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METABOLISM OF [6-^{14}C]OROTATE BY SHOOTS OF *PISUM SATIVUM*, *PHASEOLUS VULGARIS* AND *LATHYRUS TINGITANUS*

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Key Word Index—*Pisum sativum*; *Phaseolus vulgaris*; *Lathyrus tingitanus*; Leguminosae; orotic acid; pyrimidines; nucleotides; willardiine; isowillardiine; lathyrine; pseudouridine; 5-ribosyluracil; pyrimidine metabolism.

Abstract—Incorporation of radioactivity from [6-^{14}C]orotate into the pyrimidine constituents of shoots of *Pisum sativum*, *Phaseolus vulgaris* and *Lathyrus tingitanus* was examined with special reference to the unusual pyrimidine constituents. With each species, although 80% of the orotate supplied was catabolized to β-alanine, all the pyrimidine derivatives became radioactively labelled. With *Pisum*, the major part of the radioactivity incorporated into pyrimidines was located in UMP and the uracil derivatives, including the uracilyl amino acids willardiine and isowillardiine. With *Phaseolus*, UMP and the uracil derivatives were again the major radioactive products; incorporation of radioactivity into 5-ribosyluracil (pseudouridine), which accumulates in *Phaseolus* tissues, was comparable to the incorporation into orotidine and twice that found in cytidine. *Lathyrus* incorporated a substantially larger part of the presented [6-^{14}C] orotate into pyrimidine derivatives than did the other two species. CMP was the most highly radioactive product, followed next by lathyrine and UMP. Surprisingly, 20% of the total radioactivity incorporated into pyrimidines by *Lathyrus* was located in the pyrimidine amino acid lathyrine. This confirms previous evidence that lathyrine is essentially a product of the orotate pathway. The overall recovery of radioactivity in all three species was 93–95%. The data emphasize the necessity of including the less common pyrimidine constituents, as well as the common ones, in quantitative studies of pyrimidine metabolism in plants.

INTRODUCTION

Over the last 20 years, a number of papers have appeared confirming that in plants, as in animals and micro-organisms, the orotic acid pathway is the major route of pyrimidine biosynthesis (e.g. refs. [1–13]). The key product of the pathway is UMP, from which the other pyrimidines arise. Detailed information, especially quantitative information, concerning the distribution of the flow of orotate via UMP into the various pyrimidine constituents of plants is, however, still scant. Moreover, a number of unusual pyrimidine derivatives, some quantitatively important, have now been recognized to be produced via the orotate pathway in plants, e.g. willardiine and isowillardiine [14], vicine and convicine [15], and lathyrine [16]. Such compounds need to be considered in assessments of pyrimidine metabolism. The present study thus aimed to trace the metabolic fate of [^{14}C]orotate in seedlings of *Pisum sativum*, *Phaseolus vulgaris* and *Lathyrus tingitanus*. All three species produce, in addition to the full range of common pyrimidines, uncommon pyrimidine derivatives which accumulate in the tissues, viz. the uracilyl amino acids willardiine and isowillardiine in *Pisum* [14], 5-ribosyluracil (pseudouridine) in *Phaseolus* [17], and the pyrimidine amino acid lathyrine in *Lathyrus* [16]. It should be emphasized that, although it is possible to demonstrate by isotopic labelling the formation of free pyrimidine bases (e.g. uracil, cytosine and thymine) in the tissues of higher plants, these compounds do not usually accumulate to any detectable extent [18]. Early reports of the occurrence in plants of free pyrimidine bases are mostly attributable to the use of drastic extraction procedures which hydrolyse nucleic acids and nucleotides [19].

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RESULTS

Batches of 40 excised shoots from 8-day seedlings of *Pisum sativum*, *Phaseolus vulgaris* and *Lathyrus tingitanus* were allowed to take-up, completely, a solution containing [6-^{14}C]orotate (10 μCi; sp. act. 61 mCi/mmol). The procedure is outlined in the Experimental. After 24 hr, the shoots were extracted in ice-cold 0.3 M perchloric acid, and the extract clarified by centrifuging. The bulk of the ClO_4^- was removed by precipitation with potassium hydroxide at 0°. Following a preliminary purification by adsorption onto and subsequent elution from charcoal, the pyrimidine constituents of the extract were separated chromatographically and identified by UV spectrophotometry and by co-chromatography and electrophoresis with authentic reference samples.

Metabolism of [6-^{14}C]*orotate by* Pisum sativum

The distribution of radioactivity amongst the various metabolites of [6-^{14}C]orotate in shoots of *P. sativum* is shown in Table 1. All the commonly occurring pyrimidine bases were identified and shown to be radioactively labelled, as were the pyrimidine nucleosides uridine and cytidine, and the nucleotides, UMP, CMP and OMP. The

Table 1. Metabolic fate of [6-^{14}C]orotate in *Pisum sativum*, *Phaseolus vulgaris* and *Lathyrus tingitanus*

	Distribution of radioactivity					
	Pisum sativum		*Phaseolus vulgaris*		*Lathyrus tingitanus*	
Radioactive products	dpm × 10^{-3}	%*	dpm × 10^{-3}	%*	dpm × 10^{-3}	%*
Pyrimidine constituents†	211	1	104	0.6	648	4
Unidentified compounds adsorbed by Norit OL‡	973	6	205	1	249	1.5
β-Alanine	14237	86	13313	80	12326	74
Other non-pyrimidines	303	2	1857	11	2541	15
Total (i.e. total radioactivity recovered)	15724	95	15479	93	15764	95

[6-^{14}C]Orotate (16631 × 10^3 dpm) was supplied to batches of excised shoots (40 shoots per batch).

*The percentages shown are percentage recovery where 100% is the 16631 × 10^3 dpm originally supplied to each batch.

†Detailed in Table 2.

‡Mainly oligonucleotides.

isomeric pyrimidine amino acids, willardiine [β-(2,6-dihydroxypyrimidin-1-yl)alanine] and isowillardiine [β-(2,6-dihydroxypyrimidin-3-yl)alanine] were also confirmed to be radioactive products of [6-^{14}C]orotate, as had been previously observed [14].

The most striking feature of the metabolism of [6-^{14}C]orotate by *P. sativum* is the large proportion of the radioactive orotate catabolized to β-alanine. It has been known for many years that plant tissues are capable of catabolizing pyrimidines and that, as with animal tissues and micro-organisms, β-alanine is a major end product [20]. The present results (Table 1) show, however, that 86% of the [6-^{14}C]orotate supplied is degraded to β-alanine during the 24 hr experimental period. Only 1% is converted to other free pyrimidines, a further 6% to unidentified oligonucleotides, and 2% to non-pyrimidine compounds (excluding β-alanine). An analysis of the distribution of the radioactivity amongst free pyrimidine derivatives is given in Table 2. The list of radioactive compounds detected includes all the commonly occurring

Table 2. Distribution of radioactivity from [6-^{14}C]orotate amongst the pyrimidine constituents of *Pisum sativum*, *Phaseolus vulgaris* and *Lathyrus tingitanus*

	Radioactivity in pyrimidine constituents					
	Pisum sativum		*Phaseolus vulgaris*		*Lathyrus tingitanus*	
Pyrimidine constituent	dpm × 10^{-3}	% of total ^{14}C in pyrimidine fraction	dpm × 10^{-3}	% of total ^{14}C in pyrimidine fraction	dpm × 10^{-3}	% of total ^{14}C in pyrimidine fraction
OMP	19.9	9.4	15.8	15.2	15.8	2.4
UMP	105.3	49.7	35.8	34.5	126.3	19.5
CMP	14.7	6.9	11.6	11.2	157.9	24.4
Uridine	30.5	14.4	16.8	16.2	102.1	15.8
Cytidine	1.6	0.7	0.7	0.7	5.4	0.8
5-Ribosyluracil	—	—	1.4	1.3	—	—
Orotidine	8.5	4.0	1.4	1.3	4.2	0.6
Uracil	15.8	7.5	5.9	5.7	93.7	14.5
Orotic acid	5.4	2.5	6.7	6.5	4.8	0.7
Cytosine	0.1	0.1	0.1	0.1	7.0	1.1
Thymine	0.4	0.2	7.5	7.3	4.5	0.7
Willardiine	1.5	0.7	—	—	—	—
Isowillardiine	7.9	3.7	—	—	—	—
Lathyrine	—	—	—	—	126.3	19.5
Total free pyrimidines	211.6	100.0	103.7	100.0	648.0	100.0

[6-^{14}C]Orotate (16631 × 10^3 dpm) was supplied to batches of excised shoots (40 shoots per batch). Dashes indicate the absence of the compound in any detectable amount.

pyrimidine bases, ribosides and ribotides, together with the two pyrimidine amino acids, willardiine and isowillardiine. Considering the close metabolic proximity of UMP to orotate, it is not surprising that this should be the most highly radioactive pyrimidine product. Orotidine, uridine and uracil were also substantially labelled, as was also CMP.

Metabolism of [6-^{14}C]*orotate by* Phaseolus vulgaris

As with *Pisum sativum*, the major part (80%) of the [6-^{14}C]orotate supplied was catabolized to β-alanine (Table 1). The main difference between the labelling pattern of the two species concerns the non-absorbed fraction. With *Phaseolus*, the total radioactivity in the non-adsorbed fraction represents 11% of the supplied radioactivity, in contrast to 2% with *Pisum*. The higher figure for *Phaseolus* is at the expense of the adsorbed, non-identified fraction which in *Phaseolus* accounts for only 1% of the total counts supplied whereas with *Pisum*, this was 6%. The distribution of ^{14}C amongst the major pyrimidine constituents was similar to that observed for *Pisum*, the most substantially labelled compound being UMP with uridine, uracil, thymine and CMP substantially labelled. *Phaseolus*, unlike the other two plant species studied in this work, accumulated significant amounts of 5-ribosyluracil (pseudouridine) [17]. This compound was also labelled, the extent of labelling being similar to that of orotidine.

Metabolism of [6-^{14}C]*orotate by* Lathyrus tingitanus

With *Lathyrus*, as with *Pisum* and *Phaseolus*, the major portion of the supplied [6-^{14}C]orotate was catabolized to β-alanine. Some 74% of the orotate was degraded in this way (Table 2). *Ca* 4% of the supplied radioactivity was incorporated into the pyrimidine constituents of *Lathyrus*. This is significantly higher than in the other two species examined. The major part of that 4% consists of CMP, UMP, uridine, uracil and lathyrine, with CMP replacing UMP as the most highly radioactive product. The extent of radioactive incorporation into CMP was substantially higher in *Lathyrus* than in *Pisum* or *Phaseolus*. It was also noteworthy that the pyrimidine amino acid lathyrine was as highly radioactive as UMP.

DISCUSSION

Operation of the orotate pathway of pyrimidine biosynthesis in plants has been studied using wheat tissue [1–9], leaves of *Phaseolus vulgaris* [10, 11] and pea cotyledons [12, 13]. Present results are consistent with the functioning of this pathway in seedlings of *Pisum sativum*, *Phaseolus vulgaris* and *Lathyrus tingitanus*. With all three species examined, the main fate of the supplied [6-^{14}C]orotate was catabolism to β-alanine. Of the ^{14}C-labelled pyrimidine derivatives produced, those related to uracil predominated. With *Pisum* and *Phaseolus*, the most highly radioactive pyrimidine constituent was UMP. With *Lathyrus*, although a substantial part of the radioactivity in pyrimidine derivatives was also found in UMP, surprisingly, the major radioactive pyrimidine product was CMP. One of the most interesting aspects of the *Lathyrus* data is the relatively high degree of labelling in lathyrine. The total counts in this pyrimidine amino acid was the same as that in the quantitatively important orotate metabolite UMP. Furthermore, this means that of the total radioactivity incorporated into pyrimidine derivatives, 19.5% went into lathyrine. This confirms our previous finding [21] that lathyrine is predominantly produced via the orotate pathway rather than through a cyclization of β-hydroxyhomoarginine [22] and clearly shows that lathyrine is a major pyrimidine metabolite in this species.

The results show that the isomeric uracilylalanines, willardiine and isowillardiine, together account for 4.4% of the radioactivity incorporated into the pyrimidine constituents of *Pisum sativum* (Table 2). With *Phaseolus* seedlings, 1.3% of the total radioactivity incorporated into pyrimidine derivatives was found in 5-ribosyluracil. Whilst this is not a large proportion, it is substantially higher than would be expected for what is generally regarded as essentially a minor component of tRNA. The incorporation into 5-ribosyluracil was comparable with that into orotidine and twice that into cytidine.

From a comparative biochemistry viewpoint, there are some interesting differences to be seen between the three plant species. With *Lathyrus tingitanus*, substantially more radioactivity from [6-^{14}C]orotate was found in the pyrimidine constituents than with either *Pisum sativum* or *Phaseolus vulgaris* (Table 1). The reverse was true of the catabolism of orotate to β-alanine, which was significantly less in *Lathyrus* than in the other two species (Table 1). With *Pisum sativum*, more of the supplied [^{14}C]orotate was incorporated into a fraction consisting mainly of oligonucleotides than is the case with the two other species (Table 1). The overall recovery of radioactivity was very similar with all three plant species, only 5–7% of the ^{14}C originally supplied remaining unaccounted for. This would seem to be predominantly $^{14}CO_2$ since there was little or no radioactivity detectable in the insoluble residue after extraction. The results, especially those with *Lathyrus*, emphasize the need for considering the less well-known pyrimidine constituents of plants as well as the common ones in studies of pyrimidine metabolism.

EXPERIMENTAL

Materials. Seeds of *Pisum sativum* cv 'Meteor', and of *Phaseolus vulgaris* cv 'The Prince', were purchased from Suttons Seeds, U.K. Seeds of *Lathyrus tingitanus* were kindly provided by Mr. R. Isherwood, University College of Swansea Botanic Garden. All three species of seeds were germinated in moist vermiculite and grown at 25° in an alternating cycle of 16 hr light (6 k l ×) and 8 hr dark. Shoots were excised from 8-day seedlings and used as experimental material.

Analytical grade chemicals were purchased from BDH, U.K.; pyrimidine derivatives for reference purposes were obtained from Sigma, U.K.; and [6-^{14}C]orotic acid was supplied by Amersham International, U.K. Norit OL charcoal was obtained from Hopkin and Williams, U.K. and prepared for use as described in ref. [23]. A reference sample of lathyrine was prepared from *Lathyrus tingitanus* as previously described [21].

Uptake of [6-^{14}C]*orotate.* A soln (pH 5.5) of [6-^{14}C]orotate (sp. act. 61 mCi/mmol) was dispensed (0.1 ml aliquots) into Durham tubes such that each tube contained 0.25 μCi. At zero time, batches of 40 freshly excised shoots were allowed to take-up the radioactive soln (1 shoot per tube); transpiration was maximized by passing a slow current of air over the shoots at 25° and illuminating at 6 k l×. When the soln had been completely absorbed, 0.2 ml H_2O was added to each tube and this was also taken-up. Finally, for the remainder of the 24 hr experimental

period, the shoots were allowed to take up H_2O *ad libitum* under the same conditions of illumination and temp. The shoots were then removed and extracted.

Extraction of pyrimidine derivatives. The procedure was essentially that used for the extraction of free nucleotides [23] with minor modifications. The extractant was ice-cold 0.3 M $HClO_4$. After clarification by centrifuging (12000 *g* for 15 min at 4°) the supernatant was immediately neutralized with KOH and the ppted $KClO_4$ removed by centrifuging. Preliminary purification was achieved by adsorption of the pyrimidine constituents onto Norit OL charcoal and subsequent elution with ethanolic ammonia (25% aq. EtOH containing 5% v/v NH_4OH of sp. gr. 0.88) [23].

Adsorption and elution was effected using a batch procedure, allowing 1 g Norit OL/20 g (fr. wt) plant tissue. For adsorption, the suspension was continuously stirred for 2 hr at 4°. The charcoal was removed by centrifuging (5000 *g* for 30 min), washed twice by resuspension in H_2O, and collected by centrifuging. Elution was effected by suspending the charcoal in the ethanolic ammonia soln and stirring for 2 hr at 4°. After removing the supernatant by centrifuging (5000 *g* for 30 min) the charcoal was resuspended in fresh ethanolic ammonia and recentrifuged. This process was repeated × 4; the eluates were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in an appropriate vol. of H_2O for chromatographic and electrophoretic analysis.

Chromatography and electrophoresis. The following solvent systems were used for PC on Whatman No. 1 paper: (1) *n*-BuOH–HOAc–H_2O (12:3:5), (2) *iso*-PrOH–aq. NH_3 (sp. gr. 0.88)–H_2O (7:1:2), (3) *iso*-PrOH–conc. HCl–H_2O (130:33:37), (4) EtOH–aq. NH_3 (sp. gr. 0.88)–H_2O (8:1:1), (5) H_2O. High voltage paper electrophoresis was effected on Whatman 3MM paper by using an electrode potential of 3 kV (60 V/cm). For most purposes, an HCO_2H–HOAc buffer (pH 2) was used, which was 0.8 M with respect to HCO_2H and 0.7 M with respect to HOAc. Pyrimidine derivatives on chromatograms and electrophoretograms were detected by viewing in UV light; radioactive compounds were detected using a 'spark-chamber' apparatus (Birchover Instruments, U.K.). Ninhydrin reagent (0.2% in Me_2CO) was used to confirm location of lathyrine (red reaction), and willardiine and isowillardiine (purple reactions).

Identification of pyrimidine derivatives. Tentative identification of pyrimidine derivatives was made on the basis of their chromatographic behaviour together with their spectrophotometric properties in aq. soln at various pHs. Confirmation was obtained by cochromatography with authentic samples in the solvent systems listed above and by high voltage electrophoresis, as described.

Measurement of radioactivity. The radioactivity of duplicate aq. samples (0.5 ml) was determined in 5 ml NE 250 scintillant (Nuclear Enterprises, U.K.). Each sample was counted twice for 50 min in a liquid scintillation spectrometer with an efficiency for ^{14}C of 93%. Quench correction was by the external-standard channel ratio method.

Quantitative recovery. The overall recovery obtained with the extraction procedure described above was checked using UMP, CMP, uridine and orotidine. Recovery in each case was 95–96%. The loss was almost entirely attributable to the charcoal adsorption and elution steps.

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