INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO THE PROTEINS OF PLANT TISSUE HOMOGENATES[1,2]

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It has been established (2, 12) that intact tissues of higher plants readily incorporate C^{14}-labeled amino acids into their cellular proteins. In addition, evidence has been presented (11) that an isolated mitochondrial fraction from bean seedlings is capable of incorporating several amino acids. This incorporation into mitochondrial protein is of particular interest as it closely resembles the synthesis of simple peptides (14, 15, 16) in its dependence on the respiratory energy of adenosinetriphosphate (ATP). The ability of such an isolated cellular fraction to incorporate amino acids makes a further study of cell-free systems of interest.

The sole purpose of the present investigation has been to study in some detail the incorporation of amino acids into the proteins of cell-free extracts of higher plants. The present communication reports results on the nature of the incorporation process and on the partial purification and properties of a particulate system from pea seedlings that incorporates amino acids at relatively high rates.

EXPERIMENTAL

Seedlings were grown under weak red light at 26° C and 90 % humidity as described previously (13). Tissue homogenates, prepared in either sucrose-phosphate (13) or other buffers (10), were also made as described earlier. Sedimentation of cellular fractions was carried out with the techniques previously used in the study of glutamine synthesis (10). Experiments were performed in a Dubnoff Metabolic Incubator at 38° C and a shaking rate of approximately 100 cycles per minute. The basic reaction system consisted of the homogenate or cellular fraction plus 0.05 M phosphate buffer (pH 7.5) and 0.01 M glutamate-2-C^{14} or other C^{14}-labeled amino acid. The supplemental amino acid mixture used in certain experiments was composed of 0.1 mg per ml of each of the following L-amino acids: alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, and valine, plus 0.001 mg per ml of tyrosine. After incubation, proteins were precipitated and washed as described before (12). Radioactivity of each fraction was determined as previously (12) with a thin mica window Geiger-Müller tube and scaling circuit. Each value in the accompanying tables represents the average of eight separate determinations. The variation between samples was less than 5 % of the average value in every case. Approximately 0.1 to 1.0 % of the added glutamic acid-C^{14} or other labeled amino acid was taken up by the protein (depending on the conditions listed in the accompanying tables).

RESULTS

As is evident from table I, disruption of the cells of pea seedlings does not stop the incorporation of radioactive glutamate into the total protein of these cells. However, the medium used in the preparation of the homogenate exerts a marked effect on the rate of incorporation. In agreement with previous studies on organized oxidative and phosphorylative activities in plant homogenates (1, 6, 7, 8), the incorporation of glutamate-C^{14} is markedly higher in a sucrose-phosphate homogenate (in which the integrity of the particulate material is maintained) than in homogenates prepared in phosphate or tris-(hydroxymethyl)-aminomethane (Tris) buffers. Significantly, the glycolytic system does not seem to be of great importance to amino acid incorporation in the cell-free preparations studied here. As is shown in table I the glycolytic intermediates, hexose diphosphate and 3-phosphoglycerate, do not enhance amino acid incorporation. Moreover, 0.01 M sodium fluoride, a potent glycolytic inhibitor, inhibits incorporation only

TABLE I

CHARACTERISTICS OF THE PROCESS OF AMINO ACID INCORPORATION INTO THE PROTEIN OF PEA SEEDLING HOMOGENATES

GRINDING MEDIUM	ADDITION TO SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
		$\mu M/hr \times gm\ protein$
0.1 M Potassium phosphate buffer (pH 7.5)	None	0.15
0.1 M Tris-HCl (pH 7.5) ..	None	0.10
0.45 M Sucrose + 0.05 M phosphate buffer (pH 7.5)	None	0.41
"	Hexose diphosphate	0.41
"	3-Phospho-glycerate	0.43
"	NaF	0.36
"	HCN	0.05
"	Hydrogen azide	0.08
"	Nitrogen atmosphere	0.20
"	CO (dark)	0.20
"	CO (light)	0.38
"	Dinitrophenol	0.19

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.001 M each of the inhibitors or other compounds. CO/O_2 ratio was approximately 19. Light intensity incident on the experimental vessels was about 300 fc.

[1] Received January 28, 1955.

[2] Supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Company.

TABLE II

PARTICIPATION OF ATP IN THE INCORPORATION OF GLUTAMATE-C^{14} INTO PROTEIN

SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm$ protein
Untreated homogenate	0.43
" + ATP	0.38
Dialyzed homogenate	0.16
" + ATP	0.35
" + ADP	0.10
" + AMP	0.15

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), and 1.0 ml of either sucrose-phosphate homogenate or 1.0 ml of this homogenate after dialysis against sucrose-phosphate at 1° C. Where applicable, 0.001 M of the adenyl phosphates was present.

slightly. In contrast to this, the data of table I demonstrate a marked dependence of the incorporation process on oxidative activity. The incorporation is greatly inhibited by cyanide, azide, and by anaerobiosis. The marked inhibition of incorporation obtained with carbon monoxide is almost completely eliminated by light, which strongly indicates a participation of the oxidative system of peas in the incorporation process. The pronounced inhibition produced by dinitrophenol suggests, in agreement with previous results (11), that amino acid incorporation in these preparations is dependent on oxidative phosphorylation and, therefore, probably on ATP.

PARTICIPATION OF ATP IN AMINO ACID INCORPORATION: Despite the above evidence for the participation of oxidative phosphorylation (and, therefore, probably ATP) in the incorporation process, it is not possible to demonstrate a necessity for ATP unless the cell-free extracts are dialyzed (table II). The strong inhibition of glutamate incorporation obtained on dialysis can be largely restored by the addition of ATP. As is evident from table II, adenosinemonophosphate (AMP) is ineffective in restoring the activity while adenosinediphosphate (ADP) is somewhat inhibitory. These results agree both with those previously obtained with plant mitochondrial preparations (11) and with mammalian particles (16). It seems quite likely that ATP is indeed necessary for at least a portion of amino acid incorporation.

TABLE III

EFFECT OF DIVALENT IONS ON THE INCORPORATION OF GLUTAMATE-C^{14} INTO PEA SEEDLING HOMOGENATES

DIVALENT ION ADDED TO SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm$ protein
None	0.43
Mg	0.67
Mn	0.46
Ca	0.30
Co	0.29
Zn	0.06
Cu	0.03
Ba	0.01

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.01 M of each of the metal ions listed, and 1.0 ml of sucrose-phosphate homogenate in a total volume of 2 ml.

EFFECT OF DIVALENT IONS ON INCORPORATION: The apparent participation of ATP in amino acid incorporation (presumably by some transphosphorylation reaction) suggests that magnesium ions might also be required for this process as they are for amide (10) and peptide (13, 14, 15) syntheses, and in fact for most reactions involving ATP. As is evident from table III, of a number of divalent ions examined, only magnesium ions produce a significant increase in the rate of glutamate-C^{14} incorporation. Most other divalent ions are inhibitory. It is noteworthy that the effects of the different divalent ions on glutamate incorporation are qualitatively very similar to their effects on the biosynthesis of simple peptides (14).

INCORPORATION OF AMINO ACIDS OTHER THAN GLUTAMATE: The data of table IV show that incorporation by tissue homogenates is not confined to glutamate, but proceeds at easily measurable rates with each of a number of C^{14}-labeled amino acids and in cell-free extracts of three different tissues. It is noteworthy that the rates of incorporation of the various amino acids differ markedly in the same tissue. Furthermore, the rate of incorporation of any one amino acid is, in general, different in the different tissue extracts. It is not clear at present whether these differences reflect: (a) a requirement for varying amounts of the different amino acids for protein formation, (b) different rates of exchange of the amino acids into preformed protein, or (c) differences in the rates of breakdown of the different amino acids. It is possible that all three processes are involved.

EFFECTS OF OTHER AMINO ACIDS ON THE INCORPORATION OF GLUTAMATE-C^{14}: As is evident from table V, the addition of various individual amino acids to the system generally has no marked effect on the rate of glutamate incorporation. In a few cases, however, inhibition of glutamate incorporation has been observed. Such inhibition occurs in the pres-

TABLE IV

INCORPORATION OF RADIOACTIVE AMINO ACIDS BY PLANT HOMOGENATES

AMINO ACID	μM AMINO ACID INCORPORATED/HR × GM PROTEIN		
	PEA SEEDLINGS	BEAN SEEDLINGS	WHEAT SEEDLINGS
Glutamate-2-C^{14}	0.43	0.35	0.50
Aspartate-4-C^{14}	0.14	0.21	0.15
Glycine-2-C^{14}	0.59	0.37	0.48
Cysteine-S^{35}	0.36	0.43	0.21
Histidine-1-C^{14}	0.47	0.16	0.28
Leucine-2-C^{14}	0.58	0.69	0.48
Serine-1-C^{14}	0.48	0.40	0.21
Alanine-1-C^{14}	0.40	0.36	0.33

ence of aspartate, asparagine, and citrulline. It is possible that the inhibition may be due to a similarity in structure of these compounds to glutamate, a similarity which allows them to compete with glutamate for sites in the incorporating system. Especially interesting is the inhibitory action of citrulline while the analogous arginine has no effect on incorporation. Likewise, α-aminobutyrate inhibits glutamate incorporation, but γ-aminobutyrate is without effect.

The incorporation of glutamate is markedly increased by the simultaneous addition of a mixture of 17 amino acids to the system. The cause of this increase is not as yet clear. It is well known that protein synthesis in intact cells occurs only if all of the component amino acids are simultaneously present. It cannot be concluded from the present data that the enhanced incorporation of glutamate in the presence of the 17 other amino acids is a reflection of protein synthesis by the system, as no evidence for actual protein synthesis is available. Similar results, however, have been obtained by Gale and Folkes (3, 4) with bacterial breis in which apparent enzyme synthesis occurs only in the presence of an amino acid mixture similar to that used here.

In order to investigate further the promotion of incorporation by the mixture of amino acids, the ability of various antagonists of amino acids other than glutamate to inhibit the enhanced glutamate incorporation has been examined. That such antagonists are highly effective is illustrated by the data of table VI. Both *p*-fluorophenylalanine and β-2-thienylalanine, antagonists of phenylalanine, are strong inhibitors of glutamate incorporation in the presence of the mixture of 17 other amino acids. Likewise, significant inhibitions are obtained with ethionine (an antagonist of methionine) and with allyl glycine (an antagonist of cysteine). These results suggest that the enhanced incorporation of radioactive glutamate may be the result of some process in which all of the amino acids are involved.

TABLE V

EFFECT OF VARIOUS AMINO ACIDS ON THE INCORPORATION OF GLUTAMATE-C^{14} INTO THE PROTEIN OF PEA SEEDLING HOMOGENATES

AMINO ACIDS ADDED *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm\ protein$
None	0.43
α-Aminobutyrate	0.38
Aspartate	0.24
Asparagine	0.33
Citrulline	0.30
Individual amino acids **	0.43
Mixture of 17 amino acids	0.71

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.01 M each of the amino acids or 0.2 ml of the mixture of 17 amino acids and 1 ml of homogenate in a total volume of 2 ml. Incubation was for 5 hrs at 38° C.

** The following individual amino acids were without effect on glutamate-C^{14} incorporation when incubated with the above system: α-alanine, β-alanine, γ-aminobutyric acid, arginine, cysteine, dihydroxyphenylalanine, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophane, tyrosine (0.0001 M), and valine.

TABLE VI

EFFECT OF AMINO ACID ANALOGUES ON INCORPORATION OF GLUTAMATE-C^{14} INTO PROTEIN IN THE PRESENCE OF A COMPLETE AMINO ACID MIXTURE

ADDITION TO SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm\ protein$
None	0.71
p-Fluorophenylalanine	0.47
β-2-Thienylalanine	0.40
Ethionine	0.61
Allylglycine	0.58

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.01 M of each of the amino acid antagonists, 0.2 ml of the mixture of 17 amino acids, and 1 ml of homogenate in a total volume of 2 ml. Incubation was for 5 hrs at 38° C.

INCORPORATION BY VARIOUS CELLULAR FRACTIONS: It was reported earlier (12) that when bean seedlings are incubated with a radioactive amino acid, the small particle (possibly microsomal) fraction incorporated the greatest amount of radioactivity. The data of table VII show that intact tissues of bean and pea seedlings possess similar patterns of glutamate incorporation into their various cellular fractions. Furthermore, in both cases cell-free extracts prepared from the seedlings incorporated glutamate in a manner analogous to that of the intact seedlings. It

TABLE VII

INCORPORATION OF GLUTAMATE-C^{14} INTO PROTEIN BY INTACT SEEDLINGS, BY HOMOGENATES, AND BY ISOLATED CELLULAR FRACTIONS

CELLULAR FRACTION *	INTACT TISSUES	HOMOGENATE	ISOLATED PARTICLES
	$\mu M/hr \times gm\ protein$		
Pea seedling:			
Nuclear	0.41	0.48	0.13
Mitochondrial	0.68	0.63	0.31
Microsomal	1.05	1.00	0.05
Soluble	0.29	0.20	0.15
Bean seedling:			
Nuclear	0.15	0.18	0.11
Mitochondrial	0.43	0.55	0.38
Microsomal	1.18	1.83	0.09
Soluble	0.31	0.59	0.25

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), and the indicated tissue preparations. Incubation was at 38° C. The nuclear fraction includes all material sedimenting at 500 × g for 5 min. The mitochondrial fraction includes all material sedimenting between 500 and 10,000 × g for 10 min, while the microsomal fraction encompasses that material sedimenting between 10,000 and 100,000 × g for 30 min. That protein not sedimenting at 100,000 × g is designated as soluble.

should be emphasized here that the designations of the cellular fractions are entirely arbitrary and do not represent homogeneous preparations of these fractions. If the various cellular fractions are incubated separately with glutamate-C^{14}, the specific activity of every fraction is considerably reduced. The greatest reduction occurs in the case of the small particle fraction. It seems likely that one or more of the other fractions are necessary for the relatively large amount of incorporation that occurs in the small particle fraction of the whole homogenate. In order to test this, the incorporating ability of various combinations of cellular fractions has been examined. Table VIII shows that only a few of such combinations result in relatively high incorporation rates. It is noteworthy that in each of these cases the mitochondrial fraction constitutes one component of the system. In accord with results from intact cells and whole homogenates, a system composed of the mitochondrial and microsomal fractions exhibits by far the greatest specific activity. This finding is similar to the results of Siekevitz (9) and of Keller et al (5) with cellular fractions of mammalian tissues. It would appear, therefore, that an experimental system composed of these two cellular fractions offers the most logical starting place for studies on the nature of the process of amino acid incorporation and its relation to protein synthesis. The system may be still further simplified by fractionation of the microsomal material (table IX). The particulate material which sediments at $40{,}000 \times g$ has the ability (when mixed with the mitochondrial fraction) to carry out incorporation of glutamate-C^{14} at the highest rates thus far observed during these studies, while the particulate material which sediments between $40{,}000 \times g$ and $100{,}000 \times g$ has a low activity more comparable to that of the soluble fraction. Apparently only a certain type or size range of particles is involved in the very active incorporation reported here.

Effect of Supplemental Amino Acid Mixture on Differential Incorporation by Cellular Fractions: The different rates of glutamate-C^{14} incorporation by the cellular fractions have been compared in the presence and absence of the supplemental mixture of 17 amino acids (table X). It is evident that glutamate-C^{14} incorporation by the microsomal fraction is markedly enhanced by the presence of the other amino acids. The same is true, to a lesser extent, of the mitochondrial fraction. In contrast, the soluble fraction shows a marked decrease in specific activity while the nuclear fraction is little affected. These findings support the previously stated suggestion that a particulate system composed of the mitochondrial and microsomal fractions would seem to offer the most promising site for further investigations of the nature of amino acid incorporation into cellular protein in plants.

Table VIII

Incorporation of Glutamate-C^{14} by Various Combinations of Cellular Fractions of Pea Seedlings

System *	Glutamate-C^{14} incorporated
	$\mu M/hr \times gm\ protein$
Total homogenate	0.39
Homogenate – nuclear fraction	0.66
Nuclear + mitochondrial fractions	0.88
Nuclear + microsomal "	0.23
Nuclear + soluble "	0.40
Mitochondrial + microsomal "	1.28
Mitochondrial + soluble "	0.65
Microsomal + soluble "	0.20

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.001 M ATP and 1.0 ml of cellular fraction in a total volume of 2 ml. Incubated at 38° C for 3 hrs.

Table IX

Incorporation of Glutamate-C^{14} by Particulate Fractions

System *	Glutamate-C^{14} incorporated
	$\mu M/hr \times gm\ protein$
Mitochondrial + particulate fraction sedimented between:	
10,000 and $100{,}000 \times g$	1.28
10,000 and $40{,}000 \times g$	1.50
40,000 and $100{,}000 \times g$	0.73

* System contained the same components as described under table VIII.

Discussion

Two facts of potential importance have emerged from the present investigation. It has been shown that a relatively simple cell-free system which carries on the incorporation of amino acids into protein may be prepared. Like the organized respiratory systems that have been studied so extensively in both plants and animals, the activity of this system is dependent on its method of preparation. Furthermore, like various other systems which carry out biological syntheses, the amino acid incorporation system apparently involves ATP. The active incorporation system

Table X

Effect of the Presence of a Supplemental Amino Acid Mixture on the Distribution of Incorporated Glutamate-C^{14} in Pea Seedling Homogenates

Fraction *	Glutamate-C^{14} incorporated	
	Glutamate alone	Glutamate + 17 amino acids
	$\mu M/hr \times gm\ protein$	
Nuclear	0.48	0.46
Mitochondrial	0.63	0.72
Microsomal	1.00	1.25
Soluble	0.20	0.16

* Composition of system and incubation time were the same as described under table VIII.

described here may be treated operationally just as any organized complex of enzymes (such as those concerned with pyruvate oxidation or with fatty acid metabolism).

The second important finding is that the incorporation of one amino acid may be promoted by the presence of a mixture of the other amino acids normally found in protein. While it is premature to suggest that protein formation may occur in the particulate system in the presence of the amino acid mixture and a suitable energy source, the effect deserves further investigation.

Summary

The incorporation of various amino acids into the proteins of plant homogenates has been demonstrated. Incorporation rates vary greatly according to the method of preparation of the homogenate. The process is dependent on ATP and is considerably enhanced by magnesium ions, but is inhibited by most other divalent ions. Incorporation of glutamate generally is unaffected by the presence of other individual amino acids, but is inhibited by aspartate, asparagine, and citrulline. However, a mixture of 17 amino acids greatly increases glutamate incorporation. This enhanced incorporation of glutamate is strongly inhibited by antagonists of various amino acids. A particulate fraction of cellular homogenates of pea seedlings has been prepared which incorporates amino acids at high rates.

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NONVOLATILE ORGANIC ACIDS OF CROWN GALLS, CROWN GALL TISSUE CULTURES AND NORMAL STEM TISSUE[1,2]

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Measurements of the organic acids of galls, stems, and gall tissue cultures have been undertaken. Comparisons among these plant tissues are of interest because the tissues have like origins but unlike differentiation. The changes are obvious in the gross appearance of crown galls, which develop at stem wounds infected with *Agrobacterium tumefaciens*. These galls far outgrow the surrounding stem tissues and become increasingly disorganized. Crown gall tissues of several plants have been obtained bacteria-free and have been grown in sterile cultures (6). In contrast to cultures of normal stem tissues, the gall tissues grow in a simplified medium and they do not differentiate to form roots or other organs. Detailed comparisons of normal stems and crown gall tissue

[1] Received January 31, 1955.

[2] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the American Cancer Society as recommended by the Committee on Growth and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.