Phytochemistry, 1969, Vol. 8, pp. 2295 to 2299. Pergamon Press. Printed in England

THE METABOLISM OF PURINES AND THEIR DERIVATIVES IN SEEDLINGS OF *PISUM SATIVUM*

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(*Received* 15 *May* 1969)

Abstract—The pattern of metabolism of soluble purines infiltrated into pea seedlings has been investigated. Nucleotides were converted to the corresponding nucleosides and free bases. Nucleosides were converted to the corresponding free bases. Deamination of the adenine series of compounds to the hypoxanthine series occurred at nucleotide level. Oxidation of the hypoxanthine series of compounds to the xanthine series took place at free base level, providing strong evidence for the presence of xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) in these seedlings. Sub-cellular fractions from pea seedlings were prepared and tested for activity against nucleotide substrates. Hydrolytic degradation of these nucleotides by the soluble cell fraction was observed, but all particulate fractions were inactive. The possible intracellular compartmentalization of endogenous purine derivatives is discussed.

INTRODUCTION

THE METABOLISM of soluble purines and their derivatives is well understood in certain microorganisms.[1-3] The pathways of formation of adenylic acid and guanylic acid from inosinic acid are known. Control of the production, by feedback inhibition, of these two nucleic acid precursors has also been studied.[3]

While the presence of soluble nucleotides and nucleosides, but not free purine bases, in pea seeds (*Pisum sativum*) has been established,[4,5] little is known of the metabolism of these compounds in higher plant tissues. The metabolism of 5′-guanylic acid has been studied by feeding experiments with pea seedlings, where it was found that this substance was converted to guanosine and guanine, and also led to the formation of a uracil-peptide compound.[6] Adenine catabolism has also been studied in excised leaf material and in embryos of pine species.[7,8] Other investigations have shown that relatively crude cell-free extracts of pea seeds and seedlings possessed limited activity against purine substrates. Nucleotides were converted to nucleosides, but free bases were not attacked, nor were nucleosides further metabolized.[9]

This paper describes the metabolism of purine compounds introduced to pea seedlings by vacuum filtration, and the activity of sub-cellular fractions, obtained from pea seedlings, against purine substrates.

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[6] E. G. BROWN and A. V. SILVER, *Biochem. Biophys. Acta* **119**, 1 (1966).
[7] R. L. BARNES, *Nature* **184**, 1944 (1959).
[8] R. L. BARNES, *Botan. Gaz.* **123**, 141 (1961).
[9] A. V. SILVER, Ph.D. Thesis, University of Wales (1966).

RESULTS AND DISCUSSION

Metabolism of Purines Infiltrated into Seedlings

Infiltrations were conducted with 5′-adenylic acid, 5′-inosinic acid, 5′-xanthylic acid, 5′-guanylic acid, adenosine, inosine, xanthosine, guanosine, adenine, and hypoxanthine. With the exception of adenine, all infiltrated substances gave rise to products. In the case of nucleosides and free bases the infiltrated substance was not completely metabolized by the seedlings, since some was always recovered on chromatograms. Table 1 shows the conversion products identified in each of the infiltration experiments.

5′-Adenylic acid, adenosine, xanthosine and guanosine were all found to be endogenous components of the infiltrated seedlings, since these substances were always detected on chromatograms of the extract from control groups of seedlings which had been infiltrated with water in place of purine derivative. In cases where one of these substances was identified as a product of an infiltration experiment it was always present in chromatograms of the test extract in concentrations at least twice that of control extracts.

The results obtained in these infiltration experiments were consistent with the metabolic scheme depicted in Fig. 1. All the nucleotides tested, on infiltration into 6-day seedlings, gave

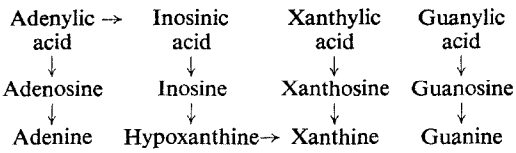

FIG. 1. SCHEME OF PURINE METABOLISM IN 6-DAY PEA SEEDLINGS INFILTRATED WITH VARIOUS PURINE COMPOUNDS.

rise to their respective nucleoside and free base. The nucleosides, likewise, gave rise to their corresponding free base (see Table 1). High activities against nucleotides were observed, since, after infiltration, no residual nucleotide was recovered on chromatograms of the test extract. With nucleosides and free bases, on the other hand, unchanged infiltrated compound was always recovered, in addition to conversion products. The only departure from the general pattern of nucleotide metabolism was found in the case of 5′-guanylic acid. Infiltration with this substance led not only to production of guanosine and guanine but also to β-(2,6-dihydroxypyrimidin-1-yl)alanine.[6, 10]

Beside the hydrolytic degradations noted above, some of the transformations observed must also have involved deamination and oxidation reactions. Thus, production of hypoxanthine derivatives from adenine derivatives involves deamination, and production of xanthine derivatives from hypoxanthine derivatives involves an oxidation step. Evidence from the infiltration experiments with adenosine and adenine indicated that deamination did not take place at nucleoside or free base level, since, in neither case were hypoxanthine derivatives detected as products. Infiltration with 5′-adenylic acid did, however, give rise to hypoxanthine (see Table 1). It would appear that deamination occurred at nucleotide level, with production of inosinic acid, which then afforded the same products as obtained on direct infiltration with this compound. Traces of a substance exhibiting selective absorption in u.v. light with a peak at 249 μm (pH 2·0) and possessing R_fs consistent with 5′-inosinic acid, were detected after infiltration with 5′-adenylic acid. If this substance was, indeed, inosinic acid, it is not surprising that only traces were detected in view of the high activity of infiltrated seedlings towards nucleotides.

[10] E. G. BROWN and B. S. MANGAT, *Biochem. Biophys. Acta* **177**, 427 (1969).

TABLE 1. CONVERSION PRODUCTS DETECTED FROM INFILTRATION EXPERIMENTS

Substance infiltrated	Products
5′-Adenylic acid	Adenosine, adenine, hypoxanthine, inosine, xanthine, inosinic acid*
5′-Guanylic acid	Guanosine, guanine, β-(2,6-dihydroxypyrimidin-1-yl)alanine
5′-Xanthylic acid	Xanthosine, xanthine
5′-Inosinic acid	Inosine, hypoxanthine, xanthine
Adenosine†	Adenine
Guanosine†	Guanine
Xanthosine†	Xanthine
Inosine†	Hypoxanthine, xanthine
Adenine†	No products
Hypoxanthine†	Xanthine

* Trace only; identification tentative.
† Some infiltrated substance recovered unchanged.
Each product was identified after chromatography in at least three solvent systems, followed by examination of u.v. spectrum and co-chromatography with standards. Control groups of seedlings were infiltrated with deionized water.

The infiltration experiments indicated that oxidation reactions occurred at free base level. Infiltration with hypoxanthine resulted in production of xanthine, as did infiltration with 5′-inosinic acid. The latter experiment, however, did not give rise to detectable amounts of xanthosine as would have been expected if oxidation occurred at nucleotide level. Similarly, infiltration with inosine, which led to formation of hypoxanthine and xanthine, again failed to produce xanthosine, thus ruling out the occurrence of oxidation at nucleoside level. It is unlikely that xanthosine was produced as an intermediate but was so rapidly metabolized that it could not be detected on chromatograms in levels greater than the controls, since infiltration with xanthosine itself, while giving rise to xanthine, always resulted in recovery of appreciable amounts of unchanged xanthosine. These data are consistent with the oxidation of the hypoxanthine series to the xanthine series taking place at free base level only.

The pattern of purine metabolism outlined in Fig. 1 and discussed above may provide information concerning the activity of xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2). This enzyme of purine catabolism has never been shown, conclusively, to be present in higher plant tissues. Previous attempts to demonstrate its presence in extracts of pea seedlings had proved negative (E. G. Brown and A. V. Silver, unpublished). The findings here, concerning the level at which oxidation of hypoxanthine to xanthine derivatives occurs, strongly suggest that infiltrated pea seedlings possess xanthine oxidase activity. It should be noted, however, that uric acid was never detected as a metabolic product in infiltrated seedlings. Nor is there any good evidence for the endogenous occurrence of this substance. Although the presence of uric acid in seeds of a number of leguminous plants has been reported,[11] Brown[5] could not find any uric acid in seeds of *Lupinus luteus*, *Phaseolus vulgaris* and *Pisum sativum*.

The results presented here contrast with reports[7] that adenine fed to excised leaves of *Acer saccharinum* L. (silver maple) was actively converted, through uric acid, to allantoin and allantoic acid, in a manner similar to the well-established sequence of purine catabolism in animal tissues. It may be noted, however, that later work on feeding experiments with embryos of various species of *Pinus*[8] showed degradation of adenine beyond the level of

[11] R. FOSSE, P. DE GRAEVE and P. THOMAS, *Compt. Rend.* **195**, 1198 (1932).

hypoxanthine was much less extensive. Furthermore, it was concluded that deamination of adenine compounds occurred at nucleoside or nucleotide level, but not at free base level. It is of interest that these latter results are more in agreement with the results presented here, since pine embryo tissues would appear more comparable with pea seedlings than would mature leaf tissue.

Activity of Sub-cellular Fractions from Pea Seedlings Against Purine Substrates

The pattern of purine metabolism revealed by the infiltration experiments was examined in the light of the observed endogenous occurrence of some of these compounds. Extracts of control seedlings contained 5′-adenylic acid, adenosine, xanthosine and guanosine, but no free purine bases. This would tend to imply that the purines introduced to seedlings by infiltration, were being metabolized at sites in the cell other than those at which endogenous purine derivatives are located. These latter were apparently avoiding the action of the degradative enzymes which produced, for example, free bases from infiltrated nucleosides. Thus it appears that the endogenous purine derivatives may be compartmentalized in the cell; possibly they are associated with one or other of the sub-cellular organelles. In this manner they could be segregated from soluble enzymes which attack the infiltrated purines.

To test this hypothesis, sub-cellular fractions were prepared and incubated with 5′-adenylic acid and 5′-inosinic acid as substrates, in an attempt to establish the intracellular location of the hydrolytic enzymes functioning in the intact seedlings. The incubations were carried out with five sub-cellular fractions obtained from 6-day seedlings, namely nuclear, chloroplast, mitochondrial, ribosomal and soluble fraction. The soluble fraction produced adenosine and adenine from substrate adenylic acid; and inosine and hypoxanthine from substrate inosinic acid. All the particulate fractions were inactive against these substrates. The experiments were repeated using fractions obtained from 3-day seedlings with identical results.

These results support the compartmentalization suggestion in that only the soluble fraction was found to be active. It may well be that the discrepancy between the content of endogenous purine derivatives and the pattern of metabolism of purines introduced by infiltration is due to the spatial separation of the endogenous purines from relatively non-specific hydrolytic activity of enzymes of the cytoplasmic fraction.

EXPERIMENTAL

Materials

Seeds of *Pisum sativum* L. var. Meteor (G. W. Yates Ltd., Manchester) were soaked in distilled water for 15 hr and grown in moist vermiculite at room temperature.

Vacuum Infiltration of Seedlings

Seedlings were harvested at the end of 6 days and sets of six seedlings were infiltrated with various purine solutions. Solutions were 15·0 mM for nucleotides, and within the range 7·0–15·0 mM for free bases and nucleosides. Six seedlings were immersed in the solution which had been brought to pH 5·5 prior to use. The pressure over the solution was lowered to 0·01 mm Hg for 15 min, then slowly allowed to return to atmospheric pressure. A control group of six seedlings was treated in the same way, using deionized water (pH 5·5) in place of the purine solution. After infiltration the seedlings were rinsed and replanted for a further 15 hr growth.

Extraction of Infiltrated Seedlings

After the 15 hr growth period following infiltration, test and control batches of six seedlings were separately extracted by a method previously described.[9]

Identification of Products from Infiltrated Substances

Comparison of the u.v. light-absorbing bands on chromatograms of control and test extracts allowed detection of metabolic products from the infiltrated substance. Such products were rechromatographed in at least two further solvent systems prior to determining their u.v. spectra. Confirmation of identity was obtained by co-chromatography with standards on thin-layer plates, using MN-cellulose powder, 300G as the absorbent layer.

Sub-cellular Fractionation of Seedlings

Seedlings were grown as previously described. Axes (30 g) from 6-day seedlings were excised, washed, and homogenized by hand in 40 ml of 5 mM-tris buffer, pH 7·1, containing 3 mM $CaCl_2$, 1 mM $MgCl_2$ and 0·45 M sucrose. The homogenate was passed through two layers of muslin and the filtrate was subjected to fractional centrifugation using an M.S.E. High Speed 18 centrifuge, all operations being carried out at 0°.

Incubation Experiments

Nuclear, chloroplast, mitochondrial and ribosomal fractions were taken up in 2·0 ml of the buffer solution used for the homogenization. 0·5 ml samples of the resulting suspensions were incubated with 0·5 ml of 0·1% (w/v) aqueous solutions of purine substrates. Controls, in which buffer replaced the substrate, and controls, in which buffer replaced the fraction, were incubated simultaneously. 0·5 ml samples of the soluble fraction were used for incubations in the same manner. Incubation was carried out at 28° for 1 hr. Reactions were halted by precipitating protein by a method previously described,[9] and aliquots of the supernatant were used for paper chromatography. Identification of products from substrates was made by the same procedures as already described.