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INCORPORATION OF RADIOACTIVE GLUTAMIC ACID INTO THE PROTEINS OF HIGHER PLANTS

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It has now been demonstrated that enzymes associated with plant mitochondria are able to utilize the respiratory energy conserved by the mitochondria in adenosinetriphosphate (ATP) (3) for the incorporation of glutamic acid into the amide, glutamine (9), and the peptides, γ-glutamylcysteine (11) and glutathione (10). During the course of these investigations, it was noted that mitochondria incorporate radioactive amino acids into their own protein (12). The incorporation process resembles that of peptide bond synthesis in its dependence on ATP. This suggests that further understanding of protein turnover in plants might be obtained by systematic study of the incorporation of labeled amino acids into plant proteins. The only previously recorded observations on radioactive tracer incorporation into plant protein have been those of Arreguin et al (1), using C^{14}-acetate, Racusen and Aronoff (7) with $C^{14}O_2$, and Boroughs and Bonner (4) with C^{14}-leucine and -glycine. In the first two cases mentioned, the substances used were probably converted to amino acids before incorporation into protein. The present investigation was concerned chiefly with establishing some of the overall characteristics of the process of amino acid incorporation in plants. Glutamic acid was utilized for these initial studies as previous results (12) indicated that it is incorporated into protein at relatively high rates even in a cell-free system.

EXPERIMENTAL

The seedlings used in the present studies were germinated and grown in the dark at 25° C as described previously (10). Certain of the mature tissues employed were obtained from local markets. The sectioned tissues were incubated in 0.05 M potassium phosphate buffer (pH 6.8) at 25° C with 1 to 100 microcuries (μc) of either 0.01 M potassium glutamate-2-C^{14} or 0.01 M uniformly labeled potassium glutamate-C^{14}. The amount of radioactivity employed was varied according to the needs of the separate experiments. At the end of the incubation period, the tissue sections were washed free of external amino acid in running water and ground in 0.05 M potassium phosphate buffer (pH 7.5) containing 0.45 M sucrose. Total activity incorporated into the cellular constituents was determined directly from the specific activity of the homogenate. Total protein was precipitated by addition of an equal volume of 12 % trichloroacetic acid at 1° C for 3 hrs. Cellular fractions were separated by centrifugation of the sucrose-phosphate homogenate as described previously (9). Glutathione formation was determined by radioactive assay on the material isolated in the form of cuprous glutathione as earlier described (10). Glutamic acid, α-ketoglutaric acid, and γ-aminobutyric acid were separated chromatographically and the relative activities of the acids estimated directly from that of the glutamic acid.

Radioactive assays were carried out by standard techniques using a thin mica window Geiger-Müller tube and scaling circuit. Protein was washed with trichloroacetic acid, with dilute alkali, and with thioglycollic acid before radioactive assay in order to remove any amino acid either adsorbed or bound through sulfhydryl linkage. These treatments generally removed 10 to 15 % of the total activity. The remainder of the activity was released only by hydrolysis of the protein.

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Results

TRANSPORT OF GLUTAMIC ACID INTO PLANT CELLS: The transport of glutamic acid into the cells of plant tissues, like the transport of metal ions and of carbohydrates, is dependent either directly or indirectly upon respiratory energy. As is illustrated in table I, the uptake of glutamic acid and its accumulation either as glutamic acid or as metabolic products of glutamic acid, is strongly inhibited by the respiratory inhibitors, hydrogen cyanide and hydrogen azide. Anaerobiosis likewise causes significant inhibition of accumulation. The marked inhibition of glutamic acid accumulation caused by dinitrophenol is of particular interest, as Bonner and Millerd (3) have shown that this substance acts as an inhibitor of oxidative phosphorylation in plants. They (3) likewise established ATP to be the product of oxidative phosphorylation in plants. It would seem, therefore, that the inhibitions of accumulation observed here are due to a decreased supply of the ATP that is apparently necessary for accumulation to occur. The lesser degree of inhibition obtained with anaerobiosis would be in agreement with this view, as it is known that small quantities of ATP are produced during glycolysis. Preliminary experiments with several other amino acids (aspartate, glycine, histidine, cysteine) indicate that the accumulation of these amino acids also is dependent upon the availability of respiratory energy.

Table I

EFFECT OF INHIBITORS OF RESPIRATION AND OXIDATIVE PHOSPHORYLATION ON THE ACCUMULATION OF C^{14} FROM GLUTAMATE BY SEVERAL PLANT TISSUES *

ADDITION TO SYSTEM	TOTAL RADIOACTIVITY IN THE TISSUE (CPM) **		
	BEAN HYPOCOTYL SECTIONS	CARROT ROOT SLICES	POTATO TUBER SLICES
None	2863	3800	1651
Hydrogen cyanide	1417	1318	801
Hydrogen azide	1220	1315	785
Nitrogen atmosphere	2126	3056	1023
Dinitrophenol	1088	889	730

* Sections were incubated for 1 hr at 25° C. Concentration of all inhibitors used was 0.001 M. The incubation medium in each case contained 0.01 M potassium L-glutamate with a total activity of 1 μc. Approximately 0.1 % of the C^{14} added as glutamate was assimilated by the controls.

** cpm = counts per minute.

METABOLISM OF ABSORBED GLUTAMIC ACID: Glutamic acid is apparently metabolized at a rapid rate in living plant cells. Previous studies (10, 11) have demonstrated that it is readily coupled with certain other amino acids to form simple peptides. Table II gives a balance sheet for the fate of glutamic acid in a short time experiment. It can be seen that the glutamic acid has entered into both degradative and synthetic reactions. It is interesting to note that under the conditions of the experiment, degradation apparently proceeded almost exclusively through the oxidative deamination of glutamate to α-ketoglutarate rather than by decarboxylation to γ-aminobutyrate. Different plants apparently have widely varying capacities for the decarboxylation of glutamate as the studies of Schales et al (8) have already indicated. The data of table II also show, however, that significant amounts of glutamate are used for the formation of peptide bonds both in glutathione and in protein. This incorporation of glutamate into protein has been found to increase steadily with time at the expense of the free glutamic acid already accumulated by the cells.

Table II

PARTITION OF RADIOACTIVITY OF GLUTAMIC ACID INTO PROTEIN AND NON-PROTEIN FRACTIONS OF BEAN HYPOCOTYL SECTIONS *

FRACTION	RADIOACTIVITY AS % OF TOTAL RADIOACTIVITY ABSORBED BY CELL
Protein	15.5
Cellular debris	4.2
Non-protein	80.3
Glutamic acid	43.0
Glutamine	18.1
Glutathione	1.3
α-Ketoglutaric acid	4.6
γ-Aminobutyric acid	0.9
α-Aminobutyric acid	0.0
Unaccounted for	12.4

* Sections were incubated for 1 hr at 25° C with 0.01 M potassium L-glutamate containing 10 μc of radioactivity. The total C^{14} assimilated gave 29,300 cpm; this is about 0.1 % of the C^{14} added as glutamic acid.

INCORPORATION BY DIFFERENT PLANTS: Table III illustrates that each of six different species of plants incorporated significant amounts of C^{14} from glutamate into their cellular protein. That this radioactivity is incorporated in the form of glutamate was established by hydrolysis of the protein and separation of the liberated amino acids by chromatography. Glutamate alone showed significant radioactivity as determined by radioautography (2).

The amino acid bound to protein by the process reported on here is undoubtedly bound through a peptide bond. Results of the treatments indicated

Table III

INCORPORATION OF RADIOACTIVE GLUTAMATE INTO THE PROTEINS OF VARIOUS PLANT TISSUES

PLANT AND TISSUE	GLUTAMATE INCORPORATED INTO TOTAL PROTEIN (μM/GM PROTEIN × HR)
Bean hypocotyl sections	0.35
Carrot root slices	0.38
Potato tuber slices	0.16
Pea seedlings	0.27
Oat seedlings	0.23
Young spinach leaves	0.07

in table IV make it seem highly unlikely that the amino acid is merely adsorbed on the protein. The failure of boiling trichloroacetic acid or of dialysis against alkali to remove significant activity argues against a physical adsorption, while the stability of the amino acid to performate treatment indicates a lack of binding to the protein through sulfhydryl groups. Likewise, the failure of ninhydrin to release radioactive CO_2 from uniformly labeled amino acid indicates a linkage involving either the carboxyl or amino group of the amino acid. Even the partial destruction of the protein with trypsin failed to release any great quantity of amino acid. In addition, the dependence of incorporation by mitochondrial protein on energy sources (ATP, Krebs cycle intermediates) and its inhibition by respiratory inhibitors (12) does not argue in favor of adsorption.

INCORPORATION INTO VARIOUS CELLULAR FRACTIONS: When intact tissue is allowed to accumulate radioactive glutamate and is then fractionated into protein fractions composed of the various cellular components, it is found that the amount of incorporated radioactivity is quite different in these different cellular fractions (table V). Most noteworthy is the relatively large incorporation into the microsomal or small particle fraction. The results indicate that the most active turnover of amino acids in the cellular proteins is associated with this fraction. The fraction designated arbitrarily here as microsomal actually encompasses particles that sediment in a number of distinct groups between 10,000 and 70,000 × g (6). Thus, it is possible that only one type of this presumably heterogeneous set of particles is so active in incorporation, but such a conclusion is dependent on further knowledge concerning the nature of the various cellular particles occurring in plants.

DISCUSSION

Several noteworthy results have been obtained during the present investigation. First, the experiments show that the accumulation of at least some amino acids by plant cells is dependent on respiratory energy and is thus possibly similar to the process of active accumulation of other substances. In addition they establish that different cellular proteins possess different capacities for incorporation, thus giving some indication of the possible sites of most active protein turnover in plant cells. In this connection, a further fact is of particular interest. The nucleus has often been suggested as the center of protein synthesis in the cell (5). If there is in fact a direct relation between nuclei and protein synthesis then it would be expected that nuclei would show marked capacities for incorporation of amino acids into protein as compared with other cellular entities. Actually, however, nuclei exhibit just the opposite behavior and have consistently shown a smaller amount of incorporation than the other cellular bodies. In contrast, the microsomes exhibit markedly great amounts of incorporation. These findings, though not definitive because they are confined to observations at one time period only, nevertheless raise a question as to the relative roles of the various cellular entities in protein synthesis. It would appear that much more information (including rate studies on incorporation by cellular fractions) is needed before any one cellular fraction can be assigned an unequivocal role in protein synthesis.

TABLE IV

STABILITY OF THE INCORPORATED AMINO ACIDS TO VARIOUS TREATMENTS OF THE ISOLATED RADIOACTIVE PROTEIN

TREATMENT	AMINO ACID ADDED *	
	GLUTAMIC ACID-C^{14}	GLYCINE-C^{14}
None	100	100
Boiled with 5% trichloroacetic acid	98	99
Dialysis against dilute alkali	100	98
Radioactivity released by ninhydrin	3	2
Dispersion in performic acid	100	100
Partial hydrolysis with trypsin	95	96

* Values given represent specific activity as a percent of the specific activity of the original protein, isolated and washed as described previously.

TABLE V

DISTRIBUTION OF THE INCORPORATED C^{14} FROM GLUTAMATE AMONG CELLULAR FRACTIONS OF BEAN HYPOCOTYL SECTIONS

FRACTION	GLUTAMATE INCORPORATED (μM PER HR PER GM PROTEIN)
Homogenate	0.36
Nuclear	0.15
Mitochondrial	0.43
Microsomal	1.18
Soluble	0.31

SUMMARY

Radioactive glutamate is accumulated by plant cells from the external medium. This accumulation is inhibited by respiratory inhibitors and by dinitrophenol and thus appears to be linked with the production of respiratory energy. Glutamate is rapidly metabolized in the cell to glutamine and to α-ketoglutarate but only slightly to γ-aminobutyrate. It is incorporated into the tripeptide glutathione and into cellular proteins. The incorporation into protein is a general reaction which occurs in a variety of plants and apparently involves the actual formation of peptide bonds. Different cellular fractions have widely different capacities for glutamate incorporation with the greatest incorporation during an hour occurring into the microsomal fraction, and the least into the nuclear fraction.

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THE EFFECT OF TEMPERATURE ON THE BEHAVIOR OF MALIC ACID AND STARCH IN LEAVES OF BRYOPHYLLUM CALYCINUM CULTURED IN DARKNESS[1,2]

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It has been known for many years that the temperature at which excised leaves of *Bryophyllum calycinum* are cultured in darkness has a considerable effect upon the extent to which the tissues become enriched in organic acids. In 1938, Wolf (14) presented data to show that, at a temperature of 7 to 8° C, the production of acid and the utilization of starch were apparently both stimulated as compared to the behavior of these substances in leaves cultured at about 22° C, and similar observations have been reported in a previous paper from this laboratory (4). Additional evidence with respect to the favorable effect of low temperature on the formation of acid has recently been given by Thomas and Ranson (5).

In the earlier work from this laboratory, however, the observations were made only after culture for 23, 66, and 142 hours at temperatures of 20°, 9°, and 1° C. Furthermore, the sampling method used at that time for collecting the leaflets was far less satisfactory than that presently employed. It has seemed desirable, therefore, to reinvestigate the matter with more detailed attention to the early phases of the chemical transformations that occur and with the use of improved sampling methods. A better opportunity has thus been afforded for the consideration of the chemical relationships between the quantities of starch and malic acid involved in the reactions, and for the study of the somewhat subtle alterations which take place in the general course of the chemical events when the period of culture under artificial conditions exceeds the normal length of a night.

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Experimental Procedure

Plantlets of *Bryophyllum calycinum* from the clone that has been maintained in this laboratory for a number of years were transplanted to sand in pots in the greenhouse March 27, 1951, and were grown with use of a culture solution. By July 9, the plants bore several pairs of well-developed 3-leaflet leaves. On this date, 12 samples were collected by the statistical method (11) from 36 plants, using the 12 leaflets from the two uppermost pairs of fully-expanded leaves. Each sample thus contained 36 leaflets, one of which was derived from each plant, and each of the 12 leaflet positions was represented three times in each sample. Collection was started at 3 P.M., the weather being clear and sunny as it had been the previous day.

The samples were at once weighed to five significant figures and sample 1 was placed in the drying oven at 4:00 P.M., this being taken as the zero hour of the experiment. Meanwhile, the leaves of the other samples were arranged along the sides of V-shaped troughs, samples 2 to 6 in a room maintained at 24° C and 75 % relative humidity, samples 7 to 12 in a room at 6° C, and enough water was added to immerse the petioles. The humidity in the cold room was uncontrolled but was fairly low. The rooms were darkened at 4:00 P.M., and the conditions were maintained for 40 hours at 24° C and for 48 hours at 6° C. Successive samples were removed at