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Characteristics and Development of Leucine Transport Activity in the Scutellum of Germinating Barley Grain[1]

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ABSTRACT

Scutella separated from grains of Himalaya barley after germination for 3 days rapidly took up L-leucine from aerated incubation media; with 1 millimolar leucine the rate varied between 4 and 14 micromoles per gram per hour and the pH optimum was at 3.5 to 5, both depending on buffer composition and prewashing time. The rate of the uptake increased with increasing concentration of leucine in a complex manner, which could be interpreted as multiphasic kinetics with apparent K_m values of 3.4 and 15.5 millimolar below and above 3 millimolar leucine, respectively. The uptake took place against a concentration difference (highest estimated ratio 270: 1) and was strongly inhibited by dinitrophenol. Uptake was apparently due to active transport requiring metabolic energy.

The development of the uptake activity during germination was studied using Pirkka barley. A low activity was present in the scutella of ungerminated grains. It began to increase after 6 hours imbibition, and the increase was biphasic, the major changes occurring during days 0 to 3 and 4 to 6. The total increase was about 20-fold.

The regulation of the development was studied by allowing separated embryos to germinate on agar gel. The increase of uptake activity was strongly inhibited by inhibitors of RNA or protein synthesis. Increase did not require the presence of the embryo proper, and was not affected by gibberellic or abscisic acid. Removal of the endosperm greatly accelerated the increase of uptake activity, and the presence of 5 or 20 millimolar glutamine counteracted the removal of the endosperm. The results suggest that the availability of glutamine or amino acids in general in the endosperm may regulate the development or the activity of the transport system.

In germinating barley grain the main site of reserve protein degradation is the nonliving storage tissue, the starchy endosperm, where the insoluble reserve proteins are hydrolyzed apparently to a mixture of amino acids and oligopeptides (14). As this "digestive compartment" of the seed is separated from the growing seedling by the scutellum, the hydrolysis products must be taken up by the scutellum before "long distance transport" to the seedling. An active transport system for oligopeptides has recently been shown to function in barley scutellum (6–9, 19–21); its properties include high transport capacity, wide specificity, and a pH optimum of 4 to 5, which is near to the pH of the endosperm.

To add to the picture of reserve protein mobilization in barley we wanted to see how free amino acids are taken up by the scutella at different stages of germination. An uptake system for one amino acid, glutamine, has been described in slices of maize scutella (22), and several other plant tissues have been shown to take up amino acids from incubation media (*e.g.* 1–4, 11–13, 15–18). Leucine was selected as the first amino acid to be tested; it comprises about 7% of all amino acid residues in the main reserve protein, hordein (10), and is generally metabolized slower than most amino acids.

MATERIALS AND METHODS

Plant Material. Himalaya barley (*Hordeum vulgare* L.; the Agronomy Club, Washington State University, Pullman, Wash., harvest of 1972) was used in the characterization of the uptake system. The grains were surface-sterilized with 1% (w/v) sodium hypochlorite and allowed to germinate under aseptic conditions on agar gel in Petri dishes in the dark at 20 C normally for 3 days (21). In experiments concerning the development of the uptake system during germination the Finnish six-row cultivar Pirkka (Lahden Polttimo Oy, Lahti, Finland) was used because the embryo could be separated from the endosperm after only 3-h imbibition. The grains were dehusked with 50% (v/v) H_2SO_4 (19) and the germinations were made in the normal way.

Uptake Assay. The standard procedure was modified from the peptide uptake assay (21). The scutella were separated from the plantlet (or embryo proper) and the endosperm with a scalpel and immersed in water at 0 C. Samples of four scutella were taken within 1 h, weighed after removal of excess water with absorbent paper, and transferred to 3 ml of 1 mM L-[^{14}C]leucine (about 0.02 μCi) in 2 mM sodium succinate buffer of pH 5 in 25-ml conical flasks without stoppers. The flasks were incubated in a shaking water bath at 30 C for 1 h. Thereafter, the scutella were rinsed in water for about 30 s and extracted with 1 ml of 6% (w/v) sulfosalicylic acid at 100 C for 5 min. The radioactivity of the extracts was measured by liquid scintillation spectrometry using a toluene-Triton X-100-based scintillation cocktail (23). The uptake rates are expressed as μmol leucine/g fresh weight·h or as nmol leucine/scutellum·h. Generally, all numerical values and points in the figures are mean values of four determinations given with the SE.

Chromatographic Separations of Leucine. The amount of leucine in the scutella both before and after the uptake was estimated by analyzing the sulfosalicylic acid extracts with a Jeol JL 6AH amino acid analyzer. In some experiments scutella were extracted either with 6% sulfosalicylic acid or with 80% ethanol (70 C), and the radioactive compounds present in the extracts were separated with TLC on Kieselgel G (Merck A.G.) using butanol-acetone-acetic acid-water (35:35:10:20) as solvent. The spots were detected by autoradiography (Curix RP1 x-ray film, Agfa-Gevaert).

Reagents. The reagents were obtained from the following sources: L-[U-^{14}C]leucine, Radiochemical Centre, Amersham; unlabeled amino acids, Fluka A.G.; peptides, Bachem Feinchemikalien A.G.; actinomycin D, 6-methylpurine, cordycepin, and

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cycloheximide, Sigma Chemical Co.; gibberellic acid (90% pure) and *cis-trans* abscisic acid, Fluka A.G.; CCC[3], technical grade, Berner Oy, Helsinki, Finland. All other reagents were of reagent grade.

RESULTS

Time Course of Uptake. When scutella separated from germinating (3 days at 20 C) grains were incubated in the standard medium (2 mM sodium succinate, pH 5, 30 C) containing 1 mM L-[^{14}C]leucine they took up radioactivity at a steady rate for about 1.5 h, whereafter the rate began to increase reaching an approximately 3-fold value after 6 h (Fig. 1). An incubation time of 1 h or less was therefore used in all later experiments.

The extent of the metabolism of leucine during the 1-h experiment was estimated in three ways. First, a set of incubations was carried out in stoppered Warburg flasks containing an O_2 atmosphere. Radioactivity absorbed by filter paper strips soaked in 2 N KOH in the central well corresponded to only 0.5% of the radioactivity taken up by the scutella. In a second set of experiments, amino acids were extracted from the scutella with either 6% sulfosalicylic acid or 80% ethanol and separated with TLC. In addition to a highly active spot in the position of leucine one other very faint spot was observed. Using a 50-fold exposure time it could be estimated that the intensity of this spot was below 2% of that of the leucine spot. To estimate the incorporation of radioactivity into protein during the standard incubation, the incubated scutella were first extracted with 6% sulfosalicylic acid at 100 C and thereafter rinsed twice with the same solution. Then the proteins were extracted with 0.5 ml of 1% SDS at 100 C for 15 min. The radioactivity in the SDS extracts was only 1.5% of the activity taken up by the scutella. The three experiments indicate that the metabolism of leucine was negligible during the standard uptake assay.

The concentrations of free leucine in the scutella were determined with an amino acid analyzer. The amount before the incubation was 3.2 μmol/g^{-1}, corresponding to 4.1 mM leucine, if the amino acid is assumed to be evenly distributed throughout the water in the tissue (79% of fresh weight; ref. 20). Corresponding concentrations after 1- and 9-h incubation were 8 and 145 mM. It is possible that the leucine uptake occurred against a concentration difference during the standard assay (1 h). After 9 h the concentration of leucine in the medium was 0.54 mM. Accordingly, the transport took place against an apparent concentration ratio of 270:1, indicating that it was probably associated with processes requiring metabolic energy. This was confirmed by testing the effects of two inhibitors of aerobic energy metabolism: 1 mM KCN and 0.25 mM DNP added 5 min before the assay reduced the uptake by 51 and 72%, respectively.

Effect of Prewashing on Rate of Uptake. When separated scutella were prewashed in water before the uptake assay, the uptake rate was unaffected by 1 h of washing but increased progressively with longer washing times (Fig. 2). After 5-h prewashing the rate was three times that for unwashed scutella. A comparison of Figures 1 and 2 shows that the activity increased in a similar way regardless of whether the washing was carried out in the presence of leucine. The increase of activity during prewashing was arrested by cycloheximide and glutamine (Fig. 2). Therefore, it seems that the increases of activity during long incubations were not due to leaching of inhibitory substances but to an actual increase in the uptake capacity.

To eliminate the prewashing effect the separated scutella were kept in water at 0 C (15–90 min) before the uptake assays; this storage had no effect on the rate of uptake.

Effects of pH and Salts. The rate of leucine uptake was relatively high between pH 2 and 6, with an optimum near pH 4 (Fig. 3). The shape of the curve and the pH optimum were, however, affected by prewashing and buffer quality; in addition to the data shown in Figure 3 the following optima were recorded: pH 3.5 in 10 mM Na-citrate and pH 4.5 in 10 mM Na-phosphate. Despite these lower pH optima the subsequent experiments were made at pH 5 as this is near to the actual pH of the endosperm.

The effects of the major physiological cations, when added at 100 mM concentration to the uptake medium (Table I), were similar to the corresponding effects on the uptake of Gly-Gly (21). Na^+, which plays a central role in various transport systems in animal cells (24), had a small inhibitory effect. Moreover, the uptake took place in water solution of leucine without any added Na^+ (data not shown). The results together with the fact that Na^+ is not essential for barley even as a micronutrient indicate that Na^+ does not take part in the uptake.

Effects of Leucine Concentration and Some Potential "Competitors" on Uptake. When the effect of leucine concentration on the rate of uptake was studied in the concentration range 0.1 to 20

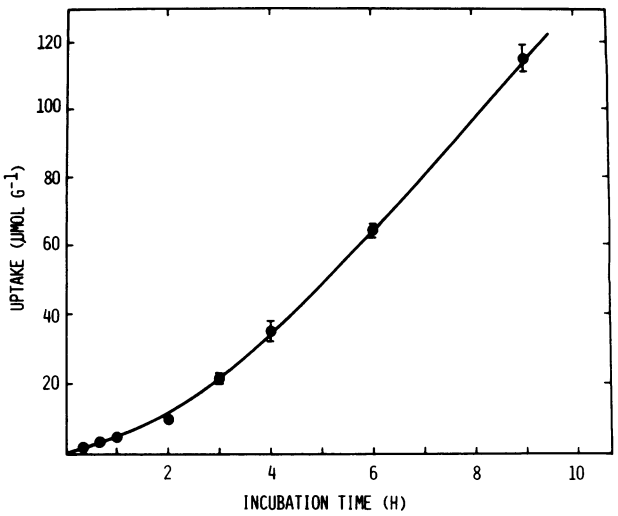

FIG. 1. Effect of incubation time on uptake of leucine. Scutella were incubated in 6 ml of substrate in the standard conditions for different times and the amount of leucine estimated.

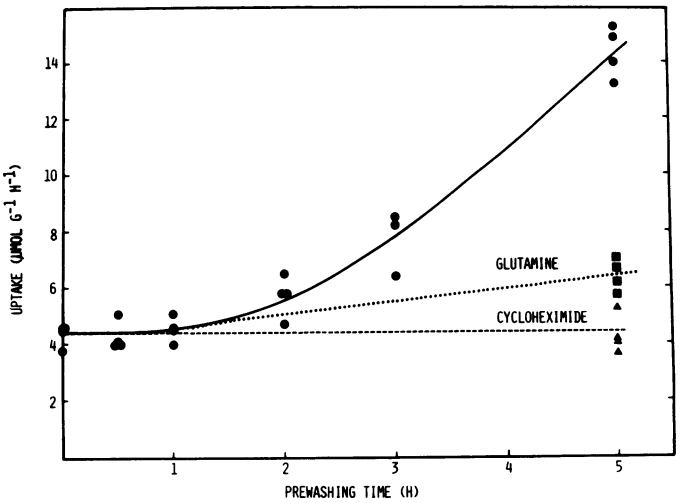

FIG. 2. Effect of prewashing time on uptake of leucine. After separation from the grain the scutella were prewashed at 23 C in 100 ml of water which was stirred by a gentle stream of O_2. After different times the uptake of leucine was measured in the standard conditions, but using only 20-min incubation time. Some scutella were prewashed in 0.3 mM cycloheximide or 20 mM glutamine. Each point represents one measurement.

[3] Abbreviations: CCC: 2-chloroethyltrimethylammonium chloride; DNP: 2,4-dinitrophenol; SAR: sarcosine; scut: scutellum.

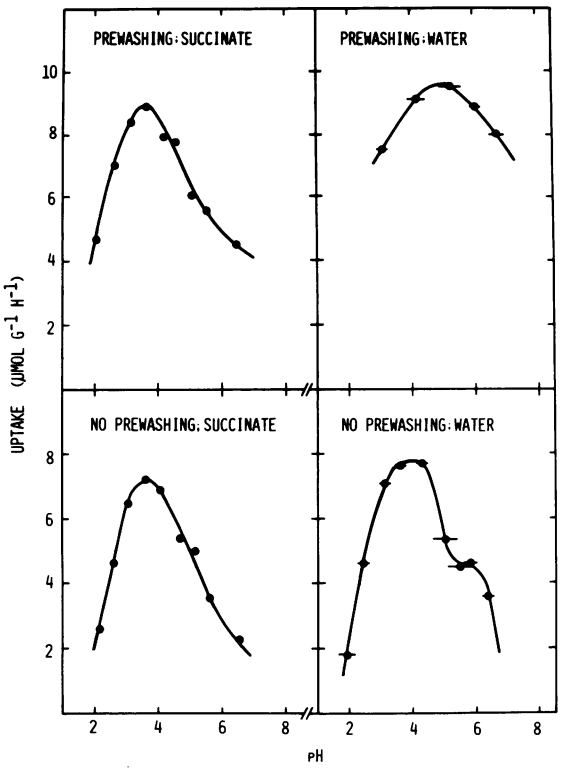

FIG. 3. Effect of pH on uptake of leucine. Uptake of leucine by prewashed (3 h) and unwashed scutella was estimated in 2 mM sodium succinate buffers with different pH values. In another corresponding experiment no buffer was used, but the pH of the leucine solution was adjusted to the desired value with HCl or NaOH. Horizontal bars indicate the change of pH during experiment. To avoid even greater changes in pH, 6 ml of substrate were used and the incubation time was 30 min.

Table I. *Effect of Some Salts on Uptake of Leucine*

Unwashed scutella were incubated for 30 min in standard substrate solutions (2 mM sodium succinate buffer, pH 5, [Na^+] about 3 mM) to which 100 mM salts were added. The pH was adjusted after the addition of the salt.

Salt	Uptake of Leucine	
	μmol g^{-1} h^{-1}	*% of control*
None	3.4 ± 0.1	
NaCl	2.5 ± 0.2	73
KCl	2.3 ± 0.1	66
$CaCl_2$	3.2 ± 0.1	94
$MgCl_2$	3.8 ± 0.5	110

mM a saturation effect was evident although the rate was still increasing at 20 mM concentration (Fig. 4 inset). An Eadie-Hofstee plot $\left(v/\frac{v}{[S]}\right)$ of the data revealed, however, that the uptake did not conform to simple Michaelis-Menten kinetics. The results can best be interpreted as indicating the functioning of a single system which changes its properties (simultaneous change in K_m and V_{max}) in response to substrate concentration (multiphasic uptake, 15, 18). In the concentration range used there was one transition point at 3 mM leucine and the phases had the following constants: K_m1 = 3.4 mM, $V_{max}1$ = 22.3 μmol g^{-1} h^{-1} (0.1–3 mM), and K_m2 = 15.5 mM, $V_{max}2$ = 65.2 μmol g^{-1} h^{-1} (3–20 mM). Another interpretation of these data would be that a high affinity and a low affinity system function simultaneously; these would have K_m1 = 1.4 mM, $V_{max}1$ = 6.0 μmol g^{-1} h^{-1}, and K_m2 = 33 mM, $V_{max}2$ = 85 μmol g^{-1} h^{-1}. When the uptake at 20 mM leucine was estimated in the presence of 0.25 mM DNP (present in the uptake solution, no preincubation), 78% of the uptake was inhibited. This confirms that the deviation from simple Michaelis-Menten kinetics was not due to nonmediated uptake.

Isoleucine, alanine, and glycine all inhibited the uptake of leucine (Table II). The simplest explanation for these inhibitions is that these amino acids compete for the same carrier(s). The effects of two peptides which are rapidly taken up by barley scutella (19) were also tested (Table II). Gly-Sar, which is hydrolyzed in the scutellum very slowly, had no effect on the uptake of leucine, whereas Leu-Leu, which is probably hydrolyzed very rapidly, unexpectedly had a stimulatory effect.

Changes in Uptake Activity during Germination. Scutella separated from ungerminated grains of Pirkka barley after 4-h imbibition in water took up leucine from a 1-mM solution at a rate of 1.3 μmol g^{-1} h^{-1} or 3.7 nmol $scut^{-1}$ h^{-1} and about 70% of the uptake was inhibited by 0.25 mM DNP (no preincubation). When

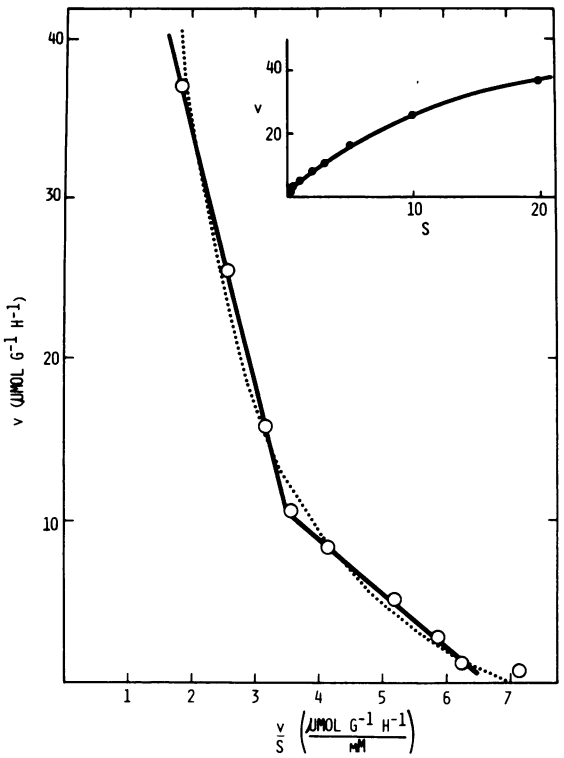

FIG. 4. Effect of substrate concentration on uptake of leucine. Uptake of leucine from 0.1–20 mM solutions was assayed in the standard conditions using an incubation time of 30 min. The figure shows the v against v/[S] plot of the original values (○), and the theoretical curve calculated on the assumption that two uptake systems function simultaneously and have the following constants: K_m1 = 1.4 mM, $V_{max}1$ = 6.0 μmol g^{-1} h^{-1}, K_m2 = 33 mM, $V_{max}2$ = 85 μmol g^{-1} h^{-1} (. . . .).

Table II. *Effect of Some Amino Acids and Peptides on Uptake of Leucine*

The uptake was measured in standard conditions (1 mM leucine) in the presence of 10 mM amino acid or peptide.

Addition	Uptake of Leucine	
	μmol g^{-1} h^{-1}	*% of control*
None	3.7 ± 0.1	
Ile	2.7 ± 0.2	73
Ala	1.3 ± 0.2	36
Gly	1.8 ± 0.2	48
Leu-Leu	4.9 ± 0.3	132
Gly-Sar	3.6 ± 0.3	96

the grains were allowed to germinate on agar gel at 20 C for 3 days the rate of uptake increased over 10-fold to 45 nmol scut^{-1} h^{-1}. A detailed study of the changes throughout the period of reserve protein mobilization was made by assaying leucine uptake both at low and high leucine concentration to detect possible differences between the high affinity and low affinity systems suggested by the kinetics of uptake (Fig. 4). At 0.2 mM leucine about 60% of the uptake should be by the high affinity system and at 10 mM leucine about 80% should be by the low affinity system (in Himalaya barley germinated for 3 days). The time course of the development was similar with both assays (Fig. 5). The activity began to rise after 6 h and increased about 10-fold in 3 days. Then there was another lag lasting about 1 day, followed by another 2-fold increase during days 4 to 6. The two curves were not identical but the differences were not conspicuous enough to confirm the presence of two separate systems.

Regulation of Increase of Uptake Activity. When separated embryos of Pirkka barley were allowed to germinate on agar gel their leucine uptake activity increased from 3.3 to 43 nmol scut^{-1} h^{-1} in 15 h. Inhibitors of RNA or protein biosynthesis arrested the increase of uptake activity by 86 to 100% (Table III). These results suggest that the carrier protein is synthesized *de novo* in the germinating embryo.

Removal of the embryo proper had only a small retarding effect (possibly due to physical damage) but removal of the endosperm greatly accelerated the increase of uptake activity (Table IV). Moreover, 5 or 20 mM glutamine effectively "simulated" the presence of the endosperm. The plant hormones GA_3 and ABA had minimal effects even at the relatively high concentration used (Table IV). A strong inhibitory effect was obtained with a very high concentration of CCC which is an inhibitor of gibberellin biosynthesis. This inhibition was not counteracted by a high concentration of GA_3. Therefore, it seems that the CCC effect was nonspecific.

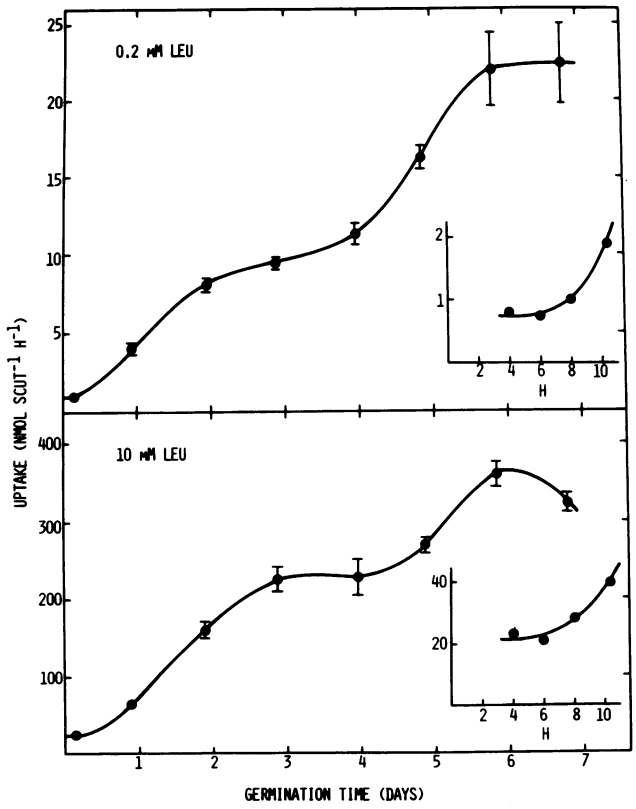

FIG. 5. Development of leucine uptake activity during germination of the grain. Grains of Pirkka barley were allowed to germinate for different times on agar at 20 C and the uptake of leucine by the scutella was measured using the standard method, except that two substrate concentrations were used, 0.2 and 10 mM. Insets show changes in uptake during the first hours of germination.

Uptake of Leucine by Rootlets and Coleoptiles. Leucine was taken up by root tips and coleoptile slices of Pirkka barley at rates roughly similar to those of uptake by resting scutella (Table V). In all cases the uptake was strongly inhibited by DNP. However, the rates were much lower than that for scutella from germinating grains. The scutellum is a compact tissue with apparently only one active side, the epithelium facing the endosperm; therefore, if the rates could have been expressed per unit of active surface area, it is probable that the difference would have been even more pronounced.

DISCUSSION

The results show that the scutellum of germinating barley grain has the capacity for rapid active uptake of the amino acid leucine from incubation media. It is improbable that this system would take up only leucine; inhibition of the uptake of leucine by three other amino acids gives support for this idea, though it does not prove it. The leucine uptake system in barley scutellum resembles the amino acid uptake systems present in maize scutellum (22),

Table III. *Effects of Inhibitors of RNA and Protein Synthesis on Development of Leucine Uptake System*

Pirkka grains were imbibed for 3–4 h at 23 C in water containing the inhibitor. Thereafter the embryo was removed and allowed to germinate at 20 C on agar containing the same concentration of inhibitor as during imbibition. The time of germination was 16 h from the beginning of the imbibition. After germination the scutella were separated and the uptake of leucine assayed in the standard conditions.

Inhibitor	Concentration	Uptake of Leucine	Inhibition[a]
	mM	*nmol scut^{-1} h^{-1}*	*%*
None		43.4 ± 0.7	
6-Methylpurine	1.0	8.8 ± 0.7	86
Cordycepin	1.0	6.4 ± 0.1	92
Actinomycin D	0.2	9.1 ± 0.3	86
Cycloheximide	0.3	3.0 ± 0.2	100

[a] Inhibition of the increase in uptake. Uptake by the scutella from nongerminated grains was 3.3 nmol scut^{-1} h^{-1}.

Table IV. *Effect of Various Treatments on Development of Leucine Uptake Activity*

Grains of Pirkka barley were imbibed for 3–4 h, treated in different ways, and allowed to germinate on agar for 15 h from the beginning of the imbibition. The chemicals were used during both imbibition and germination.

Germination Conditions	Uptake of Leucine	Development[a]
	nmol scut^{-1} h^{-1}	*% of control*
Whole grain	19.5 ± 0.9	
Endosperm removed	43.4 ± 1.5	249
Embryo removed	17.0 ± 1.2	84
Endosperm and embryo removed	39.3 ± 1.5	224
Endosperm removed + 5 mM glutamine	22.3 ± 1.4	117
Endosperm removed + 20 mM glutamine	16.7 ± 0.7	83
Endosperm removed + 500 μg/ml GA_3	36.3 ± 0.1	204
Endosperm removed + 0.1 mg/ml CCC	42.8 ± 1.2	244
Endosperm removed + 10 mg/ml CCC	14.2 ± 1.2	67
Endosperm removed + 10 mg/ml CCC + 500 μg/ml GA_3	14.0 ± 0.2	66
Endosperm removed + 10 μM ABA	39.6 ± 2.4	224
Endosperm removed + 100 μM ABA	36.5 ± 2.1	205

[a] Uptake by scutella from nongerminated grains was 3.3 nmol scut^{-1} h^{-1}.

Table V. *Uptake of Leucine by Scutella, Root Tips, and Coleoptile Slices of Pirkka Barley*

The scutella were separated from nongerminated grains imbibed for 4 h or from grains germinated for 6 days. The root tips (0.5 cm) and coleoptile tips (0.5 cm, sliced into pieces about 1 mm long) were from 4-day germinated grains. The uptake was estimated in the standard assay conditions. DNP was present in the uptake medium (no preincubation).

Organ	Uptake of Leucine	
	−DNP	+0.25 mM DNP
	$\mu mol\ g^{-1}\ h^{-1}$	
Scutella, 0 days	1.33 ± 0.09	0.38 ± 0.01
Scutella, 6 days	10.64 ± 0.30	1.49 ± 0.09
Roots	1.84 ± 0.10	0.25 ± 0.01
Coleoptiles	2.13 ± 0.22	0.44 ± 0.03

root tips (18), leaf slices (4, 13, 16, 17), *Spirodela* fronds (2), and cells cultured in suspension (1, 3, 11, 12) in several respects: in all cases the uptake is dependent on metabolic energy; the pH optimum of the uptake is generally in the range pH 3–5; the kinetics of the uptake, at least when studied over a wide concentration range, does not generally follow the simple Michaelis-Menten saturation curve; the rate of the uptake is affected by buffer composition. Unfortunately, the uptake rates have usually been measured at widely different amino acid concentrations, and the results have been expressed as rates per fresh weight (instead of per unit of effective surface area). Therefore, a quantitative comparison of the uptake rates is not possible with the data available. However, the rate obtained with "6-day scutella" at 10 mM leucine (about 70 $\mu mol\ g^{-1}\ h^{-1}$, calculated from Fig. 5) is, as far as we know, the highest rate reported for the uptake of any amino acid by any plant tissue. Other relatively high rates have been reported for the uptake of glutamine in maize scutella (about 25 $\mu mol\ g^{-1}\ h^{-1}$, Fig. 6 in ref. 22) and for the uptake of lysine in barley leaves (about 10 $\mu mol\ g^{-1}\ h^{-1}$, Fig. 3 in ref. 13).

The development of the leucine uptake system of barley scutella during germination resembles the corresponding development of the peptide uptake system (19) in several respects: (*a*) both systems have some activity in resting seeds; (*b*) a rapid increase begins after 6-h imbibition, and the total increases are of the same order of magnitude (20-fold and 35-fold); (*c*) inhibitors of RNA and protein synthesis arrest the development; (*d*) the presence of the embryo proper is not necessary for the development, and GA_3, which induces enzyme synthesis and secretion in the aleurone layer, and its antagonist ABA have no effects. There are two major differences, however. First, the leucine uptake activity develops in two steps: a 10-fold increase up to day 3 is followed by a stationary phase before another 2-fold increase during days 4 to 6. The peptide uptake system behaves in a similar way up to day 3, but thereafter there is a slow decline instead of the second increase. Second, the peptide uptake system develops at the same rate in intact grains and separated embryos during the first 12 h but the increase of the leucine uptake activity is accelerated 2.5-fold when the endosperm is removed. A similar effect seems to take place when the endosperm is removed from grains germinated for 3 days: when the separated scutella are incubated in the presence of 1 mM leucine (Fig. 1) or simply washed (Fig. 2), the leucine uptake activity remains constant for 1 h and then increases about 3-fold during the next 4 h (*versus* 2-fold increase in 72 h in intact grains). As the activity increases both during the first 15 h and after 3 days' germination are suppressed by glutamine, it seems that the activity of the leucine uptake system is regulated throughout the germination by the presence of the endosperm, which probably exerts its effect via glutamine or amino acids, in general. An analogous control of amino acid uptake seems to function in soybean and rapeseed cells cultured in suspension (11, 12). A low activity is present in cells cultured in the presence of NH_4NO_3 and a great increase occurs when the cells are transferred to a corresponding medium without any nitrogen source.

To estimate the actual rate of amino acid uptake *in vivo* in the germinating grain the concentrations of amino acids in the starchy endosperm at the site of uptake as well as the kinetics of their uptake should be known. At present such data are not available. However, Chittenden *et al.* (5) have determined the amounts of free amino acids in the starchy endosperm of wheat after 4 days' germination. Approximate calculation based on these data gave the following concentrations: leucine = 3 mM, glutamine = 8 mM, sum of all amino acids = 35 mM. Using these figures and assuming that "average amino acids" are transported at the same rate as leucine, it can be calculated that the leucine uptake system of the scutellum could take up all of the amino acid residues of the endosperm proteins in about 10 days at 20 C. The approximate calculation shows that the leucine uptake activity is of a proper order of magnitude in relation to the massive flow of amino acids which must take place during germination.

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