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AN ESTIMATE OF PROTEIN TURNOVER IN GROWING TOBACCO PLANTS

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Abstract—DL-Glutamic acid-1-^{14}C was injected into growing tobacco plants and the rate of $^{14}CO_2$ release was measured. After a period in light to allow isotope to become incorporated into protein, plants were placed in the dark to eliminate by respiration radioactivity from readily accessible pools and metabolically labile proteins. Plants were then placed in the light and growth allowed to resume. There was only negligible incorporation into polysaccharide fractions. The protein fraction contained 71% of the labelled carbon, and soluble amino acids and other metabolic intermediates accounted for 22% of the total activity of the plant. From the rate of $^{14}CO_2$ release an apparent half-life of 7·0 days was calculated for the protein fraction. This calculation assumes that both the protein fraction and the intermediates contribute isotope to the $^{14}CO_2$, but that the intermediate pool is continuously replenished from the protein. It is also assumed that a steady state of growth is re-established on the third day of illumination.

INTRODUCTION

THE WORK of Gregory and Sen[1] suggested that proteins of plants are in a state of constant breakdown and synthesis. More direct evidence for protein turnover in plants was obtained by Vickery and co-workers[2] with intact tobacco plants, and more recently by Steward and Bidwell[3] in carrot root explants. These authors, however, did not attempt to estimate the rate of turnover in their respective systems.

In growing systems, the rate of synthesis of cellular components is greater than the rate of breakdown, and it may be expected that compared to the non-growing state either degradation occurs at the same rate or is markedly depressed. In very rapidly growing carcinoma transplants, protein degradation is apparently much smaller than in normal tissue.[4] The rate of protein degradation is also extremely small in growing cultures of *Escherichia coli*.[5] In growing weanling rats on the other hand, ribonucleic acid degradation still takes place at a rate comparable to that of the slowly growing adult.[6] Therefore, in this case it is the synthetic rate that is higher than that in the adult. Thus, there is a precedent for the possibilities that growth results either from decreased degradation or results from a relative increase in synthetic capability.

We have found that the rate of protein turnover in a growing tobacco (*Nicotiana tabacum* L.) plant is quite rapid; in plants doubling in size in about fourteen days, the apparent protein turnover half-life is about seven days.

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[1] F. G. GREGORY and P. K. SEN, *Ann. Botany (London) N. S.* **1**, 521 (1937).
[2] H. B. VICKERY, G. W. PUCHER, R. SCHOENHEIMER and D. RITTENBERG, *J. Biol. Chem.* **135**, 531 (1940).
[3] F. C. STEWARD and R. G. S. BIDWELL, *J. Exp. Botany* **9**, 285 (1958).
[4] G. A. LEPAGE, V. R. POTTER, H. BUSCH, C. HEIDELBERGER and R. B. HURLBERT, *Cancer Res.* **12**, 153 (1952).
[5] A. L. KOCH and H. R. LEVY, *J. Biol. Chem.* **217**, 947 (1955).
[6] R. W. SWICK and A. L. KOCH, *Arch. Biochem. Biophys.* **67**, 59 (1957).

Measuring turnover in growing systems is technically difficult because of the problem of recycling.[7] The method used here employs as tracer a compound that is metabolically labile and has a high probability of being converted to CO_2 and is thus largely released from the plant. The tracer compound chosen was DL-[1-^{14}C]-glutamic acid. We found that after four days under a particular regimen, the remaining radioactivity is largely still in the form of glutamic acid or other compounds that can reasonably be expected to be respired to CO_2. Under these conditions the subsequent release of radioactive CO_2 is limited by the rate of protein degradation. In fact, the initial part of the experiment serves as control for the fact that the rate of release from small molecules is faster and, therefore, non-limiting.

RESULTS

Three experiments were carried out. Each experiment contributed to the conclusions drawn. The third, however, was the most extensive; and the results of this experiment are presented in this section. The time-course for the release of $^{14}CO_2$ for Experiment III is

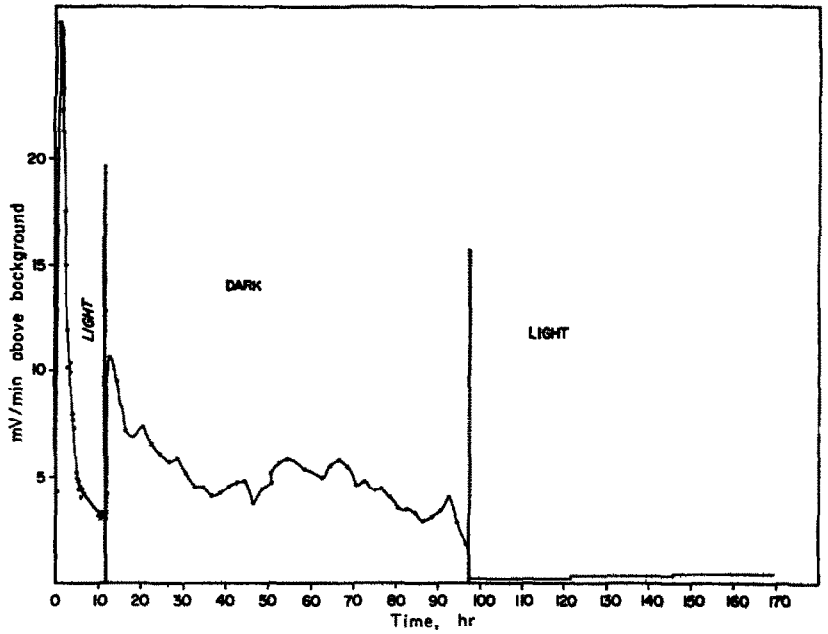

FIG. 1. THE TIME COURSE OF $^{14}CO_2$ RELEASE FROM A TOBACCO PLANT ADMINISTERED 10 μC OF DL-[1-^{14}C]-GLUTAMIC ACID

The rate of $^{14}CO_2$ release was measured continuously for the first 97 hr; for the first 12 hr, averages are for successive 15-min periods; for the remainder, averages of 2-hr intervals are given. The final three 24-hr periods in the light are averages obtained by trapping CO_2 quantitatively for each period and converting to the value that would have been observed in the flowing system.

given in Fig. 1. Quantitative data are given in Table 1. It can be seen that $^{14}CO_2$ is evolved immediately upon injection of DL-[1-^{14}C]-glutamic acid. The radioactivity rose to a peak at 1·2 hr, then fell rapidly, and subsequently more slowly. The actual rise and fall of $^{14}CO_2$ production must be considerably faster than indicated in Fig. 1 since, for the volume of the

[7] A. L. KOCH, *J. Theoret. Biol.* 3, 282 (1962).

growth chamber (37 l. empty) and the flow rate (13·5 l/hr), a considerable part of the rise and fall time may be attributed to mixing in the growth chamber. After this initial burst, radioactivity was released with a half-life of about 8·5 hr. A smaller burst of $^{14}CO_2$ commenced and quickly subsided immediately after the light was turned off. $^{14}CO_2$ production was erratic during the remainder of the dark period. With the exception of the period from 86 to 93 hr when the pumping system failed, the fluctuations appeared to be real, but they did not exhibit a daily rhythm. After the plant was again illuminated, $^{14}CO_2$ release was depressed

TABLE 1. RECOVERY OF RADIOACTIVITY FROM A TOBACCO PLANT INJECTED WITH 10μc OF DL-[1-^{14}C]-GLUTAMIC ACID

Fraction	Time (hr)	μc
CO_2	0–96	8·380
CO_2	97–120	0·0707
CO_2	121–144	0·112
CO_2	145–168	0·146
Plant top	168	1·466
		10·175

greatly. During the succeeding 24-hr periods, however, the amount released increased significantly. It is important to note that the plant was observed to grow during these three days, as evidenced by the expansion of new leaves.

After three days in the light, the plant top was analyzed. In the two other experiments, two- and four-day periods were employed. Only 1·4 and 1·7% of the activity of the administered isotope was recovered in the roots in the two experiments, respectively. In the two-day experiment, the distribution of activity in the top of the plant was determined by measuring

TABLE 2. ACTIVITY OF FRACTIONS OF ONE-GRAM PORTIONS OF WHOLE PLANT TOP HARVESTED SEVEN DAYS AFTER ADMINISTRATION OF DL-[1-^{14}C]-GLUTAMIC ACID

Fraction	Dry %	Activity %
Ether Soluble	3·7	3·2
Ethanol Soluble		
Carbon Pool	14·9	10·3
Amino Acids	5·7	11·4
Residue		
Polysaccharide	62·2	1·4
Acid Humin	9·4	18·1
Protein Amino Acids	3·4	52·8
Recovery	99·3	97·2

the activity in leaf disks taken from near the tip and base of the leaf on opposite sides of the mid-vein. The lowermost four leaves of a plant with thirteen expanded leaves contained only 4·03% of the total activity.

Table 2 shows the average composition and activity of the whole plant top in Experiment III. It is significant that only 1·4% of the radioactivity was found in the polysaccharide fraction. This finding would indicate that re-incorporation of respired $^{14}CO_2$ had been minimal. The lack of re-incorporation was not unexpected, since the rate of flow of air

through the chamber was at least twice that necessary to supply CO_2 for normal growth for the size of plant employed.[8] The sum of the amino acids in the protein and in the ethanol soluble fraction together with the acid humin fraction amounted to 82·3% of the activity present in the whole plant top. Some of each of these fractions was combined in the original proportions and treated with L-glutamic acid decarboxylase; 33·2% of the total activity was released as CO_2.

TABLE 3. ACTIVITY OF CHROMATOGRAPHICALLY SEPARATED FRACTIONS OF HYDROLYSATE OF OF ONE-GRAM ALIQUOTS OF WHOLE PLANT TOP SEVEN DAYS AFTER ADMINISTRATION OF DL-[1-^{14}C]-GLUTAMIC ACID

Fraction	Activity %
Acid Humin	12·5
Hydrolysate	
Sugars	0·4
Glutamic Acid	34·1
Arginine	12·9
Proline	4·7
γ-Aminobutyric Acid	2·8
Other Ninhydrin Positive Areas	26·1
Recovery	93·5

Table 3 shows the activity of various fractions after hydrolysis of an aliquot of the whole plant top and chromatographic separation of the hydrolysate. Chromatographically identifiable glutamic acid accounted for 34·1% of the total activity, agreeing well with 33·2% obtained by glutamic decarboxylase measurement of the mixture of the soluble fractions and the total protein hydrolysate. Thus it is evident that virtually all the free and combined glutamic acid left in the plant is of the L configuration. Therefore, all the D form originally injected has been inverted or metabolized by this time.

DISCUSSION

The results of all isotope tracer experiments must be interpreted at four levels: the level of intermediary metabolism, the level of macromolecular turnover, the cellular level, and the physiological level.

Intermediary Metabolism

DL-Glutamic acid is quickly metabolized in the light. A portion is rapidly converted to CO_2. As pointed out above, the rate of this process cannot be measured because of the length of time required to flush the chamber. The amount can, however, readily be measured. This phase is variable, possibly depending on the precise nature of the injection site. It was 8% in the experiment considered in detail and higher (about 30%) in the other two experiments. The remaining glutamic acid was converted into substances which turn over at a slower rate (8 hr half-life in all experiments). In the dark, the plant uses these substances for respiration, and another immediate burst of $^{14}CO_2$ was seen. Subsequently, $^{14}CO_2$ release must be dependent on the breakdown of macromolecular substances. Since polysaccharides were

[8] V. F. C. GLASSTONE, *Plant Physiol.* 17, 267 (1942).

substantially unlabelled, glutamic acid is not an important precursor of carbohydrates in the light. Instead, labelled carbon is found in great part in free and combined glutamic acid, arginine, and proline. In the light or in the dark, it may be expected that, when released from protein, these amino acids and their metabolic products will be rapidly respired to CO_2. Presumably, the fraction so metabolized will be much larger in the dark. Photosynthetic fixation of CO_2 produced in the initial burst is not important because the burst represents a small portion of the activity and also the flow of air maximized the removal of $^{14}CO_2$ from the chamber.

Another factor should be mentioned. In the first 96 hr, 86% of the DL-[1-^{14}C]-glutamic acid was respired. In the experiments of two and four days' duration, 77 and 89% of the administered radioactivity was recovered as $^{14}CO_2$. Therefore, D-glutamic acid must have been rapidly metabolized. In fact, little remaining radioactivity can be D-glutamic acid since similar values were obtained for the chemically isolated glutamic acid and the CO_2 produced by the sterospecific L-glutamic decarboxylase.

Macromolecular Turnover

Since illumination was given before the experiment commenced and continued for the first 12 hr of this experiment, the plant was growing during the incorporation phase. All macromolecular substances characteristic of growing tobacco plants were being synthesized, in particular, cellulose. Consequently, if its precursors had become labelled in the course of synthesis, cellulose necessarily would be labelled. Cellulose is virtually insoluble once formed; in fact, it is not in dynamic equilibrium and does not break down.[9] The small degree of labelling found shows unequivocally that precursors of this polysaccharide were not significantly labelled during this time period.

In contrast, precursors of protein were labelled; and, if there were any proteins that are as metabolically inert as cellulose, they would have become labelled and would also retain the label indefinitely. Thus, eventually such proteins would be the only labelled proteins in the plant. In essence, the extended dark period should have served as a period of starvation during which proteins that are metabolically more labile could be catabolized by the plant, leaving behind those metabolically less labile. The breakdown in the subsequent light period, during which active growth is taking place, can only be representative of the turnover of those proteins of the cell as a normal process taking place during growth.

It is, of course, implicit that the metabolically labile components of the plant, whether protein in nature or not, may contain an appreciable fraction of the radioactivity of the cell as a result of the process of recycling of labelled compounds.[7,10] For our purpose, we must consider all the radioactivity of the plant, after sufficient time, to be derived from the metabolically more inert components, even though a portion of the activity may be associated at that time with acid-soluble pools or truly labile proteins, etc.

Cellular and Physiological

After injection of the isotope into the mid-vein of a leaf, the isotope was translocated chiefly to the younger leaves and stem apex of the plant. These tissues served as the source of radioactive $^{14}CO_2$ during the final three-day light period. During this time the leaf tissue was growing by expansion, not by division,[11] and thus the turnover observed is not a concomitance

[9] J. BONNER, *Plant Biochemistry*, p. 89, Academic Press Inc., New York (1950).
[10] R. W. SWICK, A. L. KOCH and D. T. HANDA, *Arch. Biochem. Biophys.* 63, 226 (1956).
[11] G. S. AVERY, *Am. J. Botany* 20, 565 (1933).

of cell division. Because of the small amount of radioactivity found in lower leaves which might be approaching senescence, this source can contribute only a negligible amount to the overall rate of protein turnover as estimated from these experiments.

Estimation of Protein Turnover

The biological half-life (τ) of a substance is usually obtained by the following relationship:

$$N = N_0 e^{-0.693\, t/\tau} = N_0 2^{-t/\tau}$$

where N is the activity (total activity for a growing system) at the time, t, and N_0 is the activity at time zero. Alternatively, the differential equation that yields the above relation

$$\frac{dN}{dt} = \frac{0.693\, N}{\tau} \quad \text{or} \quad \tau = \frac{0.693\, (N)}{dN/dt}$$

may be employed. This form is appropriate for the present experiment. The total proteins of the plant are the only *macromolecular* source of the isotope, hence in the steady state dN/dt can be taken as being equal to the observed rate of release of $^{14}CO_2$ (i.e. 0·146 μc/day in the last 24-hr period).

The value for N of the whole plant top harvested seven days after administration of glutamic acid-1-^{14}C must include the 52·8% "protein amino acids", the 18·1% acid humin, and the 11·4% in the ethanol-soluble "amino acids", since the turnover of this pool is so rapid that the radioactivity present can only be accounted for by the breakdown of protein. Probably, the 10·3% activity associated with the ethanol-soluble "carbon pool" should also be included since the bulk of this isotope must come from degradation products of the amino acids and not polysaccharides. Only the 1·4% polysaccharide of the radioactivity of the seven-day plant can clearly be considered neither protein nor derived from protein, and should be subtracted from the 97·2% total activity recovered. In the middle of the last 24-hr period, N was larger by one half the activity of $^{14}CO_2$ respired during the 24-hr period (Table 1); thus,

$$\tau = 0.693\, \frac{(0.972\text{–}0.014)\ 1.466 + (0.5)\ (0.146)}{0.146} = 7.0 \text{ days.}$$

This value is the time required to decrease the radioactivity in the metabolic system of proteins, amino acids, etc., where the main determining factor is the rate of protein degradation. The true half-life, i.e. the time in which half the radioactivity would leave protein in the absence of recycling, is related to the apparent half-life by

$$\tau_{app.} = \frac{V_1 + V_2}{V_1} \tau_{true}$$

where V_1 is the rate of entry of materials into intermediary labile metabolites of the system from outside, and V_2 the rate of entry from protein breakdown.[7] The present experiment does not allow an accurate estimate of these rates; however, an approximation based on the average specific activities of the amino acids of the ethanol extract compared to the residue suggests that about four new molecules enter from photosynthesis for every one from protein. The τ_{true} in the light would be, therefore, about four-fifths of 7 days, or about 5·6 days. This value

is then to be compared with 14 days, the length of time required for plants this size to double in size.[12]

The smaller release of $^{14}CO_2$ on the fifth day as compared to the seventh day may have several explanations. The amino acid pool depleted in the dark may have been replenished during the first and second-day light period. Similarly, certain proteins may be depleted in the dark, and rapidly formed in the light. Both of these phenomena would temporarily increase the amount of recycling and decrease temporarily the release of $^{14}CO_2$.

It is of some interest to consider the turnover in the dark period. Obviously, the apparent half-life varies erratically as shown in Fig. 1. The differential calculation is more sensitive to physiological variations in the biological material since much of the variation would not be apparent if we had computed N from these data and plotted it against time. In the last 24-hr period in the dark, the half-life was 1·3 days; in the immediately preceding 24-hr period, 1·43 days; and, in the one before that, 2·36 days. Figure 1 and these values show that the rate of protein degradation increases during the dark period. In a steady state condition, as is well-known, the apparent half-life can only decrease with time as the more rapid components come to isotopic equilibrium. This suggests that as starvation proceeds, the plant utilized increasing kinds of proteins that are then broken down for metabolic energy needs.

MATERIALS AND METHODS

Three plants were employed in this work. Similar conditions and results were obtained in each experiment except for the initial burst of $^{14}CO_2$. The details of Experiment III are described below. Ten μc of DL-[1-^{14}C]-glutamic acid (8·26 μmoles in 0·01 M phosphate buffer at pH 6) were injected into the mid-vein of the sixth macroscopically visible leaf from the apex of a 12-week-old tobacco plant (*Nicotiana tabacum* var. Hick's Broadleaf). The plant was placed in a 37-l. glass chamber through which air was drawn at the rate of 13·5 l/hr. After leaving the chamber, the air was passed through a 250-ml ionization chamber of a vibrating reed electrometer and then through a series of 1 N NaOH scrubbers. The rate of $^{14}CO_2$ released from the plant was recorded continuously (Fig. 1). The plant was kept in the light for 13 hr and then placed in the dark to deplete radioactivity from readily accessible pools. After 3·5 days (84 hr), the plant was returned to the light. In this second light period, $^{14}CO_2$ production was very slow; therefore, the average rate of $^{14}CO_2$ released by the plant was estimated from radioactivity in NaOH scrubbers employed for successive 24-hr periods. The total $^{14}CO_2$ for each period was measured with the vibrating reed electrometer by the rate of charge method.[13] Electrometer readings were converted to μc with the use of a calibration factor determined by measuring the $^{14}CO_2$ produced by the action of L-glutamic decarboxylase[14] on an aliquot of the labelled glutamic acid (1 mv/min $= 9 \cdot 6 \times 10^{-5}$ μc). The L-glutamic decarboxilase method was also used for estimation of labelled glutamic acid in certain samples.

After 3 days in the second light period, the plant top was cut into small pieces and dried at 80° for 24 hr. The dried plant tissue was then ground to pass 80 mesh in a Wiley mill. Samples of dried ground tissue were extracted in a Soxhlet with ether for 8 hr followed by ethanol for 16 hr. The residue was hydrolyzed with 6 N HCl in a vacuum-sealed glass tube at 104° for 17 hr. Both the ethanol extract and the filtered, dried, redissolved hydrolysate were fractionated on Dowex 50 into a non-adsorbed fraction and an adsorbed fraction. In the case of the

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[13] B. M. Tolbert, Univ. Calif. Radiation Lab. (UCRL-3499), Berkeley (1956).
[14] E. F. Gale, *Biochem. J.* **41**, vii (1947).

ethanol extract, these two fractions were designated as carbon pool fraction and amino acid fraction. In the case of the hydrolysate, these were designated as polysaccharide fraction and protein amino acid fraction. It is recognized that these are crude fractions and will contain other substances. The adsorbed fractions were eluted with 1 N NH_4OH. All the various fractions were plated on planchets as "infinitely" thin samples and counted in a gas flow counter operated in the Geiger region. As an alternative method of fractionation, samples of dried, ground tissue were refluxed for 24 hr with an excess of 6 N HCl[15] and the filtered, dried, redissolved hydrolysate separated into three amino acid fractions on Dowex 50 according to the method of Thompson *et al.* [16] The fractions were chromatographed in butanol: acetic acid: water (250:60:250), v:v:v) in duplicate series. One series was developed with ninhydrin; areas on the other series corresponding to the ninhydrin-positive spots were cut out and the adsorbed substances were eluted and counted as infinitely thin samples in a gas flow counter operated in the Geiger region. The acid lumin fractions were also counted as infinitely thin samples.

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[16] J. F. THOMPSON, C. J. MORRIS and R. K. GERING, *Anal. Chem.* **31**, 1028 (1959).