Interference of L-α-Aminooxy-β-Phenylpropionic Acid with Phenylalanine Metabolism in Buckwheat

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L-α-Aminooxy-β-phenylpropionic acid (AOPP), a potent competitive inhibitor of phenylalanine ammonia-lyase (PAL), blocked light-induced phenylpropanoid synthesis in excised buckwheat hypocotyls and produced an up to 40-fold increase in the endogenous phenylalanine concentration, while the level of all other amino acids was hardly affected.

After a 24 h incubation in the light in the presence of 0.3 or 1 mM AOPP phenylalanine alone constituted about 25% of the total soluble amino acids, compared to appr. 1% in the controls.

In the presence of AOPP illuminated hypocotyls accumulated nearly 3 times more phenylalanine than hypocotyls kept in the dark, indicating an enhancing effect of light on the flow of carbon through the shikimate pathway. Exogenously added [^{14}C]phenylalanine was extensively metabolized by control tissue, but accumulated in AOPP treated tissue. In the presence of AOPP radioactivity from [^{14}C]shikimate accumulated predominantly in phenylalanine, and the flow of shikimate into tyrosine and phenylalanine was not affected by the inhibitor. Therefore, under these conditions no feedback control of phenylalanine and tyrosine synthesis from shikimate is apparent in buckwheat hypocotyls.

Introduction

Structural analogues of amino acids have been widely used in biochemical and biological studies of metabolism and development [1–3]. In particular, analogues of phenylalanine, such as *e. g.* p-fluoro-phenylalanine, have been of interest with respect to their activation and incorporation into proteins [4] as well as tools in investigations of aromatic amino acid biosynthesis [3]. In higher plants phenylalanine is the immediate precursor of cinnamic acid, which is the parent compound of a multitude of phenolic substances, including lignins and flavonoids. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the first committed step in the biosynthesis of these substances, and the biochemistry and physiology of this enzyme have been studied in considerable detail [5, 6]. As outlined elsewhere [7], specific inhibitors of this enzyme would be of value in the elucidation of various aspects of the biochemistry and physiology of phenylpropanoid metabolism. We have recently introduced the aminooxy analogue of phenylalanine, L-α-aminooxy-β-phenylpropionic acid (AOPP) as a highly potent competitive inhibitor of PAL [8], which appears to have the desired specificity to be applicable in *in vivo* studies [7, 9–11]. Thus, it was shown that seeds germinate and develop normally in the presence of concentrations of AOPP, which suppress phenylpropanoid synthesis [9, 12], but apparently did not interfere with protein synthesis. In fact, Norris *et al.* [13] have shown that AOPP is neither activated by nor does it inhibit phenylalanyl-tRNA-synthetase from *Phaseolus aureus* seeds. It is known, however, that AOPP interferes with the transamination of phenylalanine in extracts from *Phaseolus aureus* seedlings [11], and also inhibits pig heart glutamate-oxalacetate transaminase as well as glutamate-pyruvate transaminase (Amrhein and Strüber, unpublished). The potency of inhibition was, however, far less pronounced than in the case of phenylalanine deamination. Nevertheless, the interference of AOPP *in vivo* with the formation and metabolism of amino acids other than phenylalanine has to be taken into account. We, therefore, decided to investigate the effect of AOPP on the levels of soluble amino acids in buckwheat hypocotyls. Furthermore, we hoped to gain insight into the regulation of the shikimate pathway under conditions, which prevent the flow of phenylalanine into the phenylpropanoid pathway.

Abbreviations: AOA, α-aminooxyacetic acid; AOPP, L-α-aminooxy-β-phenylpropionic acid; PAL, phenylalanine ammonia-lyase (EC 4.3.1.5).

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Materials and Methods

Plant material

Buckwheat (*Fagopyrum esculentum* Moench) seeds were germinated in moist vermiculite and grown in the dark for 6 days at 23.5 ± 1 °C. For the experiments described in Table III seeds of the various species were allowed to germinate for 5 days on filter paper soaked with either H_2O or 0.3 mM L-AOPP in plastic dishes in a growth chamber under a 16 h photoperiod at 22 ± 1 °C. Mung bean seeds had previously been soaked overnight in running tap water. The leaf material was from plants grown in the greenhouse (wheat, barley, tobacco) or in the Botanical Garden (*Catalpa*).

Chemicals

The enantiomers of α-aminooxy-β-phenylpropionic acid and its N-benzyloxycarbonyl derivatives as well as the enantiomers of α-hydrazino-β-phenylpropionic acid were synthesized according to procedures kindly made available to us by Dr. J. S. Morley, ICI Pharmaceuticals Division, Macclesfield, Cheshire, U. K. Aminooxy-acetic acid, semihydrochloride, was obtained from Sigma, St. Louis, MO., and *Rhodotorula glutinis* PAL, Cat. No. 0810, from P-L Biochemicals Inc., Milwaukee, WI. L-[U-^{14}C]-Leucine (sp. A. 354 mCi/mmol), L-[U-^{14}C]phenylalanine (sp. A. 513 mCi/mmol), and D-[2,3,4,5(*n*)]-[^{14}C]shikimic acid (sp. A. 84 mCi/mmol) were provided by the Radiochemical Centre, Amersham, U. K. The radiopurity of the compounds was checked by paper and thin layer chromatography. Chemicals and biochemicals in reagent grade were purchased from Merck, Darmstadt, Dowex 50 W × 8 from Serva, Heidelberg.

Incubation of buckwheat hypocotyls

Excised hypocotyls from 6-day-old buckwheat seedlings were incubated in petri dishes as described previously (for references see [8]). Hypocotyls for incubations in the dark were prepared under a dim green safety light. For feeding experiments 20 derooted 6-day-old seedlings were placed with the cut lower hypocotyl end in a glass scintillation vial containing 2 ml of either 0.1 M potassium phosphate buffer, pH 5.5, or, in addition, 1 mM L-AOPP. After a 4 h preincubation the radioactive precursor was added and the incubation continued for 24 h at 25 °C and under continuous illumination from fluorescent tubes (5000 lux). Leucine and phenylalanine were fed at 1 μCi/vial (leucine concentration: 1.4 μM; phenylalanine concentration: 0.98 μM), shikimic acid at 0.2–0.5 μCi/vial (concentration: 1.2–3 μM).

Extraction and measurement of amino acids

Buckwheat hypocotyls, entire seedlings, or leaf disks (fresh weights 0.1–1.2 g) were extracted twice for 20 min with 20 ml of boiling 80% ethanol under reflux. The extracts were dried in an air stream and residues taken up in 8 ml H_2O. After acidification with 1 N HCl and three extractions with 4 ml ethylacetate the aqueous phases were transferred to 1 × 5 cm columns of Dowex 50 W × 8 equilibrated with 0.01 N HCl. The columns were washed with 30 ml 0.01 N HCl followed by 5 ml H_2O. Amino acids were eluted with 50 ml 2 N NH_4OH, and the eluates were dried in an air stream. The residues were taken up in 1 ml H_2O, and aliquots of 20 to 40 μl were subjected to amino acid analysis in a BC 200 amino acid analyzer (LKB, München). An amino acid standard chromatogram is found in [14]. Incorporation of radioactivity into the aromatic amino acids was determined either after separation of the amino acids by paper chromatography in *n*-propanol: NH_3 (7 : 3, v/v) or in the eluates of the amino acid analyzer as described in ref. [14].

Other measurements

Radioactivity in ethanol-extracted dried hypocotyls was determined after combustion in a Mikromat-Analyzer, Berthold-Frieseke, Wildbad. Incorporation of radioactivity into protein was determined as the activity released by hydrolysis of the ethanol-extracted hypocotyls in 6 N HCl at 110 °C for 48 h.

Radioactivity was determined in a Berthold Frieseke BF 5000 scintillation counter. Anthocyanin extractions and measurements were made according to ref. [15]. Apparent inhibitor constants for buckwheat PAL were determined as described in ref. [8].

Results

Effect of AOPP on amino acid levels in excised buckwheat hypocotyls

When hypocotyls of 6-day-old etiolated buckwheat seedlings were excised and incubated for 24 h

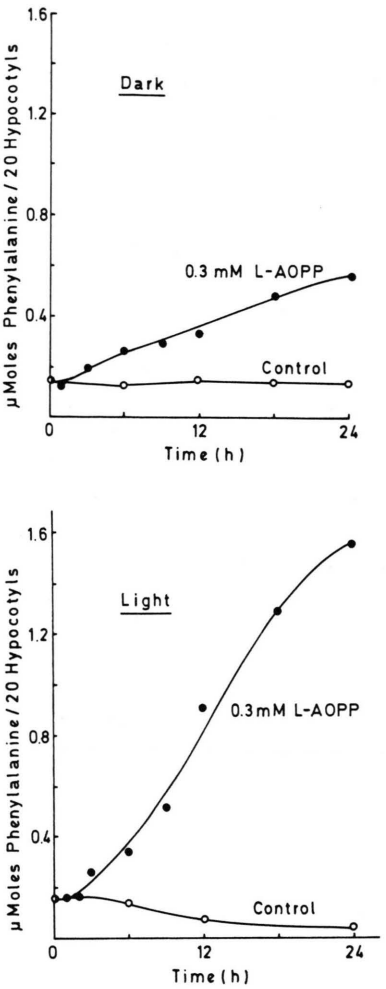

Fig. 1. Change of phenylalanine levels in excised buckwheat hypocotyls in darkness (a) and in the light (b) in the absence and presence of 0.3 mM AOPP.

in buffer alone, total 80% ethanol soluble amino acids decreased. The decrease was more pronounced in the light than in the dark (Table I). Phenylalanine levels were stable in the dark (Table I, Fig. 1 a), but gradually decreased in the light (Table I, Fig. 1 b). Due to a small amount of unknown ninhydrin-positive material, which eluted from the amino acid analyzer column between tyrosine and phenylalanine, accurate measurements of low phenylalanine amounts were impeded. Levels of phenylalanine in the light controls are, therefore, likely to be slight overestimates.

Determination of phenylalanine levels by two other independent methods – the enzymatic procedure of Uchiyama *et al.* [16] using *Rhodotorula* PAL, and the fluorescence assay of Wong *et al.* [17] – produced similar values within a range of ± 30%, but difficulties arose also with low phenylalanine concentrations. Moreover, AOPP from AOPP-treated plants severely interfered with the enzymatic procedure, because it is a potent inhibitor of *Rhodotorula* PAL [8]. AOPP gives, however, only a slight reaction with ninhydrin and did not interfere with the determination of phenylalanine in the amino acid analyzer.

Amino acid measurements in general were the same within the variations given in Table I, independent on whether the hypocotyls were floated in the incubation solutions in petri dishes or placed with the cut lower ends in the solutions in small vials. The latter mode of incubation, which deviates from our standard procedure [18], became necessary, when it was found that labelled shikimic acid was taken up effectively only with the transpiration stream. When 1 mM L-AOPP was included in the incubation medium, phenylalanine levels rose dramatically, while all other amino acids were hardly affected (Table I). Only the tyrosine level was slightly elevated, too.

Time course studies showed that phenylalanine levels were raised significantly within a 3 h incubation in AOPP (Fig. 1), and continued to increase during the next 20 h. After a 24 h incubation in 1 mM L-AOPP in the light phenylalanine alone constituted up to 30% of the total soluble amino acid pool, compared to 0.5 to at most 2% in the controls, and its concentration in the tissue was in the range of 1–2 mM. In the dark the phenylalanine level rose to less than one third of the level produced by AOPP in the light (Fig. 1). As little as 1 µM was sufficient to produce an elevated level of phenylalanine, and there was a nearly linear relationship between the logarithm of the exogenous AOPP concentration and the endogenous phenylalanine concentration (Fig. 2). Anthocyanin production was inversely related to the AOPP concentration, but inhibition of anthocyanin synthesis became apparent only at AOPP concentrations larger than 3 µM.

Effect of other inhibitors on the level of phenylalanine and tyrosine in buckwheat hypocotyls

We had previously shown that both enantiomers of AOPP, as well as their N-benzyloxycarbonyl derivatives, effectively inhibit anthocyanin synthesis

Table I. 80% Ethanol soluble amino acids in buckwheat hypocotyls as affected by a 24 h incubation period in darkness or in light ± 1 mM AOPP *.

	Initial amino acid content (nmol/g fresh weight)	24 h Dark (in % of initial content)	24 h Light	24 h Light + 1 mM AOPP (in % of 24 h light control)
asp	488 ± 54 ***	67	42	98
thr	433 ± 117	96	66	94
ser **	1109 ± 249	67	67	98
glu	847 ± 191	74	67	94
gly	172 ± 46	79	42	118
ala	264 ± 78	86	76	102
val	553 ± 181	97	32	126
ile	272 ± 29	73	17	147
leu	395 ± 47	71	38	100
tyr	228 ± 39	56	26	217
phe	116 ± 34	106	34	**2408**
trp	159 ± 78	95	65	127
his	454 ± 110	93	50	114
lys	90 ± 34	91	52	103
arg	112 ± 21	70	50	99
sum	5692 ± 555			
% of initial	100	79	58	130 (in % of 24 h light control)

* met and cys not determined.
** ser, asp · NH_2, and glu · NH_2 not separated in the system used.
*** Standard deviations from 5 separate experiments.

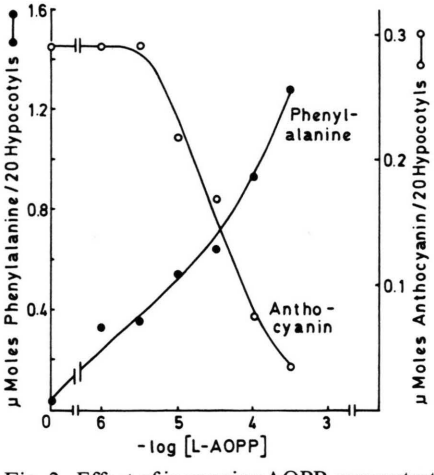

Fig. 2. Effect of increasing AOPP-concentrations on phenylalanine levels and anthocyanin production in excised buckwheat hypocotyls. Measurements were made after a 24 h incubation in the light.

in excised buckwheat hypocotyls [9], and aminooxyacetic acid was also successfully employed as an inhibitor of phenylpropanoid synthesis in buckwheat [19]. In addition, the hydrazino-analogue of phenylalanine is also known as a potent inhibitor of PAL [20]. It was, therefore, of interest to study the effect of these compounds on phenylalanine levels in buckwheat hypocotyls. As Table II shows, the N-benzyloxycarbonyl derivatives of D- and L-AOPP effectively produced high phenylalanine levels, too, while the hydrazino analogues were less effective. The D-enantiomers generally possess less enhancing activity than the L-enantiomers, and aminooxyacetic acid had only a relatively small effect on phenylalanine and tyrosine levels. For comparison, K_i-values of the compounds for buckwheat PAL and I_{50}-values for inhibition of anthocyanin synthesis in buckwheat hypocotyls are also given in Table II.

Effect of AOPP on the levels of phenylalanine and tyrosine in various seedlings and leaves

Seeds of various species were germinated and seedlings were grown for five days on filter paper soaked with 0.3 mM AOPP. 80% Ethanol extracts of the entire seedlings were then analyzed for their content of phenylalanine and tyrosine. Similarly, segments from various leaves were incubated for 24 h in the light with or without 0.3 mM AOPP. It is evident from Table III that of all the seedlings tested, buck-

Table II. Effect of various inhibitors on phenylalanine and tyrosine levels in excised buckwheat hypocotyls (A), on buckwheat PAL (B), and on anthocyanin biosynthesis in excised buckwheat hypocotyls (C). Amino acid levels were determined after a 24 h incubation in 0.3 mM inhibitor in the light. Apparent K_i-values were determined for PAL in crude acetone powder extracts from illuminated buckwheat hypocotyls [8], and I_{50}-values are those concentrations of the inhibitors required to produce 50% inhibition of anthocyanin synthesis in excised buckwheat hypocotyls during a 24 h incubation in the light [11].

	A *		B	C
	phe (nmol/20 hypocotyls)	tyr	app. K_i for PAL [M]	I_{50} for inhibition of anthocyanin synthesis [M]
Control	48	168	–	–
L-α-aminooxy-β-phenylpropionic acid	1712	263	1.4×10^{-9}	$2-5\times10^{-5}$
D-α-aminooxy-β-phenylpropionic acid	1230	275	2.5×10^{-8}	$2-5\times10^{-5}$
N-Benzyloxycarbonyl-L-α-aminooxy-β-phenylpropionic acid	2101	237	– **	$2-5\times10^{-5}$
N-Benzyloxycarbonyl-D-α-aminooxy-β-phenylpropionic acid	1548	241	– **	$5-10\times10^{-5}$
L-α-hydrazino-β-phenylpropionic acid	271	268	1.5×10^{-7}	$5-10\times10^{-4}$
D-α-hydrazino-β-phenylpropionic acid	100	239	1.8×10^{-5}	$5-10\times10^{-4}$
α-aminooxyacetic acid	59	144	1.2×10^{-4}	$6-10\times10^{-5}$

* mean of two replicates.
** The N-benzyloxycarbonyl derivatives probably inhibit PAL only after the release of the protective group [9].

Table III. Levels of phenylalanine and tyrosine in various 5-day-old seedlings grown in the absence or presence of 0.3 mM AOPP and in leaf segments incubated for 24 h in the absence or presence of 0.3 mM AOPP.

	phe (nmol/g fresh weight)		tyr	
	– AOPP	+ AOPP	– AOPP	+ AOPP
Seedlings				
mungbean	1530	2519	766	771
gherkin	686	1436	396	315
rye	397	188	376	162
white clover	1615	3656	594	774
red cabbage	391	2529	317	621
buckwheat	88	3620	188	379
barley	136	431	115	237
white mustard	499	2918	837	1226
Leaves				
wheat	310	1015	66	487
barley	229	725	30	322
Catalpa bignonioides	55	2674	125	331
tobacco	291	916	243	692

Table IV. Protein amino acids in buckwheat hypocotyls after a 24 h incubation in the light ± 1 mM AOPP.

	(μmol/g dry weight) *	
	– AOPP	+ AOPP
asp	143	154
thr	78	74
ser	104	95
glu	161	150
pro	87	87
gly	146	134
ala	136	125
cys/2	21	22
val	93	84
met	12	9
ile	63	58
leu	130	119
tyr	42	48
phe	61	62
trp	–	–
his	35	31
lys	105	99
arg	66	60
sum	1483	1411

* Values not significantly different at 95% confidence level.

wheat seedlings responded with the greatest phenylalanine accumulation to the AOPP treatment. 29% of the soluble amino acid pool of the seedlings grown on 0.3 mM AOPP consisted of phenylalanine alone (control: 1%), and the concentration of phenylalanine in the tissue was 3.62 mM. In some seedlings, such as mungbeans and white clover, the relative increases in the phenylalanine levels were low, but the absolute increases were considerable.

Leaves of *Catalpa bignonioides* responded particularly well to AOPP with a nearly 49 fold increase in phenylalanine, and in wheat and barley leaves, which have high tyrosine ammonia-lyase activity [21], AOPP treatment preferentially raised tyrosine levels.

The lack of an effect of AOPP on the phenylalanine and tyrosine levels of rye seedlings is reflected in the inability of AOPP to inhibit anthocyanin synthesis in the seedlings (Amrhein, unpublished). Insufficient uptake or inactivation of AOPP may be the explanation for this anomaly.

The effect of AOPP on insoluble amino acids in buckwheat hypocotyls

To check if under conditions of phenylalanine overproduction more phenylalanine is channeled into proteins, the 80% ethanol insoluble residue of the hypocotyls was subjected to acid hydrolysis and the hydrolysate was subjected to amino acid analysis. Table IV demonstrates that the amino acid composition of hypocotyl tissue treated with AOPP is not significantly different from that of the control.

Metabolism of labelled phenylalanine in AOPP-treated buckwheat hypocotyls

During a 24 h feeding period in the light control hypocotyls took up 88% of the labelled phenylalanine, while the uptake in the AOPP-treated hypocotyls was reduced to 64%. While little activity remained in the 80% ethanol soluble fraction in the control, more than 70% of the activity absorbed by the AOPP-treated hypocotyls remained in this fraction and was largely recovered in the amino acid fraction (Table V). Chromatographic analysis revealed that the activity in the amino acid fraction consisted entirely of unmetabolized phenylalanine. Combustion of the 80% ethanol insoluble residues showed that the controls had incorporated twice as much of the total absorbed activity into this fraction. Less than one half of this activity in the controls was released after acid hydrolysis as phenylalanine, while in the AOPP-treated tissue the entire activity in the insoluble fraction was recovered as phenylalanine after hydrolysis and had thus exclusively resided in the protein fraction. The non-hydrolysable activity in the control tissue most likely resided in ligneous material. Considering the accumulation of phenylalanine in AOPP-treated tissue (Fig. 1 a) one should expect a considerable dilution of the exogenously added radioactive phenylalanine by the endogenous pool and hence an apparent reduction of the incorporation of radioactive phenylalanine into protein. The results in Table V show that this was not the case, and it is assumed, that the exogenously supplied phenylalanine is channeled into protein without prior equilibration with the bulk of the cellular phenylalanine.

The fate of labelled shikimate in AOPP-treated buckwheat hypocotyls

[^{14}C]shikimate was fed to AOPP-treated and control hypocotyls to trace the fate of endogenously produced aromatic amino acids in the presence of the inhibitor. It was of particular interest to determine, if under conditions of high endogenous phenylalanine levels produced by AOPP a feed back control of shikimate incorporation into phenylalanine occurs.

Shikimate was readily taken up with the transpiration stream when supplied to the cut lower ends of hypocotyls, to which the cotyledons had been left attached. (The seed coats had been removed.) Only hypocotyls were subjected to analysis, however. Fig. 3 shows the kinetics of the metabolization of shikimate. The uptake of shikimate by the hypocotyls was somewhat reduced in the presence of AOPP (Fig. 3 a, Table VI). The average reduction of uptake in a total of 8 experiments was 18% (Table VI). The rate of accumulation of radioactivity into the 80% ethanol fraction slowed down in the controls after 8 to 10 h, and radioactivity in this fraction then declined in the controls, while it continued to increase in the presence of AOPP (Fig. 3 b). A similar tendency was observed for the fraction of the ethanol soluble fraction that did not adsorb to the Dowex-column (neutral and acidic fraction, Fig. 3 c).

Chromatographic analysis of this fraction revealed that it consisted almost entirely of shikimic acid. Pronounced differences were found for the incorporation of shikimate into the amino acid fraction (Fig. 3 d), and after separation of phenylalanine and tyrosine by paper chromatography it became evident that AOPP preferentially enhanced the accumulation of labelled phenylalanine (Fig. 3 e), while the incorporation into tyrosine was hardly affected until the 18th h of incubation (Fig. 3 f). Incorporation of shikimate into phenylalanine and tyrosine in the controls was in the same order of magnitude (Fig. 3 e, f – note the different scales of the ordinates!).

No incorporation of shikimate into tryptophan was detectable under the conditions of this experiment. We have found, however, that exogenous

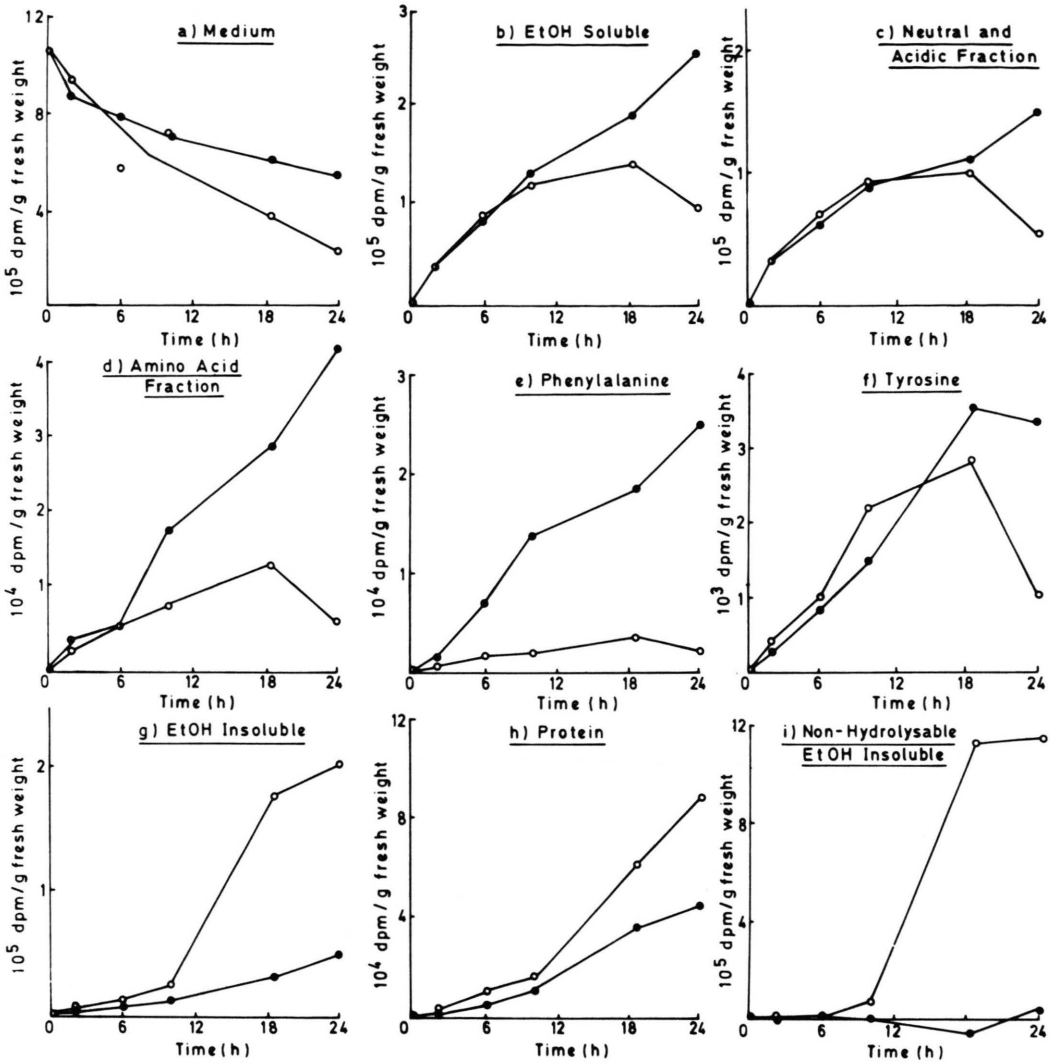

Fig. 3. Uptake and metabolic fate of [^{14}C]shikimate in buckwheat hypocotyls during a 24 h incubation period in the light. Open symbols: controls; closed symbols: 1 mM AOPP. Incorporation of radioactivity into the non-hydrolysable 80% ethanol insoluble fraction (i) was determined as the difference between incorporation of radioactivity into the 80% ethanol insoluble fraction (g) and into the protein fraction (h).

radioactive shikimate can be channeled into tryptophan, when phenylalanine is supplied simultaneously in the incubation medium (Holländer and Amrhein, unpublished).

Incorporation of shikimate into the ethanol insoluble fraction of the control tissue was highest between the 10th and 18th h of incubation (Fig. 3g), and then declined. AOPP drastically reduced the incorporation of shikimate into this fraction (Fig. 3g). Upon acid hydrolysis of the insoluble fraction radioactivity was released, which consisted entirely of phenylalanine and tyrosine and the origin of which is therefore attributable to protein. AOPP also inhibited shikimate incorporation into this fraction, but to a lesser degree (Fig. 3h). In agreement with this apparent inhibition of shikimate incorporation into protein the specific activities of phenylalanine and tyrosine in the hydrolysates were reduced by appr. 40% in the AOPP-treated hypocotyls (Table VII). Incorporation of shikimate into the non-hydrolysable insoluble fraction (presumably ligneous material) was completely suppressed by AOPP (Fig. 3i). In

Table V. Distribution of radioactivity from [^{14}C]phenylalanine in fractions of buckwheat hypocotyls after a 24 h feeding period in the light ±1 mM AOPP.

Radioactivity	– AOPP		+ AOPP	
	[dpm]	in % of absorbed radio activity	[dpm]	in % of absorbed radioactivity
Absorbed from medium	1 824 000	100	1 371 000	100 (=75.2% of control
80% EtOH	143 570	7.9	969 750	70.7
Amino acid fraction	9 160	0.5	532 230	38.8
Total 80% EtOH insoluble fraction	442 335	24.2	175 033	12.8
Protein	194 390	10.7	199 500	14.5

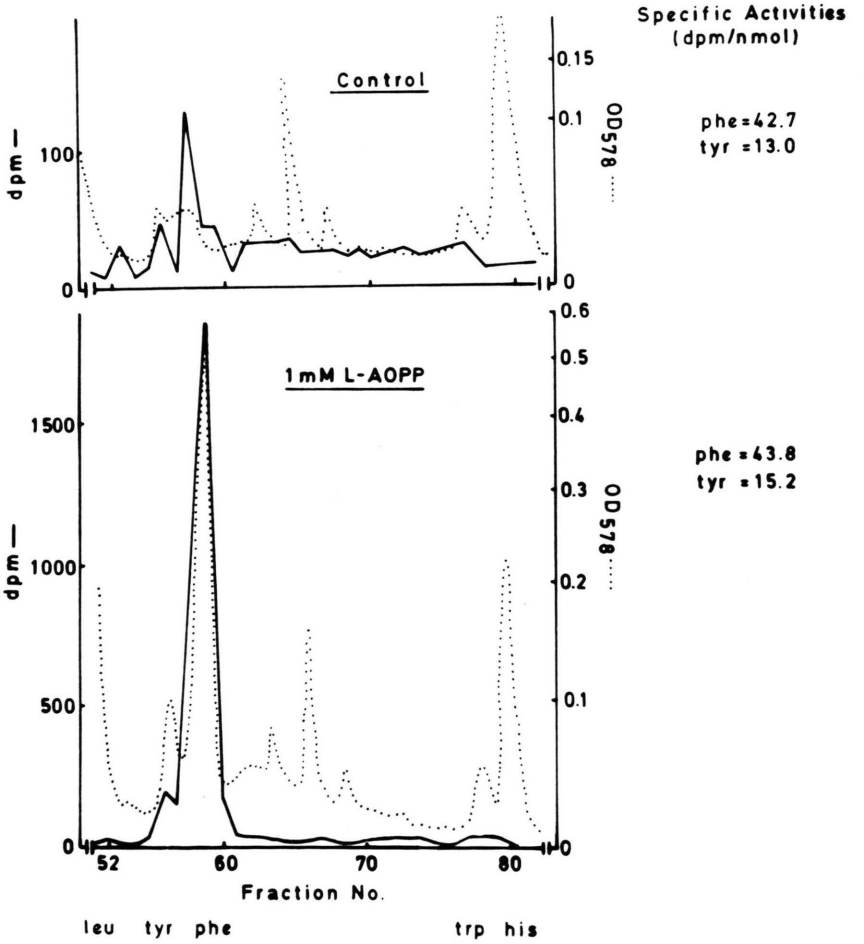

Fig. 4. Incorporation of radioactivity from [^{14}C]shikimate into the aromatic amino acids of buckwheat hypocotyls. Derooted buckwheat seedlings were incubated for 24 h in the light with [^{14}C]shikimate in the absence or presence of 1 mM AOPP. The soluble amino acid fraction of the hypocotyls was subjected to amino acid analysis in the amino acid analyzer, and fractions were assayed for radioactivity (solid lines) after staining with ninhydrin. (Dotted lines: amino acid analyzer tracing).

Table VI. Distribution of radioactivity from [^{14}C]shikimate in fractions of buckwheat hypocotyls after a 24 h feeding period in the light ± 1 mM AOPP.

	Control	AOPP
% Radioactivity absorbed from the medium	83.95 ± 9.26%	68.86 ± 11.45 (= 82% of control)
Radioactivity in AOPP-treated hypocotyls (in % of controls)*		
80% EtOH fraction		325.2 ± 115.8
Neutral and acidic fraction from Dowex column		304.5 ± 117.3
Amino acid fraction from Dowex column		784.5 ± 161.8
Tyrosine		311.5 ± 141.1
Phenylalanine		2075.4 ± 547
80% EtOH insoluble fraction		41.5 ± 10.8
Protein fraction		63.7 ± 20.36

* Values corrected for reduced uptake of shikimate in AOPP-treated hypocotyls. Standard deviations were calculated from 8 separate experiments.

Table VII. Specific activities of phenylalanine and tyrosine in hydrolysates of the EtOH insoluble residues of control and AOPP-treated hypocotyls after a 24 h feeding period with [^{14}C]shikimate.

	[dpm/nmol]	
	Control	AOPP
phe	26.1	16.3
tyr	26.6	15.2

Table VI the collected data of 8 shikimate feeding experiments are given. In spite of the variations between the individual experiments the preferential incorporation of shikimate into phenylalanine in the presence of AOPP is well established by these experiments. When soluble amino acids from shikimate-fed hypocotyls were separated on the amino acid analyzer and the region of the eluate, which contained the aromatic amino acids, was analyzed for radioactivity (Fig. 4), the results of the paper chromatographic analysis (Fig. 3 e, f) were fully confirmed. With the aid of the amino acid analyzer the specific activities of both phenylalanine and tyrosine were determined (Fig. 4) and found to be nearly identical in control and AOPP-treated tissue. This result clearly indicates that under the conditions of the experiment the flow of shikimate into phenylalanine was not impeded by the accumulated amino acid and that feedback inhibition was, therefore, not apparent.

Metabolism of labelled leucine in AOPP-treated buckwheat hypocotyls

The fact that the incorporation of exogenous labelled phenylalanine into protein was not impeded by AOPP (Table V), while incorporation of labelled shikimate was reduced (Fig. 3 h, Tables VI, VII), even though the specific activities of soluble phenylalanine and tyrosine in control and AOPP-treated tissues were nearly the same (Fig. 4), prompted the question, whether AOPP, in fact, interfered with protein synthesis. We, therefore, tried to evaluate the effect of AOPP on protein synthesis by feeding radioactive leucine along with AOPP. In a 24 h feeding period control hypocotyls incorporated 74% of the absorbed radioactivity into the EtOH insoluble residue, while the incorporation was 60% for the AOPP-treated hypocotyls. Analysis of the acid hydrolysate gave leucine as the only labelled constituent. Germination and prolonged growth of seedlings in the presence of AOPP (Table III and [9]) make it unlikely that AOPP inhibits protein synthesis to a critical extent.

Discussion

The selective accumulation of phenylalanine in buckwheat hypocotyls treated with AOPP (Fig. 1) clearly indicates that AOPP blocks phenylalanine ammonia-lyase *in vivo* and interferes little, if at all, with the metabolism of other amino acids (Table I). Tyrosine ammonia-lyase (TAL)-activity is not detectable in extracts from buckwheat hypocotyls [18, 22]; furthermore, exogenous tyrosine is incorporated into buckwheat lignin to only a very small extent [23, 24] and cannot replace phenylalanine in the reversion of AOA-mediated inhibition of cyanidin synthesis in buckwheat hypocotyls [19]. The twofold increase in tyrosine concentrations in AOPP-treated hypocotyls (Table I) may thus either reflect low TAL-activity *in vivo* or a positive effect of phenylalanine on tyrosine synthesis [25]. Unpublished experiments from this laboratory have shown that AOPP inhibits TAL-activity from maize or *Rhodotorula glutinis* with a similar potency as PAL-activity, and the pronounced accumulation of tyrosine in AOPP-treated wheat and barley leaves

(Table III) can most likely be attributed to the inhibition *in vivo* of TAL-activity.

Buckwheat has been a particularly fortunate choice as experimental material for these investigations, because it responds more strikingly than most other tissues to the application of AOPP with an increased phenylalanine accumulation (Fig. 1, Tables I – III). The extent to which phenylalanine will accumulate in a tissue treated with AOPP clearly depends on a number of parameters: 1) there must be a sufficiently high PAL-activity, which normally diverts phenylalanine to cinnamic acid and its metabolic products; 2) related to this, there must be a high turnover rate of the endogenous phenylalanine pool(s), or, in any event, a high rate of production of phenylalanine; and 3) there must be little or no feedback control of phenylalanine synthesis. It is obvious, especially from the shikimate feeding experiments (Table V, Figs 3, 4), that in buckwheat there is little, if any, apparent feedback control of phenylalanine production from shikimate under our experimental conditions, since shikimate is incorporated into phenylalanine in the presence of AOPP at the same rate as in the absence of AOPP. Even though regulatory forms of chorismate mutase, which are subject to feedback inhibition by phenylalanine and tyrosine and feedback activation by tryptophan *in vitro*, have been reported in higher plants [26 – 30], the high phenylalanine concentrations that build up in the presence of AOPP are either not sufficiently high to effect feedback inhibition or are sequestered so that phenylalanine concentrations at the site of chorismate mutase are never high enough to be inhibitory. Alternatively, an aromatic amino acid-insensitive chorismate mutase [27 – 30] might be functional in buckwheat. Unpublished results (Holländer and Amrhein, in preparation) show, however, that in the presence of exogenous phenylalanine (3 mM) shikimate incorporation into phenylalanine and tyrosine is reduced and incorporation into tryptophan is enhanced, so that, at least under these conditions, a feedback inhibition *in vivo* can be demonstrated.

Recently, Berlin and Vollmer (manuscript in press) also did not find evidence for feedback inhibition of phenylalanine synthesis, when they fed shikimate to tobacco cell suspension cultures in the presence of AOPP. Increased levels of phenylalanine in AOPP-treated soybean seedlings were also recently reported by Duke and Hoagland [31].

Compartmentation of aromatic amino acid biosynthesis and storage has also to be taken into consideration. The data of Table V indicate that in the presence of AOPP exogenous radioactive phenylalanine is incorporated into protein without the dilution of radioactivity which one would expect if the exogenous amino acid fully equilibrated with the elevated pool of the endogenous amino acid. This finding is in agreement with the concept of functionally different pool of amino acids in plant cells, of which one (cytosolic?) is small, rapidly equilibrates with exogenous amino acids and provides precursors for protein synthesis, while the other (vacuolar?) is expandable and equilibrates only slowly with exogenous amino acids (see ref. [32] for a detailed discussion). Our data would indicate that in the presence of AOPP phenylalanine accumulates in the expandable (vacuolar?) pool. The situation may, however, be even more complex, because the synthesis of aromatic amino acids in higher plants may, completely or in part, occur in plastids [25, 33 – 36]. Unless the intracellular site of phenylalanine accumulation in buckwheat hypocotyls is found, all conclusions drawn from our results on the regulatory mechanisms operative in the control of the shikimate pathway in this tissue must remain speculative.

The finding that in the presence of AOPP buckwheat hypocotyls accumulate more phenylalanine in the light than in the dark (Fig. 1) is of considerable interest with respect to the control by light of phenylpropanoid metabolism [37]. Increased production of flavonoids and chlorogenic acid in illuminated plant tissues is generally thought to be related to light-induced increases in PAL-activity (see [37, 38] for references). However, Margna [38] has challenged this concept and contended that phenylalanine supply, rather than PAL-activity, is the limiting factor in the control of phenylpropanoid accumulation. Direct evidence for a light-induced increased production of phenylalanine has so far not been provided, however. *In vivo* inhibition of phenylalanine utilization via PAL, brought about by AOPP, has now provided this evidence (Fig. 1). As AOPP is a competitive inhibitor of PAL [8] and as the PAL-activity increases transiently about 6-fold during the light-treatment [8], there is in all likelihood relatively less AOPP available for PAL inhibition in the light than in the dark, and the stimulatory effect of light on phenylalanine production may even be more than three-fold as found in the present investigation.

While we would hesitate to entirely agree with Margna's hypothesis [38], our results clearly indicate that increased substrate (phenylalanine) supply might be involved in light-induced phenylpropanoid synthesis.

A comparison of the time course of phenylalanine concentrations in excised buckwheat hypocotyls in the light and in darkness (Fig. 1, controls) with our previously published data [18] shows a discrepancy: we had formerly reported that phenylalanine levels remain constant during illumination, while we now found that they, indeed, decrease to about one third of the initial level in the light, while they remain constant in darkness. In the former study we were not aware of the ninhydrin-positive material, which elutes from the amino acid analyzer column in the region of tyrosine and phenylalanine. Improved techniques in the present investigation have now allowed to determine phenylalanine more precisely, but, nevertheless, the determination of low phenylalanine levels in buckwheat is impeded by the presence of this interfering substance(s). No such complications were encountered with the other plant tissues that were analyzed. Duke and Naylor [39] found decreases in the free pools of phenylalanine and tyrosine in illuminated previously dark-grown maize seedlings and attributed these changes to increased levels of PAL.

Previous calculations [18] have shown that the small pool of phenylalanine has to turn over at least 20-fold during a 24 h incubation of buckwheat hypocotyls in the light in order to provide sufficient substrate for increased phenylpropanoid production. It would now seem that the tissue is not fully capable to meet this increased demand for phenylalanine, and the decline in phenylpropanoid synthesis after an appr. 20 h illumination [15] may partially be the result of phenylalanine depletion.

While the results presented here were obtained with the L-enantiomer of AOPP, the analogue of the natural PAL-substrate, Table II indicates that the D-enantiomer, as well as the N-benzyloxycarbonyl derivatives of both enantiomers, which are intermediates in the chemical synthesis of the free acids, also effectively promote phenylalanine accumulation. The higher efficiency of the L-enantiomer is reflected in its lower K_M for PAL, but is less evident from experiments on the inhibition *in vivo* of anthocyanin synthesis [9]. The variability of anthocyanin production has so far not allowed the determination of precise I_{50}-values for the two enantiomers and their N-benzyloxycarbonyl derivatives [9].

As the D-enantiomer of AOPP and its N-benzyloxycarbonyl derivative are less specific *in vivo* than the L-enantiomer [9], application of the L-enantiomer is clearly preferable to the D-enantiomer or the racemate. It is quite probable that replacement of the N-benzyloxycarbonyl group by other groups will produce compounds that might be even more potent inhibitors of phenylpropanoid synthesis *in vivo*.

Replacement of the aminooxy-group of AOPP by the hydrazino group reduces the potency of the compound to inhibit PAL *in vitro* or *in vivo* (Table II) and results in relatively poor inhibition of anthocyanin formation. AOPP is thus unexcelled as the most potent PAL-inhibitor presently known. AOA, even though an efficient inhibitor of anthocyanin synthesis (Table II and [19]) is a relatively poor inhibitor of PAL [11] and has little effect on phenylalanine levels (Table II). AOA is, however, a very efficient inhibitor of phenylalanine transamination [11] and transamination reactions in general, (see [11] for references), and inhibition of phenylpropanoid synthesis by AOA may thus be due to its interference with both the transamination and deamination reactions, *i.e.* production and utilization of phenylalanine.

In conclusion, our results provide evidence for the preferential inhibition of phenylalanine deamination *in vivo* by AOPP, and we have now a tool to study the flow of substrate through the shikimate pathway in the absence of extensive utilization of phenylalanine in phenylpropanoid synthesis. For various aspects of aromatic and alicyclic metabolism in higher plants application of AOPP should provide us with meaningful answers.

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