Catabolism of Pyrimidines in Rape Seedlings [1, 2, 3]

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Thymine-2-C^{14} and uracil-2-C^{14} were previously shown by Evans and Axelrod (5) to be reductively catabolized by germinating rape seed to the corresponding dihydropyrimidines, dihydrothymine and dihydrouracil, which were in turn hydrolyzed to give β-ureidoisobutyric acid and β-ureidopropionic acid (BUP) respectively. By analogy with the reactions observed in animal tissues and microorganisms (3, 4, 8, 9, 12) it was to be expected that corresponding β-amino acids, β-aminoisobutyric acid (BAIB) and β-alanine would be formed. They were not detected in the above experiments with rape seedlings because the labelled C was lost as CO_2 following decarbamylation. Subsequently it was shown in this laboratory by the use of thymine-H^3, that in Iris BAIB originated from thymine (6).

The objectives of the experiments described here were to extend the observations with the rape seedlings using suitable labelled pyrimidines, to confirm the existence of the indicated catabolic pathways and to investigate the subsequent metabolism of β-alanine in the seedlings. While the present work was underway, Barnes and Naylor demonstrated that β-alanine arose from uracil in pine tissues (2).

Materials

The rape seeds (*Brassica napus* L. var. Dwarf Essex) were surface sterilized by immersion in 0.5 % NaOCl for 5 minutes and dried prior to use. The radio-isotopically labelled compounds were of commercial origin. Specific activities of the C^{14} compounds were of the order of 1 to 5 mc/mmole; the H^3 compounds were 100 to 500 mc/mmole. All radioactive compounds were checked for purity by paper chromatography before use. Where not specifically stated, the amount of labelled compound used in the germinations depended on its specific activity. In general, about 1 to 5 μmoles of C^{14} labelled compounds and less than 1 μmole of tritiated compounds were used per experiment. Nonradioactive carriers were not added.

Methods

Germination. Germination was carried out in covered petri dishes, 5 cm in diameter. The surface-sterilized seed (0.5 g) was placed on filter paper discs moistened with 5 ml of an aqueous solution of the labelled compound. The temperature of germination was not controlled and varied from 22° to 27°.

Homogenization and Fractionation of the Seedlings. Seedlings were washed thoroughly with distilled water and 75 % ethanol and homogenized (5 ml of 75 % ethanol) in a Potter-Elvehjem homogenizer cooled in an ice bath. The homogenate was centrifuged at $750 \times g$ for 10 minutes. The supernatant which contained the nitrogenous catabolic products and the organic acids is referred to as the "ethanol extract." The residue was washed twice with cold 100 % methanol, twice with cold 5 % trichloroacetic acid, once with cold 95 % ethanol, once with warm (80°) ethanol, twice with acetone and finally twice with ether. The warm ethanol, acetone and ether washings were combined to give the lipid extract. The washed residue was air-dried overnight to give the seedling powder from which the proteins and nucleic acids were subsequently extracted.

Separation and Identification of Nitrogenous Catabolic Products and Organic Acids. The ethanol extract was concentrated to 1.0 ml and shaken with hexane to remove any remaining lipoidal materials. In order to separate and identify the catabolic products an aliquot was chromatographed on 18 inch lengths of Whatman No. 1 filter paper. The following solvents were used: (I) *t*-butanol-methylethylketone-H_2O-HCOOH (44: 44: 11: 0.26) (8) and (II) *n*-butanol-HCOOH (95: 5) saturated with H_2O (1). Pyrimidine bases were detected by fluorescence quenching with UV light (2537 Å). Dihydropyrimidines were located with successive sprays of NaOH and *p*-dimethylaminobenzaldehyde and β-ureido acids with *p*-dimethylaminobenzaldehyde (10) and β-amino acids by dipping in 0.25 % ninhydrin in acetone. Radioactive regions were located with a Forro Radiochromatograph scanner, or a Vanguard Automatic Scanner.

For the isolation of organic acids, another aliquot of the hexane-extracted concentrate was passed through a column of cation exchange resin (Dowex 50W − H +) with the aid of gentle suction. The effluent was made 2 N with respect to H_2SO_4 and extracted continuously for 24 hours with ether in a liquid-liquid extractor. The organic acids thus obtained

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were separated by chromatography on a Celite column (21). The acid contents of the eluates were determined by titration with NaOH, and the radioactivities determined, in case of C^{14}, with a Geiger tube fitted with a Micro-mil window, or in the case of H^3, with a windowless counter. Organic acids were also separated by paper chromatography with the following solvents: (III) *n*-butanol-H_2O-HCOOH (21: 3: 1), (IV) ethanol-H_2O-NH_4OH (8:1:1) and (V) ethyl acetate-acetic acid-H_2O (3: 1: 1). The acids were located by spraying with bromophenol blue and by scanning for radioactivity.

Separation of the Lipids. The solvent in the lipid extract was removed by evaporation. The residue was redissolved in hexane. The hexane solution was washed several times with H_2O and dried with anhydrous Na_2SO_4.

Extraction and Hydrolysis of Protein. The seedling powder was dispersed in 0.2 N NaOH and stirred for 5 minutes. The suspension was centrifuged at 750 × *g* for 5 minutes, and the resultant supernatant was neutralized and mixed with an equal volume of cold 10 % (w/v) trichloroacetic acid. The suspension was held at 4° for 2 hours after which the precipitate was collected and washed twice with cold 5 % TCA. Nucleic acids were decomposed by heating the precipitate with 5 % trichloroacetic acid in a boiling water bath for 15 minutes. After this procedure was repeated 3 times, the precipitate was washed with 95 % ethanol and acetone and air-dried. When required, the protein was hydrolyzed by heating with 6 N HCl in a sealed tube at 120° for 20 hours. Amino acids were separated and identified by paper electrophoresis (Spinco, Model R-Durrum type) (17) or by paper chromatography with the following solvents: (VI) methanol-H_2O-pyridine (20: 5: 1) (23), (VII) 2,6-lutidine-ethanol-H_2O-diethylamine (55: 25: 20: 2) (25).

Results

Catabolism of Pyrimidine Bases. That the catabolic pathway for uracil in rape seedlings does indeed proceed through the expected β-amino acids is evident from the scanning diagram of the radiochromatogram obtained in an experiment in which uracil-5,6-H^3 was supplied to the seedlings (fig 1). The results obtained with uracil-2-C^{14} are shown in the same figure. In the latter case, as noted previously (5) no β-alanine was detected. Identity of the putative β-alanine was established by cochromatography with the authentic material, using 6 different solvents (solvents I, II, III, IV, V and VI). When thymine-H^3 was furnished to the seedlings, BAIB was detectable (fig 2).

In contrast to tritiated or C^{14}-labelled uracil and thymine, cytosine-5,6-H^3 did not form any detectable breakdown products, when tested in the above manner.

Formation of Organic Acids from Uracil-5, 6-H^3, Thymine-H^3, β-Alanine-1-C^{14} and β-Alanine-2-C^{14}. A new radioactive region, in addition to that attributed to the ureido amino acid, appeared when tritiated thymine or uracil was fed (fig 1 and fig 2). These new

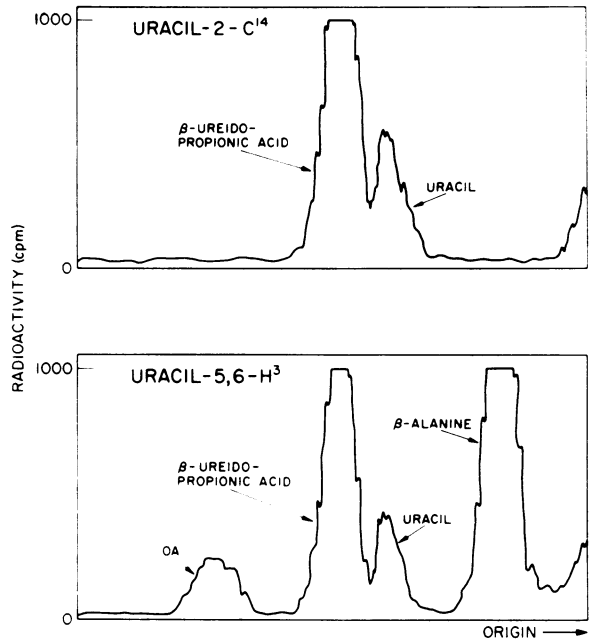

FIG. 1. Scanning diagram of radioactive catabolic products obtained from radioactive uracil. Upper curve, uracil-2-C^{14}; lower curve, uracil-H^3. Rape seed (0.5 g) was germinated with 5 ml of an aqueous solution of the pyrimidine for 72 hours. Approximately one tenth of the total reaction mixture was chromatographed on Whatman No. 1 filter paper (solvent II). OA refers to organic acid derived from uracil-H^3.

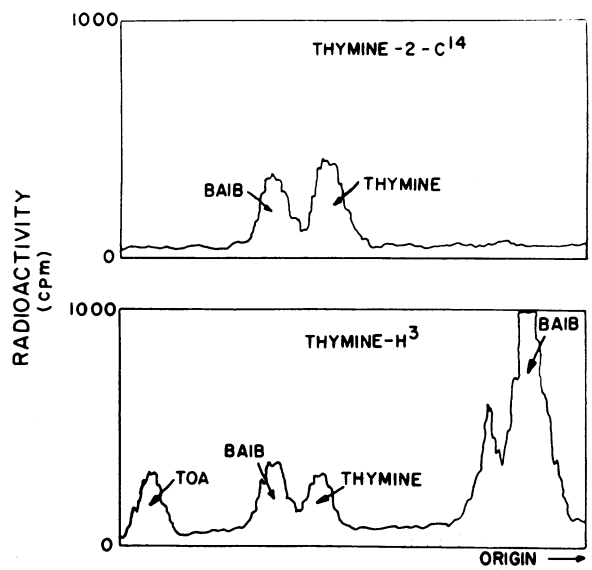

FIG. 2. Scanning diagram of radioactive catabolic products derived from radioactive thymine. Upper curve, thymine-2-C^{14}; lower curve, thymine-H^3. BAIB, β-aminoisobutyric acid; TOA refers to organic acid derived from thymine-H^3. Conditions as given for figure 1, but solvent I was used.

products, TOA, from thymine, and OA, from uracil, apparently contained acidic groups but were not amino acids for they were retained by an anion exchange resin (Dowex 1) but not by a cation exchange resin (Dowex 50). The fact that no such labelled substances were formed from the pyrimidines labelled at C-2 implied that the β-amino acids were further metabolized to organic acids. The ether-extractable acids, prepared as described above, from seedlings receiving uracil-5, 6-H^3, were chromatographed on paper, yielding 2 products, UOA_1 and UOA_2 (fig 3). When β-alanine-2-C^{14} was given in a similar experiment 3 radioactive areas shown as BOA_1, BOA_2 and BOA_3 were detected (fig 3). The mobilities of

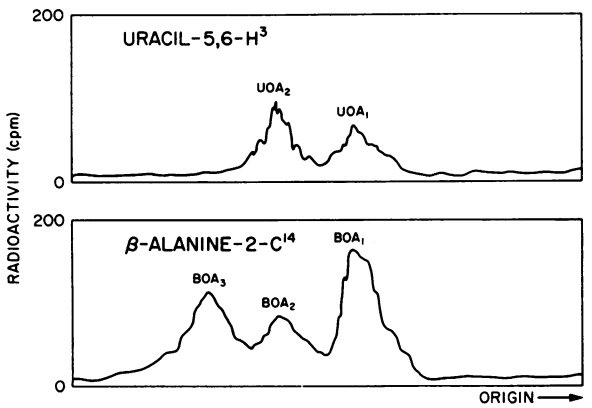

FIG. 3. Scanning diagram of acidic catabolic products obtained from uracil-H^3 (upper curve) and β-alanine-2-C^{14} (lower curve). An aliquot (about 100 μl of the ethereal extract) (see text) obtained from 0.5 g rape seeds, germinated as above in the appropriate solutions, was chromatographed with solvent III. UOA_1 and UOA_2 refer to organic acids derived from uracil-H^3; BOA_1, BOA_2 and BOA_3 to organic acids derived from thymine-H^3.

UOA_1 and UOA_2 matched those of BOA_1 and BOA_2, respectively.

An aliquot of the ethereal extract obtained from rape seedlings receiving β-alanine-C^{14} was chromatographed on Celite with the results as shown in figure 4. A somewhat similar elution pattern was obtained when uracil-H^3 was fed. Chromatographic comparisons with knowns indicated, in both cases, the presence of pyruvic acid in the peak P material and citric acid in the peak C material. Paper chromatography with solvent I showed that the mobility of a high proportion of the radioactivity in peak M resembled BOA_1 and that of peak B resembled BOA_2. Furthermore, BOA_1 from its behavior on Celite chromatography as well as on paper chromatography appeared to be malic acid. Its identity as malic acid was confirmed by 2-dimensional cochromatography with radioactive BOA_1 using solvents III and IV. Perfect correspondence of the autoradiographic image with the acid region as revealed with phenol blue, was

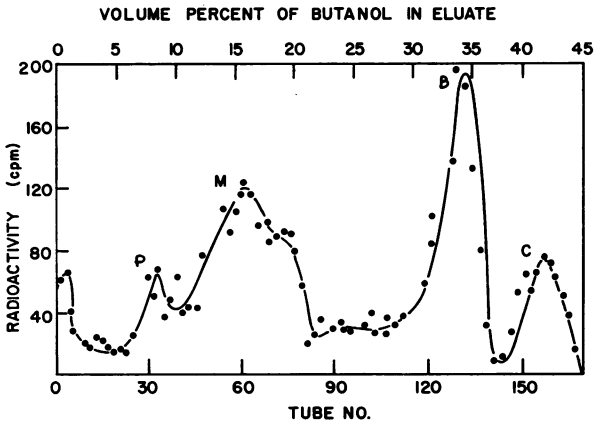

FIG. 4. Fractionation of organic acids arising from β-alanine-2-C^{14}. Chromatography was carried out on Celite as described under Methods. Ether-extracted material, corresponding to 0.25 g seed (original weight) was used. The seed was germinated with 2.5 μmoles of β-alanine-2-C^{14}.

observed. To confirm the identity of the unknown, BOA_1 was converted to the hydroxamate (19) which was found to migrate together with authentic malic acid hydroxamate when chromatographed 2-dimensionally on paper using solvent III.

The fact that BOA_3 was formed from β-alanine-2-C^{14} but not from uracil-5, 6-H^3 suggested that labilization of the labelled hydrogens must have occurred during the transformation. When β-alanine-1-C^{14} was fed, only labelled BOA_2 was detected. The identities of BOA_2 and BOA_3 were not established.

Formation of Lipids from β-Alanine-2-C^{14} and Acetate-1-C^{14}. The lipid fraction obtained from rape seedlings which had been germinated in β-alanine-2-C^{14} was separated by silicic acid chromatography into sterol esters, triglycerides, sterols, fatty acids and phospholipids by the procedure of Fillerup and Mead (7). All 5 fractions were found to be labelled, the sterols and phospholipids being highest with 31 % and 34 % of the radioactivity, respectively (fig 5). The fact that lipids, in general, and sterols in particular, were formed from β-alanine, suggested that the amino acid must have been first converted to acetate. In support of the foregoing argument, the same pattern was observed (fig 5) when the experiment was repeated with acetate-1-C^{14}. When however β-alanine-1-C^{14} was fed, the relative amount of radioactivity found in the lipid fraction was greatly reduced. A predominant portion of the activity was found in the phospholipid fraction. If indeed β-alanine were being converted to acetate, then it should undergo appreciable decarboxylation in the process. From inspection of figure 5, it is apparent that the phospholipid derived from β-alanine-1-C^{14} was highly labelled compared to the other lipid fractions. The possibility that a portion of the radioactivity of the phospholipid was associated with the nonfatty acid portion was examined. Phospholipid fractions derived

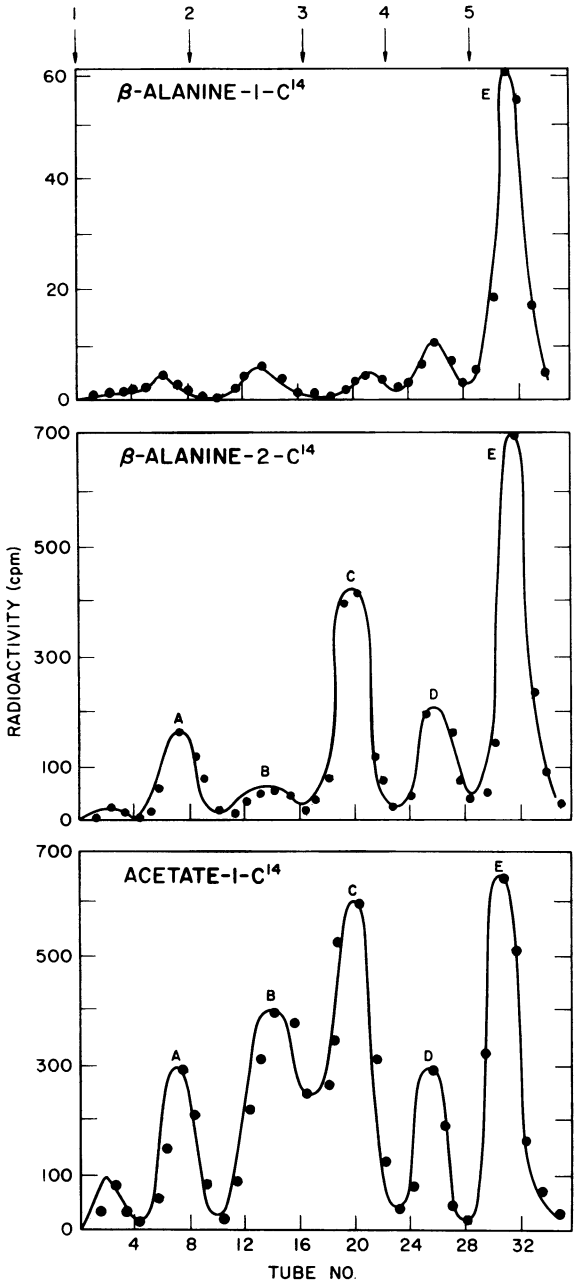

FIG. 5. Separation of radioactive lipid fractions derived from β-alanine-2-C^{14}. The lipid fraction which was obtained from 1.0 g of rape seed which had been germinated 72 hours in 2 ml H_2O containing 3.5 μmoles (2.1×10^6 cpm) of β-alanine was further fractionated on a silicic acid column. (See Methods). Arrows indicate where eluant was changed. 1, 1 % ether in petroleum ether. 2, 4 % ether in petroleum ether. 3, 10 % ether in petroleum ether. 4, 50 % ether in petroleum ether. 5, 25 % methanol in ether. (Middle curve) Conditions same as for upper curve except that 5.0 μmoles (2.2×10^6 cpm) of β-alanine-2-C^{14} were used. (Lower curve) Conditions same as for upper curve except that 0.11 μmole (2.8×10^6 cpm) of sodium acetate was used.

from β-alanine-1-C^{14}, β-alanine-2-C^{14} and acetate-1-C^{14} were isolated as above and hydrolyzed by heating with 6 N methanolic HCl at 80° for 3 hours. After removal of the methanol and the HCl, the hydrolytic products were partitioned between water and chloroform, and the radioactivities of the 2 fractions were measured. The relative activity of the water-soluble fraction was considerably greater with β-alanine-2-C^{14} or acetate-1-C^{14} (table I), despite the fact that

Table I. *The Distribution of Radioactivities in Aqueous and Chloroform Phases after Hydrolysis of Phospholipids*

Compound fed	Phase	Percentage of radioactivity
Acetate-1-C^{14}	Water	6.5
	Chloroform	93.5
β-Alanine-2-C^{14}	Water	11.8
	Chloroform	88.2
β-Alanine-1-C^{14}	Water	28.2
	Chloroform	71.8

the efficacy of labelling of phospholipid with β-alanine-2-C^{14} was estimated to be 6 times greater than with β-alanine-1-C^{14}. Similar comparisons between acetate-1-C^{14} and the β-alanines were not made because pool sizes were not determined.

Incorporation of β-Alanine-1-C^{14}, β-Alanine-2-C^{14} and Acetate-1-C^{14} into Protein. The isolation of labelled organic acids, including malic acid, suggested the possibility that uracil could participate in the formation of amino acids, presumably by formation of keto acids which could be transaminated. The protein fractions were extracted from the seedling powders from plants fed β-alanine-C^{14}, β-alanine-2-C^{14} and acetate-1-C^{14}. The degree of label incorporated into protein is shown in table II. Again β-alanine-2-C^{14} was a more effective precursor than β-alanine-1-C^{14}. Comparisons between the effectiveness of β-alanine and of the acetate as precursors were not made because pool sizes were not determined. A preliminary examination of the protein hydrolyzate showed that activity actually resided in the α-amino acids and was not due to contamination by β-alanine. After paper electrophoresis at pH 6.4, the neutral and acidic regions were found to be radioactive. The acid fraction after elution was cochromatographed with authentic amino acids using solvents VI and VII, and shown to contain labelled glutamic and aspartic acids.

Demonstration of Cytidine Deaminase Activity in Rape. Four-day-old rape seedlings (30 g fr wt) were ground in a mortar with 30 g acid-washed sand and 30 ml Na citrate buffer (pH 6.0, 0.05 N). The liquid obtained by squeezing the ground mass through cheese cloth was centrifuged at $900 \times g$ for 15 minutes to remove the particulate matter and the oily material. A qualitative test for cytidine deaminase was performed by mixing 0.2 ml of the extract with 4 mg of cytidine in 0.2 ml H_2O and incubating at 24° for 20 hours. Toluene was added as a preservative. The reaction mixture was spotted on chromatographic

Table II. *Incorporation of Radioactive β-Alanine in Protein*

Rape seeds (0.5 g) were germinated in 1.0 ml solution of the indicated radioactive β-alanines for 72 hours. Isolation of the protein is described in Methods.

	Radioactivity cpm		Percentage of input incorporated
	Input	Incorporated	
β-Alanine-2-C^{14}	2.10×10^6	5.10×10^3	0.24
β-Alanine-1-C^{14}	1.10×10^6	0.58×10^3	0.05

paper at zero time and after 20 hours' incubation. The chromatograms were developed in butanol and in isopropanol-H_2O-HCl (24). A substance corresponding to uridine in mobility was formed during the incubation. The absorption spectra of this substance in H_2O and in dilute NaOH matched those of authentic uridine. Approximately 10 % of the original cytidine substrate was converted to uridine. Uridine was not detected at zero time nor when cytidine was omitted. Cytosine tested under similar conditions did not undergo deamination. No deaminase activity was detected in extracts prepared from ungerminated rape seeds.

Demonstration of β-Alanine-L-glutamic Acid Transaminase in Rape Seedlings. Rape seedlings, 4 days old, were extracted by grinding with equal weights of K phosphate buffer (0.067 mole, pH 7.4) and sand. The brei was filtered through cheese cloth and centrifuged for 10 minutes at $748 \times g$. The precipitate and the fatty floating layer were discarded and the extract dialyzed against rapidly flowing tap water for 3 hours. Transaminase activity was qualitatively demonstrated in the following reaction mixture: 0.2 ml extract, 0.04 μmole pyridoxal phosphate, 20 μmoles β-alanine and 20 μmoles α-ketoglutarate in a final volume of 0.7 ml which was adjusted to pH 7.4. Several drops of toluene were added as preservative. Samples of 10 to 20 μl of reaction mixture were chromatographed on paper together with appropriate reference amino acids using phenol-NH_4OH (20). Incubation of the reaction mixture for 90 minutes at 23° resulted in the production of readily detectable quantities of glutamic acid plus traces of γ-aminobutyric acid. The latter compound presumably arose by decarboxylation of glutamic acid by glutamic acid decarboxylase. No glutamic acid was detected when β-alanine, α-ketoglutarate, or extract was omitted. When α-ketoglutarate was replaced by pyruvate, oxalacetate or phenylpyruvate, the corresponding amino acids did not appear.

Discussion

It thus seems to be clearly established that in rape seedlings uracil and thymine can not only be used as a source of polynucleotide pyrimidines (5) but they can also be degraded through the dihydropyrimidines to the corresponding β-amino acids via the Fink pathway. The subsequent metabolism of β-alanine proceeds as if this compound were degraded to acetate by the loss of the carboxyl carbon. This belief is based on the following observations: Radioactive fatty acids and steroids which are known to arise from acetate, are formed from β-alanine-2-C^{14}. The gross distribution of the radioactivity among the several lipid fractions separated by Celite chromatography after germination with β-alanine-2-C^{14} resembles that found with acetate-1-C^{14}. β-Alanine-1-C^{14} is much less efficacious than β-alanine-2-C^{14} as a source of radioactivity in the lipid formed.

The formation of radioactive malate, lipids and organic acids from uracil-5, 6-H^3 is consistent with the concept that the β-alanine derived from the uracil gives rise to labelled acetate. The labelling of malate and citrate by acetate can occur with the aid of the Krebs cycle enzymes, and malate may also be formed by intervention of the glyoxylate pathway.

The pathway leading from β-alanine to acetate (or acetyl CoA) in higher plants is not yet elucidated. Some years ago Pihl and Fritzon (18) discovered that β-alanine was metabolized by the rat to acetate and CO_2. More recently it was shown by Hayaishi et al. (15) that in *Pseudomonas fluorescens* β-alanine was converted by transaminase action to formylacetic acid and then by oxidative decarboxylation in the presence of diphosphopyridine nucleotide and CoA to acetyl CoA. The present finding that a β-alanine transaminase appears in rape seeds on germination reinforces the speculation that the metabolism of β-alanine may occur by a similar pathway. Indeed, Hatch and Stumpf (14) observing CO_2 formation and β-alanine formation from C^{14} labelled propionate in several plants, postulated that formylacetic acid was an intermediate and suggested the existence of a β-alanine transaminase. β-Alanine transaminases have not been previously reported in higher plants. In mammalian tissues (15) and in *Clostridium propionicum* (13) the transamination partner can be either L-α-alanine or L-glutamic acid while in *Pseudomonas fluorescens* only the former is effective. In rape seedlings only L-glutamic acid is the partner. The pathway could explain the formation from β-alanine-2-C^{14} of labelled acetate, malate, citrate and pyruvate, lipids and sterols, and asparate and glutamate. It could also explain the superiority of the 2-C^{14} isomer compared to the 1-C^{14} isomer as a precursor of radioactive lipids, and finally it could explain the formation of radioactive malate from uracil-5, 6-H^3, inasmuch as the latter has been shown to give rise to β-alanine.

In the course of the growth and development of the organism it is to be expected that polynucleotides and sugar nucleotides will be broken down. It can be imagined that the presence of pyrimidines (or their

simple derivatives) may interfere with the control mechanisms concerned with the formation of new polynucleotides and sugar nucleotides, if the appearance of these pyrimidines is not subject to the controls regulating their synthesis. The degradative pathways which provide for the disposal of the waste pyrimidines may therefore have the important function of preventing interference with, or circumvention of, the regulatory mechanisms. However cytosine, unlike the hydroxypyrimidines, uracil and thymine, is not degraded by the seedlings when externally supplied. The failure to observe degradation is not due to lack of absorption by the seedling for it has been shown that added cytosine is incorporated into polynucleotide (22). It is suggested that cytidine deaminase, whose presence has been shown in rape seedlings, converts the cytosine moiety to uracil which is subsequently freed and degraded by the pathway described.

Summary

Externally added thymine and uracil were catabolized by rape seedlings through the 5,6-dihydropyrimidines and β-ureidoamino acids to give β-aminoisobutyric acid and β-alanine, respectively.

Uracil-5, 6-H^3 and β-alanine-2-C^{14} gave rise to labelled carboxylic acids, including malic, pyruvic and citric acids.

β-Alanine was also incorporated into lipids and sterols. Results of comparative studies with β-alanine-1-C^{14} were explicable on the basis of the conversion of β-alanine to acetate by a mechanism involving loss of its carboxyl carbon.

The possibility that the degradation of β-alanine proceeds through formylacetic acid was supported by the demonstration of a β-alanine-L-glutamic acid transaminase in the rape seedlings. This is apparently the first report of a β-alanine transaminase in higher plants.

No degradation products were detected when cytosine was furnished to the seedlings. Cytidine deaminase was demonstrated in the seedlings but cystosine deaminase was not detected. No cytidine deaminase was found in the ungerminated seed.

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