ments were examined by combining plants treated for different durations and at different stages of development, it was found that exposure to 32° C (90° F) reduced yield more than 29° C (85° F) and 29° C (85° F) more than 27° C (80° F).

Exposure to high temperature for 6-hour periods in the middle of the light period on five consecutive days reduced yield more than three days, and three days more than one day.

Plants treated at five days after full bloom were most severely affected by high temperature.

Plants treated five days after full bloom at 27°, 29°, or 32° C (80°, 85°, or 90° F) and for one, three, or five days were lower yielding than comparable treatments at other stages of development.

Within each of the four stages of development studied, fresh weight of peas was reduced more with increasing temperature from 27° to 29° to 32° C and with increasing duration from one to three to five days.

Within each temperature, the longer the treatment, the less the yield and for each of the durations, the higher the temperature, the lower the yield.

The practical application of results obtained in this study are discussed in view of breeding heat-resistant varieties of peas and selecting suitable areas for growing peas on a commercial scale.

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ORNITHINE, CITRULLINE, AND ARGININE METABOLISM IN WATERMELON SEEDLINGS[1]

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Arginine occurs widely both in the free form and in proteins of higher plants but there is little information on its metabolism in plants. The discovery and isolation of citrulline from the watermelon fruit by Wada (18), and the studies of Krebs and Henseleit (11) led to the formulation of the ornithine cycle. Since then much attention has been devoted to arginine metabolism in animals and a number of microorganisms (12), and its catabolic and anabolic reactions are in some cases well established. Coleman and Hegarty recently reported upon the metabolism of ornithine in potassium deficient barley (4, 5).

Ornithine, citrulline, arginine, and urea have been found in some plants but, except as cited above, their relationships to each other and metabolic interconversions have not been studied. The purpose of this investigation was to gain information concerning the interconversion of these compounds in the watermelon seedling.

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Methods

Watermelon seedlings (var. Klondike) were used for this study. Seeds previously treated with "Semesan" fungicide were soaked for 48 hours in aerated tap water and germinated on cheesecloth as described in an earlier publication (9). The combined sprouts and roots were vacuum infiltrated with solutions of labeled and unlabeled compounds. Extracts of the plant material were studied for both qualitative and quantitative differences.

Vacuum Infiltrations: Four to 10 g fresh weight of seedlings were cut into approximately 2-cm pieces

and immersed in solution in a vacuum desiccator. A vacuum of about 10-mm pressure was produced, held 10 to 15 minutes and air was allowed to re-enter the container rapidly. The procedure was repeated. The plant material was rinsed with water and placed in a moisture-saturated atmosphere in a north window for a suitable incubation period. The plant material was extracted with boiling 70 % ethanol and the extracts were prepared for analysis by evaporation to dryness and resolution in water as described previously (9). Non-labeled compounds, DL-ornithine monohydrochloride, L-citrulline, L-alanine, DL-aspartic acid, ammonium chloride, and guanidinoacetic acid were used as 0.01 M solutions in sufficient volume to immerse the plant parts. The labeled compounds prepared as 0.001 M solutions were: DL-ornithine monohydrochloride-2-C^{14}, L-arginine-C^{14} (Random), and urea-C^{14}.

HOMOGENATE PREPARATION: For experiments in which homogenates were used, material was prepared in a blender according to the procedure of Mueller, Quinn, and Reuckert (13). Twenty five to fifty grams of plant tissue were ground for two to three minutes in a chilled blender with an equivalent weight of water at 0° C. The resultant homogenate was filtered through several layers of cheesecloth and the filtrate used immediately.

ANALYTICAL METHODS: Qualitative comparisons were made with 1- and 2-dimensional chromatography using the methods and indicator reagents described (9). The chromatographic solvents most generally used were *N*-butanol : acetic acid : water (4 : 1 : 5), water saturated phenol, and 95 % ethanol : NH_4OH (95 : 5). Citrulline and arginine were separated from each other on chromatograms with the solvent system methanol : triethanolamine : water (80 : 4 : 16). The multiple descending chromatographic technique suggested by Block, et al, (2) with the non-aqueous phase from a mixture of *N*-butanol : phenol : acetic acid : water (20 : 20 : 8 : 40), was used to separate ornithine, lysine, histidine, glycine, serine, and aspartic acid.

In order to determine the specific activities of amino acids labeled with C^{14}, they were usually first separated by elution from a Dowex-50 (cation) column with hydrochloric acid according to the methods of Stein and Moore (14). The fractions that contained a particular amino acid were bulked and evaporated in vacuo at 50 to 60° C, or by hot air. The residues were further purified by band paper chromatography. The area containing the amino acid was cut out and eluted from the paper with water. Each amino acid was purified in this manner with at least two suitable solvents and the final products were characterized further on 2-dimensional chromatograms with color tests (9).

Arginine was recovered from infiltrated samples in some cases by precipitation from the extract solutions by the flavianic acid method of Vickery (16). Precipitations were carried out in small tapered centrifuge tubes and the precipitates were recovered by centrifugation. The washed precipitates were dissolved in 1 ml of 4 M HCl, further purified by band paper chromatography in butanol : acetic acid : water with subsequent recovery of arginine by elution from the paper with water. Citrulline was obtained from the supernatant solutions after the precipitation of arginine by adding excess $Ba(OH)_2$ to precipitate barium flavinate. Excess barium was removed as the carbonate and the solution was treated with 4 % phosphotungstic acid according to the procedure used by Wada (18). The supernatant solution was band chromatographed in each of the three solvents, phenol, butanol : acetic acid : water, and phenol : isopropanol : water (70 : 5 : 25). The product on chromatography in a number of solvents, gave single ninhydrin- and *p*-dimethylamino-benzaldehyde (7) spots and negative reactions to the vanillin (6) and Sakaguchi (1) tests.

The ninhydrin method of Troll and Cannon (15) was used for quantitative estimation of single amino acids for specific activity determinations. A standard curve and suitable blanks were run simultaneously for each amino acid. Duplicate determinations were made, which gave variations between duplicates of $\pm$ 10 %.

The quantities of ornithine and proline were measured by the method of Chinard (3). Citrulline was determined by the carbamidodiacetyl method of Knivett (10). Arginine was measured in plant extracts or purified solutions by a modification of the method of Sakaguchi (8).

RADIOACTIVITY MEASUREMENTS: Radioactivity determinations were made with a thin end-window Geiger tube. Aliquots of known concentration were evaporated on copper planchets and counted to give a probable counting error of 2 % or less. The weights of the compounds on the planchets were negligible so that no corrections were made for self absorption.

RESULTS

INFILTRATION OF UNLABELED COMPOUNDS: Preliminary qualitative and quantitative comparisons were made of extracts from seedlings infiltrated with citrulline, ornithine, or water. No appreciable effect of this treatment on the concentration of arginine was noted and the qualitative distribution of amino acids was unchanged except for the presence in the water infiltrated seedlings of an unknown Sakaguchi-positive substances having an R_f value on butanol chromatograms half that of arginine. This substance was absent in the citrulline and ornithine infiltrated samples.

INFILTRATION OF LABELED COMPOUNDS: Since preliminary studies with N^{15} labeled ammonium ion indicated that a comparatively rapid interconversion of these amino acids did take place, various treatments were made with C^{14} labeled ornithine, arginine, and urea.

DL-ORNITHINE-2-C^{14}: A number of amino acids were isolated from the ornithine-infiltrated seedlings and their specific activities determined. The results

TABLE I

SPECIFIC ACTIVITIES OF AMINO ACIDS ISOLATED FROM WATERMELON SEEDLINGS INFILTRATED WITH 0.001 M DL-ORNITHINE-2-C^{14} *

AMINO ACID	SPECIFIC ACTIVITY (CPM/μG-ATOM CARBON)	CONCENTRATION MICROMOLES/G FRESH WEIGHT
Ornithine	22,600	...
Citrulline	238	0.9
Arginine	36.6	0.9
Proline	36.0	0.2
Lysine	11.0	0.9
Glutamic acid	11.2	1.0
Alanine	10.3	2.6
Threonine	7.3	0.5
Glycine	10.5	0.9
Valine	1.6	0.7
Serine	< 5.0	1.5
Histidine	< 0.2	0.3
Aspartic acid	< 1.5	1.1

* Incubation time: 24 hr.

are tabulated in table I along with the concentrations of free amino acids found in the seedlings.

The majority of the amino acids contained less than 30 cpm per micromole radioactivity. Compounds known to be metabolically related to ornithine in other organisms were highly labeled. Citrulline contained the largest amount of radioactivity, about 6.5 times that of arginine on a per mole basis. In the seedlings there were approximately equal quantities of arginine and citrulline in the free form.

Glutamic acid and lysine were labeled to an intermediate degree. The specific activity of proline was about three times that of glutamic acid but the latter was present in concentrations five times those of proline.

L-ARGININE-C^{14} (U): Uniformly-labeled L-arginine was infiltrated and the specific activities of some of the amino acids, including ornithine and citrulline, were determined. After infiltration two lots were incubated 3 and 24 hours respectively. The results are presented in table II.

By the end of three hours appreciable activity had appeared in a number of amino acids. Ornithine was most highly labeled with a specific activity almost 50 times that of citrulline. Proline was also somewhat higher in activity than citrulline and lysine and histidine had significant labeling.

After 24 hours, considerable equilibration had taken place so that the differences between ornithine and citrulline were no longer evident.

Urea was isolated from the plant extract of the three-hour lot after the addition of 0.5 micromole of authentic urea. The compound was separated on a Dowex-50 column and further purified by band paper chromatography in butanol : acetic acid : water and *N*-butanol : 95 % ethanol : water (80 : 20 : 20) on Whatman no. 1 papers previously washed with 1 % oxalic acid and water. Paper chromatography with *p*-dimethylamino-benzaldehyde treatment of extracts from non-infiltrated seedlings showed there was less than 0.02 micromole urea per gram of seedlings. The urea isolated from the arginine-infiltrated seedlings contained a small but definite radioactivity, which was estimated to be 140 cpm per micromole of in situ urea.

Urea-C^{14} was infiltrated and after 24 hours incubation time the free amino acids were isolated and their specific activities determined. The results are shown in table III.

Appreciable quantities of the radioactive isotope appeared in a number of amino acids. The long incubation time required resulted in considerable randomization of carbon so that it is difficult to discern the pathway of entry. The comparatively high label in citrulline (approximately 8 times that of ornithine or arginine) certainly suggests that CO_2 fixation was primarily responsible for incorporation of the label.

EXPERIMENTS WITH HOMOGENATES: Additional experiments conducted with crude homogenates indicated that isolation of reactions involved in these interconversions is possible. The extent to which ornithine is converted to citrulline and arginine is far below the activity level of intact leaf disks. Transfer of label from ornithine to proline proceeds readily.

Efforts were made to evaluate the role of carbamyl phosphate in the synthesis of citrulline from ornithine by crude homogenates. The non-enzymatic formation of citrulline was sufficiently rapid, however, to mask any enzyme effects. A further evaluation of this and other reactions of the homogenates is contemplated.

DISCUSSION

Although ornithine and citrulline have been demonstrated to occur in a number of plants (9, 4, 5, 17) the metabolic role of these amino acids in the plant is not clear. Coleman and Hegarty (5) have demonstrated the conversion of carbamyl labeled citrulline to arginine by barley and white clover and it appears

TABLE II

SPECIFIC ACTIVITIES OF AMINO ACIDS ISOLATED FROM WATERMELON SEEDLINGS INFILTRATED WITH 0.001 M L-ARGININE-C^{14} (UNIFORMLY LABELED)

AMINO ACID	SPECIFIC ACTIVITY PER CARBON (CPM/μG-ATOM CARBON)	
	3 HR INCUBATION	24 HR INCUBATION
Arginine	12,500	1,150
Ornithine	547	163
Citrulline	11.2	150
Proline	52.0	...
Lysine	16.7	< 3.0
Histidine	14.3	< 3.0
Glutamic acid	2.5	16.6
Alanine	4.0	9.0
Threonine	...	18.0
Aspartic acid	...	6.0
Glycine	...	< 5.0
Serine	...	< 0.3

TABLE III

SPECIFIC ACTIVITIES OF AMINO ACIDS ISOLATED BY DOWEX-50 COLUMN AND PAPER CHROMATOGRAPHY FROM WATERMELON SEEDLINGS INFILTRATED WITH 0.001 M UREA-C^{14} *

AMINO ACID	SPECIFIC ACTIVITY (CPM/MICROMOLE)
Arginine	8.5
Ornithine	8.0
Citrulline	71
Alanine	320
Proline	320
Lysine	75
Aspartic acid	38
Serine	37
Glutamic acid	30
Leucines	28
Glycine	23
Histidine	22
Valine	39
Threonine	29

* Incubation time: 24 hr.

evident from the foregoing experiments that the complete sequence of Krebs-Henseleit conversion takes place in the watermelon seedling. Although both the formation and utilization of urea is demonstrated, it would appear unlikely that the role of this reaction sequence has much in common with its function in animals since the elimination of catabolic nitrogen via urea is not to be considered a normal plant function. It appears that ornithine and citrulline are intermediate in the synthesis of arginine in the plant, the latter presumably being utilized in protein synthesis. The role of arginase and urea formed by its action, however, remains obscure. Because with the specific activities a comparatively long incubation time was necessary to demonstrate significant conversion of infiltrated ornithine or arginine in the plant, a considerable randomization of the label occurred. Nevertheless, the distribution of activities observed after an appropriate incubation period serves to indicate that the reaction sequence is similar to that in animals. When isotopically labeled urea was infiltrated, the label appeared not only in citrulline to a considerable extent, but also in other amino acids which could be expected to become labeled as a result of CO_2 fixation. It is suggested that CO_2 from the breakdown of urea is responsible for the label appearing in arginine rather than a reversal of the arginase reaction. The disparity between the specific activities of proline and glutamic acid is not explained.

When seedlings were infiltrated with arginine and incubated for only three hours, proline was highly labeled in comparison with glutamic acid. The conclusion is implied that proline was derived from some intermediate other than glutamic acid, presumably glutamic semi-aldehyde, although this was not demonstrated directly. Moreover, it is impossible to ascertain from experiments of this type the comparative sizes of the various amino acid pools and the fractions of these pools which are metabolically active. Only the general close metabolic relationship between proline and citrulline can be inferred.

SUMMARY

1. Watermelon seedlings and homogenates were incubated with isotopically labeled ornithine, arginine, and urea in order to follow the course of synthesis and breakdown of Krebs-Henseleit amino acids in that plant.

2. A rapid interconversion of arginine, ornithine, and citrulline takes place. Urea is split from arginine and presumably is further hydrolyzed to CO_2 and ammonia. These can then be fixed with the formation of citrulline and arginine from ornithine via reactions analogous with those in animals.

3. On the basis of comparative specific activities, the close metabolic relationship of glutamic acid and proline to ornithine in higher plants is inferred. Specific activity of these amino acids precluded glutamic acid being intermediate between ornithine and proline.

4. Relationships between these reactions and similar reactions in animals and microorganisms are discussed.

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TRIPHOSPHOPYRIDINE NUCLEOTIDE DIAPHORASE FROM WHEAT GERM[1,2]

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The term "diaphorase" has been used in a variety of ways and consequently has taken on a number of different meanings. It was originally applied by Straub to a solubilized flavoprotein, obtained from mammalian heart muscle. The enzyme catalyzed the transfer of electrons from reduced diphosphopyridine nucleotide (DPNH) by way of flavin-adenine-dinucleotide (FAD) to an artificial electron acceptor, such as methylene blue or an indophenol derivative (6, 13, 19, 20, 21). The important point was that neither cytochrome c nor oxygen nor any other naturally occurring substrate could readily serve as an electron acceptor in place of the dyes. Subsequently, other purified flavoproteins have been found to oxidize pyridine nucleotides and to reduce dyes. In virtually all of these systems, however, natural substances could replace the synthetic acceptor. Thus the term "diaphorase activity" has been applied by some investigators to such enzymes as cytochrome c reductase when a dye was used as the electron acceptor in place of cytochrome. Strictly speaking, this use is not in keeping with the original designation of the term, since the enzyme can use a naturally occurring acceptor, namely cytochrome c.

The important problem concerning diaphorase, however, is the question of its role in the living cell. Minor modifications of Straub's isolation procedure have been found to yield a cytochrome c reductase (8, 16). Edelhoch et al (8) suggested that the native diaphorase protein may have cytochrome c reductase activity but that this is lost during the purification procedure of Straub. Mahler et al (16) have indicated that cytochrome c reductase and diaphorase proteins differ only in the steric arrangement around the site of binding cytochrome c to the enzyme. The differences were also tentatively ascribed to a removal of iron from the cytochrome c reductase to yield a diaphorase (14). While the relationship between these two systems is still not clear, it is possible that one is derived from the other.

Until very recently evidence for the occurrence of a reduced triphosphopyridine nucleotide (TPNH) diaphorase was almost completely lacking, Curran et al (6) having mentioned a very low activity of Straub's diaphorase with TPNH. Abraham and Adler (1) compared the DPNH- and TPNH-diaphorase activities of the enzyme prepared from heart muscle and adrenal gland according to Straub's purification procedure and observed very little activity of the enzyme with TPNH. Mahler et al (16) reported that TPNH-cytochrome c reductase will also reduce dyes. The first truly specific TPNH-diaphorase was found in spinach leaf chloroplasts by Avron and Jagendorf (2, 3). Evans (9) reported on a TPNH-diaphorase from soybean leaves at the 1956 American Institute of Biological Sciences meetings.

The present paper elaborates on an earlier report (4) given at the same meetings describing the purification and characterization of a TPNH-specific diaphorase from wheat germ. A DPNH-specific diaphorase is also present. There appears to be no requirement for flavin or metallic ions. Evidence is also presented to show that a heat stable factor(s) in wheat germ will convert the TPNH-diaphorase and the DPNH-diaphorase into enzymes having cytochrome c reductase activity. Menadione (the nucleus of vitamin K) and a number of other quinones can also serve in place of the naturally occurring substance(s).

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