organs or through impermeable surfaces. Even in as simple a tissue as a near-apical segment of a bean root, most of the ABA enters through the cut ends rather than through the thin cuticle covering the epidermal cells (1). The other advantage of using *D. viscosa* suspension culture cells was that they lacked photosynthetically active plastids so that illumination would not cause their stroma to become alkaline and act as an extra anion trap for weak organic acids inside the cytosol.

It is not only the permeation rates and mechanisms of uptake of (+)- and (−)-ABA that are different, but the pathways and rates of metabolism are also different. Experiments in which ABA accumulates into tissues over time periods longer than a few minutes require that the ABA within the cells must be separated from its metabolites before it can be assayed.

The results reported here show that the results of experiments on uptake, transport, and accumulation of ABA can be seriously inaccurate unless two criteria are satisfied: (a) (+)-ABA must be used, and (b) the material taken up must be separated from metabolites to show how much of the labeled material is present as ABA; ideally the amount of endogenous, unlabeled as well as the labeled, exogenously derived compound should be determined. The racemic ABA used for most of the work reported in the literature, and the frequent measurement of radioactivity only, means that the conclusions drawn from such experiments may be incorrect.

Even if a chiral column is unavailable to resolve racemic, labeled ABA, the natural enantiomer can be readily labeled with $^3H$ by exchange in $^3H_2O$ or stably deuteriated by exchange in $MeO^2H$ (33).

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