2,3,6,6′-tetraacyltrehalose 2′-sulfate. The approximate molecular formula $C_{145}H_{275}NO_{20}S \pm$ (5C, 10H), molecular weight about 2384 was advanced for NH_4SL-I on the basis of microanalysis and molecular weight determination through utilization of the spontaneous desulfation which characterizes the ammonium sulfolipids. The apparent unsaturation was believed attributable in part to ketonic functions within the acyl groups; however, further study showed this not to be the case. The empirical formula is now revised to a more precise expression as indicated in Figure 12, and is in excellent accord with the now demonstrated distribution of the acids which have been described herein. The actual placement of the individual substituents has not yet been ascertained; this is an effort currently in progress.

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Protein Metabolism in Cultured Plant Tissues. Calculation of an Absolute Rate of Protein Synthesis, Accumulation, and Degradation in Tobacco Callus *in Vivo**

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ABSTRACT: During exponential growth of cultured tobacco cells, amino acids present in the intracellular soluble pool were used to synthesize proteins. The mean pool size (0.1 μmole/g fresh weight for leucine) remained unchanged during growth. Radioactive leucine was taken up from the medium and incorporated into protein and the soluble leucine pool. Labeled leucine was not converted into other amino acids in the pool; all of the radioactivity found in protein was contained in leucine. When tissues were labeled on radioactive medium the specific radioactivity of the soluble leucine pool remained unchanged from at least 1 hr through 7-hr labeling and incorporation of radioactivity into protein was linear over the same time period. These data suggest that incorporation was a function of the specific radioactivity of the soluble leucine pool. A simple differential equation was developed to relate these data to observations of synthesis.

The rate of accumulation of total and soluble protein (7.1 mg of protein × g of protein $^{-1}$ × hr^{-1}) was measured directly and equaled the rate of growth of the tissue (7.1 mg of tissue × g of $tissue^{-1}$ × hr^{-1}). Kinetics of uptake and incorporation of radioactivity were used to calculate an absolute rate of protein synthesis. Comparing that rate (18 mg of protein × g of $protein^{-1}$ × hr^{-1}) to the rate of accumulation a rate of protein degradation was estimated (11 mg of protein × g of $protein^{-1}$ × hr^{-1}).

Although it is generally accepted that amino acids (not peptides) serve as precursors for protein synthesis, the role of the measurable amino acid pool within the cell remains unclear. The assumption that the measurable pool represents the protein biosynthetic precursor pool has been seriously challenged by findings in yeast (Cowie and McClure, 1959; Halvorson and Cohen, 1958), a slime mold (Wright and Anderson, 1960), mammalian cells (Kipnis *et al.*, 1961), and higher plant cells (Bidwell *et al.*, 1964). These studies indicate that only a small portion of the soluble amino acid pool is available for protein synthesis.

The objectives of this study were to determine: (1) whether

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an absolute rate of protein synthesis for cultured tobacco cells can be calculated from uptake and incorporation data; and (2) whether the amino acid pool is the obligatory precursor pool for protein synthesis. We also consider those problems that occur in calculating an absolute rate of synthesis and present a method of estimating protein degradation or turnover.

Materials and Methods

Callus tissue derived from pith of *Nicotiana tabacum* var. Wisconsin no. 38 was maintained on Linsmaier and Skoog's medium (Linsmaier and Skoog, 1965). Kinetin and indoleacetic acid were supplied at 1 and 11.5 μM, respectively. This tissue was obtained from the standard "tight" stock maintained by Helgeson (Helgeson and Upper, 1970). Growth rate determinations were made as described by Helgeson *et al.* (1969).

Exponentially grown tissues were labeled by carefully removing them from the growth medium and placing them on an identical agar medium containing 0.33 μM L-[4,5-^{3}H]-leucine (60 Ci/mmole, New England Nuclear).[1] Generally, no less than 5 ml nor more than 20 ml of labeling medium was used per g fresh weight of tissue. The exogenous leucine concentration, therefore, ranged from 0.3 to 1.2% of that of the soluble pool in the tissue. Therefore, even if quantitative uptake of leucine occurred there would not be a significant change in the soluble pool concentration.

For pulse-chase experiments the tissues were transferred from the radioactive medium to identical nonradioactive growth medium containing L-[^{1}H]leucine.

In those experiments where radioactive sucrose was added, the concentration of sucrose in the medium was reduced from 88 to 20 mM to conserve radioactive material. Tissues were preconditioned 2 days on 20 mM unlabeled sucrose medium before labeling on medium containing 20 mM uniformly labeled [^{14}C]sucrose (1.2 Ci/mmole, New England Nuclear). The growth rate of the tissue was unaffected by the change to 20 mM sucrose (unpublished data).

After labeling for the times specified, tissues were immediately homogenized with a Duall tissue grinder in an equal volume (w/v) of cold 0.03 M potassium phosphate buffer (pH 7.0) containing 20 mM β-mercaptoethanol. The homogenate was centrifuged for 10 min at 12,000g and the clear supernatant liquid retained as the soluble protein and soluble amino acid pool fractions.

The supernatant liquid was separated into soluble and precipitated fractions by the additions of an equal volume of 10% (w/v) trichloroacetic acid. After 30 min at 4° the precipitated material was removed by centrifugation and washed once with cold 10% trichloroacetic acid. The soluble fraction (soluble amino acid pool) and wash were combined and radioactivity measured. The washed precipitated fraction (soluble protein) was dissolved in a volume 0.5 M sodium hydroxide equal to the volume of the original supernatant liquid and a portion removed for protein determination and radioactivity measurements. Protein was estimated by a modified Lowry procedure (Rutter 1967). Radioactivity was measured in a Nuclear-Chicago Mark II liquid scintillation spectrometer using a scintillant solution containing 110 g of naphthalene, 5.5 g of 2,5-diphenyloxazole, and 56 mg of 2-(α-naphthyl)-5-phenyloxazole dissolved in 1 l. of p-dioxane. Counting efficiency was 50% for ^{3}H and 90% for ^{14}C. All samples were counted to a two σ error of less than 3% for ^{14}C and 1% for ^{3}H.

To determine amino acid composition, the soluble protein fraction was heated to 110° for 20 hr in an evacuated tube containing 6 N HCl. The hydrolysates were evaporated to dryness in a rotary evaporator, dissolved in distilled water, and evaporated again to dryness. The residue was dissolved in 0.2 N sodium citrate buffer (pH 2.2), centrifuged to remove sediment, and analyzed. Specific radioactivity of leucine in trichloroacetic acid soluble (amino acid pool) and precipitable fractions (soluble protein) was determined using a Beckman Model 120B amino acid analyzer after the protein fraction was hydrolyzed (Crestfield *et al.*, 1963). The acid and neutral amino acids were measured using Beckman PA 28 resin and the basic amino acids using PA 35 resin. The change in pH, due to the presence of trichloroacetic acid in the soluble fraction, retards the rapidly eluted amino acids about 30 min on the PA 28 resin and prevents the separation of threonine and aspartic acid. Fractions from the analyzer were collected every 4 min and radioactivity was measured in an aliquot as described above. Total radioactivity under the leucine peak was estimated and compared to the quantity of leucine measured by its absorbance at 570 nm after reaction with ninhydrin. Specific radioactivity was expressed as curies of radioactivity in leucine per mole of leucine.

Total protein was estimated from the sum of soluble protein, base-extracted protein, and residue protein. The pelleted material which remained after removal of the soluble protein (45% of the total protein) and soluble amino acid pool from the original homogenate was extracted twice with 0.5 M sodium hydroxide. The sodium hydroxide extracted protein was separated from the residue pellet by centrifugation and reprecipitated by adding two volumes of 10% (w/v) trichloroacetic acid. The final precipitation of the sodium hydroxide extracted protein was necessary to remove traces of β-mercaptoethanol which appear to interfere with protein determination. Protein was determined in an aliquot after the precipitated fraction was redissolved in 0.5 M sodium hydroxide. Generally 45–50% of the total protein was extracted from the pellet by sodium hydroxide. Therefore, the combination of soluble protein and sodium hydroxide extracted protein represents about 90–95% of the total cellular protein. The residue pellet was heated to 110° in 6 N HCl for 20 hr under a vacuum to hydrolyze any remaining protein. The quantity of amino acids liberated from this residue were measured on the amino acid analyzer. Residue protein (5–10% of the total) was estimated from the sum of all of the micromoles of amino acids present in this fraction.

Results

Protein Accumulation. The growth of callus tissue is illustrated in Figure 1. For at least 20 days there was an exponential increase in fresh weight, soluble protein, and total protein of the tissue, all having a doubling time equal to 96 hr. Previous examination of callus tissue (Helgeson and Upper, 1970) demonstrated that dry weight also increased exponentially under these conditions.

The rate of accumulation of either total protein or soluble protein as calculated from the doubling time equaled 7.1 mg of protein accumulated $\times$ g of protein$^{-1} \times$ hr^{-1}. Since the rates of accumulation of protein were identical with the

[1] Mention of a trademark name or a proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

TABLE I: Amino Acid Composition of Soluble Protein and Soluble Pools in Tobacco Callus Tissue.

Residue	Cellular Fraction[a]			
	Soluble Amino Acid Pool[b]		Soluble Protein[b]	
	μmoles/g Fresh Wt	% Total	μmoles/g Fresh Wt	% Total
Glutamic acid	0.99 ± 0.15[c]	26.1	2.69 ± 0.30	12.5
Aspartic acid	0.26 ± 0.07	6.8	2.37 ± 0.30	11.0
Threonine			1.08 ± 0.04	5.0
Serine	0.24 ± 0.03	6.3	1.12 ± 0.14	5.2
Proline	0.12 ± 0.04	3.2	1.16 ± 0.08	5.4
Glycine	0.24 ± 0.02	6.3	1.83 ± 0.12	8.5
Alanine	0.56 ± 0.10	14.7	1.85 ± 0.09	8.6
Valine	0.06 ± 0.007	1.6	1.40 ± 0.17	6.5
Methionine	0.04 ± 0.01	1.1	0.38 ± 0.04	1.8
Isoleucine	0.04 ± 0.007	1.1	1.05 ± 0.08	4.9
Leucine	0.09 ± 0.007	2.4	1.72 ± 0.08	8.0
Tyrosine	0.03 ± 0.009	0.8	0.62 ± 0.06	2.9
Phenylalanine	0.03 ± 0.009	0.8	0.84 ± 0.04	3.9
Lysine	0.05 ± 0.02	1.3	1.70 ± 0.10	7.9
Histidine	0.02 ± 0.005	0.5	0.43 ± 0.04	2.0
Arginine	0.03 ± 0.01	0.8	1.03 ± 0.14	4.8
Glutamine	1.0 ± 0.20	26.3		
Total	3.80	100.0	21.27[d]	100.0

[a] Clarified cell homogenates were fractionated with cold trichloroacetic acid into a soluble amino acid fraction and a precipitated protein fraction. [b] The soluble amino acid fraction was placed directly on an amino acid analyzer whereas the protein fraction was first hydrolyzed 20 hr in 6 N HCl at 110° under a vacuum. [c] The standard deviation was calculated from the average of five runs. [d] Based on a weighted-average molecular weight of 128 and a total of 21.27 μmoles of amino acids the quantity of soluble protein present was 2.72 mg of protein/g fresh weight. This value is similar to protein measured directly by the Lowry procedure (see Figure 1).

rate of accumulation of fresh weight of tissue, the concentration of both total protein and soluble protein would be constant within the tissue throughout its exponential growth phase.

Amino Acid Distribution. To calculate an absolute rate of protein synthesis, it was necessary to measure the specific radioactivity of the amino acid in the biosynthetic precursor pool and the same amino acid in the synthesized protein. Table I presents the amino acid composition of the hydrolyzed soluble proteins and soluble amino acid pools in callus tissue. The standard deviation for individual amino acids ranges from 4 to 14% of the average for protein and from 8 to 32% of the average for the soluble pool. The standard deviations for leucine are 5% in soluble protein and 10% in the soluble amino acid pool. The concentration of leucine is 0.09 μmole/g fresh weight in the soluble pool and 1.72 μmoles/g fresh weight in soluble protein. Because neither soluble protein nor soluble pool concentrations change during growth, the ratio of leucine in soluble protein to leucine in the pool is 19 to 1.

If significant protein hydrolysis occurred during sample preparation the values for the amino acid pools might be abnormally high. Equal amounts of tissue were prepared by two procedures; the first as described in Methods and the second by grinding the tissue to a fine powder in an equal volume of 10% trichloroacetic acid under liquid nitrogen. In neither case did the values for any amino acid pool vary significantly from those presented in Table I.

The amount of soluble protein present in callus, using data in Figure 1 estimated by the Lowry method, was calculated and equaled 2.8 mg of protein/g fresh weight. Using the total number of micromoles of amino acids liberated from the soluble protein, as measured in Table I, the amount of soluble protein was calculated and equaled 2.7 mg of protein/g fresh weight. These methods agree well within experimental error. Thus there was no major contamination of the protein fraction with pool amino acids.

Several investigators have reported the presence of a radioactive fraction (likely aminoacyl-tRNA) in labeled cells which was precipitated by cold trichloroacetic acid but solubilized by a 90° treatment with trichloroacetic acid. Stephenson *et al.* (1956) reported that 20–30% of the radioactivity found in trichloroacetic acid precipitated material of plant tissue labeled with [^{3}H]leucine was tightly bound material removed by dissolving the precipitated fraction in sodium hydroxide followed by a second precipitation with trichloroacetic acid. To test these possibilities callus tissue was labeled with [^{3}H]leucine and a homogenate prepared as described in Methods. Approximately 0.6% of the cold trichloroacetic acid precipitable radioactivity was solubilized by 90° treatment with trichloroacetic acid (Table II); and less than 2% of the radioactivity remained soluble after dissolving in sodium hydroxide and reprecipitating with trichloroacetic acid.

The distribution of radioactivity in the soluble amino acid pool and in the protein fractions is presented in Figure 2A,B, respectively. More than 95% of the radioactivity in the soluble protein fraction cochromatographed with

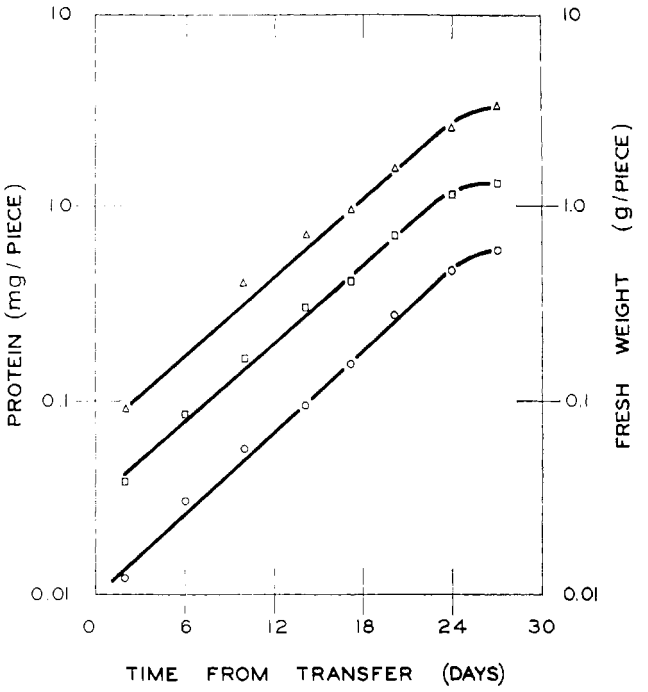

FIGURE 1: Growth and protein content of tobacco callus tissue on media containing 88 mM sucrose, 11.5 μM indoleacetic acid, and 1.0 μM kinetin. Fresh weight points (○—○—○) represent the average fresh weight of 24 pieces (6 pieces/plate). Soluble protein (□—□—□) represents that fraction of the total extractable protein (△—△—△) which is solubilized by homogenization of tissue in buffer (see Methods for details).

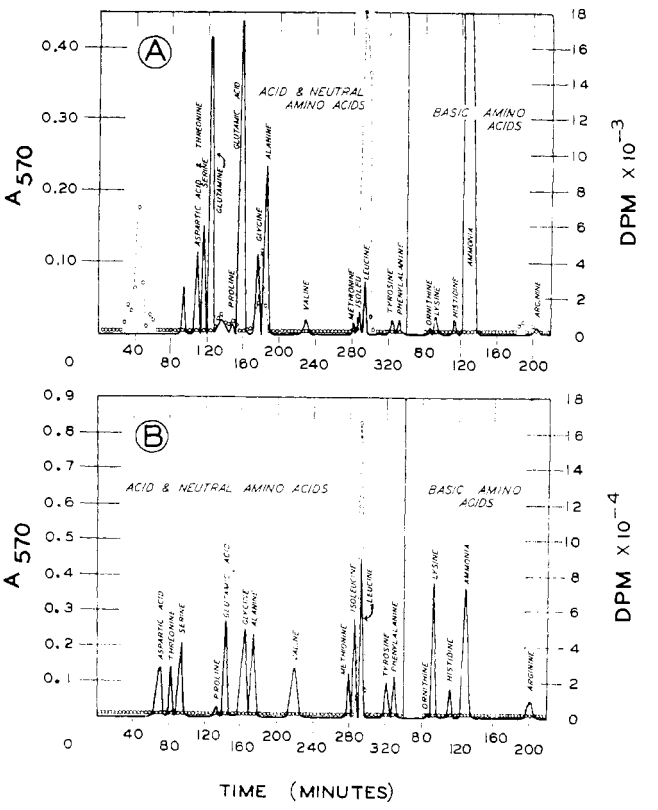

FIGURE 2: Chromatography of amino acids present in the soluble amino acid pool (A) and hydrolyzed soluble protein (B). Ninhydrin-reacting material (—) is measured at 570 nm. Radioactivity (- - -) is measured in an aliquot of each fraction collected from the amino acid analyzer using a liquid scintillation spectrometer. Tissues were labeled 7 hr on media containing [^{3}H]leucine. Fractions were obtained as described in Methods.

leucine and less than 0.5% of the radioactivity was associated with any other amino acid peak (Figure 2B). Leucine was the only amino acid in the soluble pool fraction to contain tritium (Figure 2A). Thus, no conversion of [^{3}H]leucine into other amino acids had occurred. However, only 40% of the total radioactivity applied to the analyzer was coeluted with leucine. An additional 45% of the radioactivity was eluted from the column largely with unidentified, ninhydrin-negative materials. Approximately 15% was not eluted under our conditions of chromatography.

Fractions collected from the amino acid analyzer lost 50% of their radioactivity upon standing for 18 hr. A likely explanation of this phenomenon is that the radioactive products formed by the ninhydrin reaction are volatile. The reaction product formed from leucine is presumably the volatile liquid isovaleraldehyde which boils at 92° (Virtanen *et al.*, 1940). If the ninhydrin pump on the analyzer was turned off so that the fractions were collected in buffer, no significant radioactivity was lost, even after standing 48 hr. We recommend that two separate runs be made on the analyzer, one for measuring ninhydrin-positive material and one for measuring radioactivity. If a single run were desired, the radioactivity in the fractions should be measured immediately. Both procedures yield similar results.

TABLE II: Amount of Trichloroacetic Acid Soluble ^{3}H Material Contained in the Labeled Soluble Protein Fraction.

	Trichloroacetic Acid[a]		
Temp (°C)	Soluble dpm × 10^{-3}	Insoluble dpm × 10^{-3}	% Soluble
4	5	810	0.6
90	10	840	1.2

[a] A trichloroacetic acid precipitated fraction of soluble protein was prepared as described in Methods. The precipitate was treated with 10% trichloroacetic acid at 4 and 90° for 10 min.

Synthetic Rates. Pieces of logarithmically growing tissue of three different sizes were placed on medium containing [^{3}H]leucine, and removed for analysis at various times. The amount of tritium in the soluble protein fraction of these tissues is shown in Figure 3. Pieces weighing 330 mg each have a rate of incorporation equal to 280 dpm × μg of protein^{-1} × hr^{-1}, those weighing 110 mg each have a rate of 450, and the 40-mg pieces a rate of 700. In each case incorporation with respect to time remained linear from 0.5 to 7 hr. The 2.3-fold difference in incorporation rate, between tissues weighing 330 mg and those weighing 40 mg, suggests a similar difference in the rate of protein synthesis. Such a conclusion would be valid only if there were no change in the specific radioactivity of the precursor pool with respect to both piece size and time.

Figure 3 shows the accumulation of radioactivity in the total soluble amino acid pool fraction and in the leucine pool as measured with the amino acid analyzer. There was a very rapid accumulation of radioactivity in the total soluble amino

TABLE III: Differences in Specific Radioactivity of the Soluble Leucine Pool and Soluble Protein between Three Sizes of Tissue, All of Which Have the Same Rate of Protein Synthesis.

Specific Radioactivity of Leucine[a]			
Tissue Fresh Wt[c] (mg)	Soluble Pool (Ci/mole)	Protein Bound (Ci/mole)	Rate of Synthesis[b]
40	25	3.5	20
110	16	2.2	19
330	10.5	1.4	19

[a] Tissues were labeled for 7 hr and specific radioactivity of leucine measured as described in Methods. [b] Rates of protein synthesis (mg of protein synthesized × g of protein^{-1} × hr^{-1}) were calculated from eq 2. [c] Fresh weight of any piece of tissue varied as much as 20%, however, the average of ten or more pieces was as reported.

acid pool fraction during the first 30 min on [^{3}H]leucine containing medium, followed by a greatly reduced rate of accumulation. The accumulation of radioactive material in the total soluble pool, during the time radioactivity in leucine remained constant, can be accounted for by the accumulation of the unknown materials, identified only as nonninhydrin-reactive peaks from the analyzer.

Even though the specific radioactivity of the leucine pool remained unchanged with time it did change according to the size of the piece of tissue used (Table III). However, the ratio of radioactivity in leucine of the soluble protein to that in the soluble amino acid pool at any given time after labeling was the same for tissue pieces of varying weights. This suggests that, although the total amount of radioactive leucine incorporated into protein varied by 2.3-fold, the fraction incorporated per unit time (*i.e.*, the rate of protein synthesis) was the same no matter what the size of the tissue.

The decrease in specific radioactivity of the soluble leucine pool, and hence a decrease in the rate of incorporation of radioactivity into protein occurring as the size of the tissue increases, appears to be related to the geometry of the tissue on the labeling medium and, therefore, probably not to changes in metabolism. The volume of the tissue (V), the height of the tissue above the medium (h), and the area of the tissue in contact with the medium (A), were measured and related to one another by $(V/A) \times h$. SA pool (see eq 1) and incorporation rates were inversely proportional to $(V/A) \times h$ over a tenfold range of $(V/A) \times h$ and a 100-fold range of weight. Data for the three tissue sizes presented in Table III are given in Table IV. These data suggest the following relationship

$$\text{relative incorporation rate} = \frac{1}{\frac{V}{A} \times h} = \frac{A}{V \times h}$$

As the area in contact with the medium increases, or as the volume (weight) of the tissue decreases, the specific radioactivity of the soluble pool, and hence the rate of incorporation, will increase.

Since the specific ratioactivity of the leucine pool does not

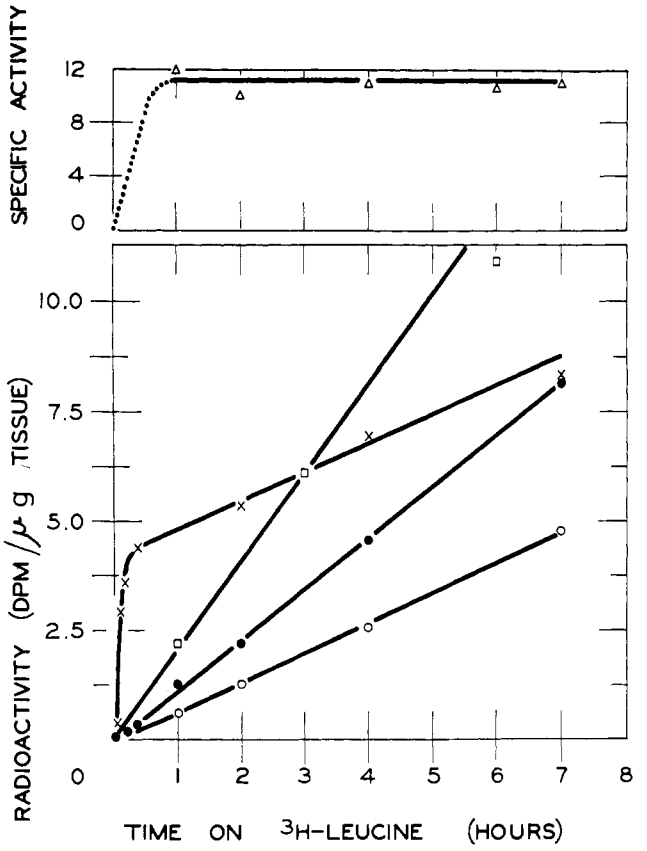

FIGURE 3: Quantity of radioactivity in soluble protein and soluble amino acid pool fractions at times indicated. The amount of radioactivity in the soluble protein fraction is shown for tissue of 40 mg (□—□), 110 mg (●—●), and 330 mg (○—○) fresh weight. Total soluble amino acid pool fraction (×—×) is presented for 110-mg size pieces. The inset represents the specific radioactivity (Ci/mole) of the soluble leucine pool in 330-mg size pieces.

change during the labeling period, and incorporation of [^{3}H]leucine into protein is constant with time, it is possible to calculate the rate of protein synthesis from the eq 1,

$$\frac{\text{d(SA protein)}}{dt} = R \times \text{(SA pool)} \qquad (1)$$

where R = rate of protein synthesis, (SA protein) = specific radioactivity of leucine in protein, (SA pool) = specific radioactivity of leucine in the soluble precursor pool, and t = time.

After integrating eq 1 between the limits of $t = 0$ and $t = T$, eq 2 is derived.

$$R = \frac{\text{(SA protein) at } T}{\text{(SA pool)} \times T} \qquad (2)$$

The calculations of absolute rate of protein synthesis for the three size pieces used in Figure 3 are presented in Table III. The rates of synthesis were all equal to 19 ± 1 mg of protein synthesized × g of protein^{-1} × hr^{-1}.

These rates are calculated for only the soluble proteins which constitute approximately 45% of the total protein; therefore, it is of interest to examine the remaining 55% of the cellular protein. For this reason tissues were labeled for 7 hr then prepared as usual. The soluble protein fraction

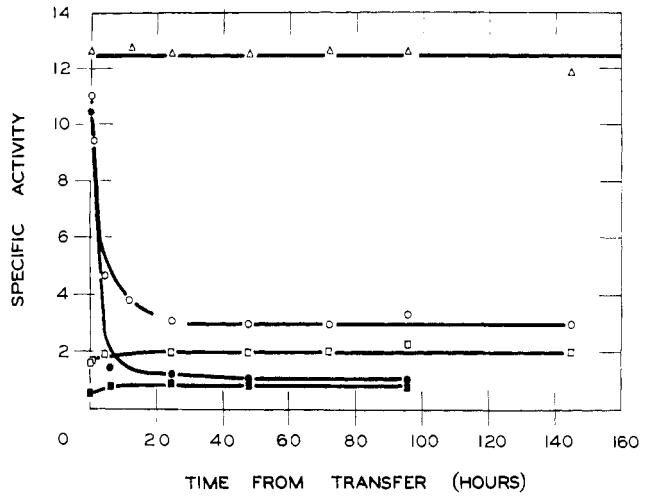

FIGURE 4: Specific radioactivity of leucine after transferring to nonradioactive media. Tissue was placed on medium containing [^{3}H]-leucine for 2 hr (closed symbols) and 7 hr (open symbols) and then transferred to nonradioactive media for the time indicated. After harvesting, tissues were prepared and specific radioactivity of the soluble leucine pool (circles) and soluble protein (squares) fractions were determined as described in Methods. The total radioactivity (μCi $\times$ g fresh weight^{-1} $\times$ 2) of tissue (labeled 7 hr) is represented by the open triangles. All data points were corrected for dilution due to growth by multiplying each by the ratio of its final fresh weight/initial fresh weight. Weight measurements were taken when tissue was transferred to nonradioactive media and again after harvesting. Tissue grew during these periods with a generation time of 4 days.

(in this case 43% of the total protein) had a specific radioactivity of 1.7 Ci/mole of leucine. The extractable protein fraction (solubilized by treatment with 0.5 M sodium hydroxide, 48% of the total protein) had a specific radioactivity of 1.6 Ci/mole. The residue (9% of the total protein) had a specific radioactivity of 1.4 Ci/mole of leucine. Leucine ranged from 7.7 to 8.0% of all three protein fractions. The weighted-average specific radioactivity for all protein was 1.6 Ci/mole. These values suggest there may be some preferential synthesis of the more easily solubilizable proteins. Although differences may not be great, the rate of synthesis based on only soluble protein may be higher than that based on total protein by 6%. The average value for the rate of soluble protein synthesis was 19 $\pm$ 2 mg of protein synthesized $\times$ g of protein^{-1} $\times$ hr^{-1} calculated from ten experiments performed with tissues of 40–300 mg/piece. Rates of synthesis of extractable and residue protein were not calculated for these experiments. However, if we assume that synthesis of total protein will average 6% slower than that of soluble protein, the average rate of synthesis of total cellular protein would be 18 $\pm$ 2 mg of protein synthesized $\times$ g of protein^{-1} $\times$ hr^{-1}. This rate would be equivalent to a doubling every 39 hr if there were no turnover of protein. This of course is much greater than the rate of 7.1 mg/hr at which protein accumulates, as measured in Figure 1; this suggests significant protein turnover. The difference between the rate of accumulation and synthesis will be taken as the average rate of protein degradation. For callus tissue grown under our conditions this rate equals 18.0 $-$ 7.1 = 10.9 mg of protein degraded $\times$ g of protein^{-1} $\times$ hr^{-1}.

TABLE IV

Tissue Size (mg)	Incorporation Rate (dpm/μg of Protein)	(Rel)	$(V/A) \times h$
40	700	18	7
110	450	11	12.7
330	280	7	17.9

TABLE V: Comparison of Protein Synthesis Rates Based on Either Exogenous Leucine or Sucrose as the Source of Radioactivity.

Radioactive Source	Specific Radioactivity of Leucine[a] Soluble Pool (Ci/mole)	Protein Bound (Ci/mole)	Rate of Synthesis[b]
[^{14}C]Sucrose	0.075	0.009	17
[^{3}H]Leucine	22.0	3.0	19

[a] Tissues were labeled for 7 hr on the radioactive sources listed and specific radioactivity of leucine measured using the amino acid analyzer. [b] Rates of protein synthesis (mg of protein synthesized $\times$ g of protein^{-1} $\times$ hr^{-1}) were calculated from eq 2.

Source of Radioactive Label. We compared rates of synthesis calculated from data obtained by labeling tissues with either [^{14}C]sucrose or [^{3}H]leucine as exogenous sources in the medium. All procedures were the same as those described in Methods except that the concentration of sucrose in the labeling medium was reduced from 88 to 20 mM for both sucrose- and leucine-labeled tissue. The specific radioactivity of leucine in protein and in the pool vary by a factor of 10^3, depending on whether labeled sucrose or leucine were added (Table V). Leucine, as described earlier, was not converted into other common amino acids and as a result all radioactivity precipitated from a homogenate by trichloroacetic acid was present in leucine of protein. Sucrose, on the other hand, was converted into other amino acids along with many other biosynthesized cellular components. Although the distribution of radioactivity and the specific radioactivity of leucine fractions were very different from [^{14}C]sucrose to [^{3}H]leucine, the ratio (SA protein)/SA pool) for leucine remained constant. Therefore, the observed rate of protein synthesis was independent of the exogenously added compound used to label the tissue. As will be discussed later these data suggest that the measured leucine pool is the true precursor pool for protein synthesis.

Pulse-chase experiments further strengthened the hypothesis that the experimentally measured leucine pool and the true precursor pool are identical. In these experiments tissues were labeled for either 2 or 7 hr then transferred to fresh nonradioactive medium. Total radioactivity within a piece of tissue remained constant during a chase period of 6 days (Figure 4). Total radioactivity per gram fresh weight, on the other hand, should decrease, owing to dilution by growth. This value however, becomes constant when corrected for growth by multiplying by the ratio of final/initial fresh weights (Figure 4). Specific activities of leucine in either soluble protein or soluble amino acid pool will change, owing to both growth

and protein metabolism. Again the correction for dilution by growth was made and the results shown in Figure 4. There was an increase of 23% in the specific radioactivity of the protein, and a decrease of 75% in the specific radioactivity of the leucine pool, for tissue labeled 7 hr; and there was a 50% increase in the specific radioactivity of the protein and a 90% decrease in the specific radioactivity of the pool for the 2-hr labeling period. In both cases equilibrium appears to be established after 40 hr; and the specific radioactivity of the pool is approximately 30% higher than that of the protein.

Discussion

Conclusions concerning *in vivo* rates of protein synthesis in both plants and animals are often drawn from rather incomplete data. Radioactive incorporation of exogenous, labeled amino acids into acid-precipitable material is repeatedly used to calculate at least a relative (if not an absolute) rate of synthesis. Many times such data are compared from one experiment to the next and differences interpreted as changes in rates of protein synthesis when in fact they may be due to differences in rate of synthesis of a nonprotein, acid-precipitable material or to a change in the distribution of radioactivity within precursor pools (including precursors of material other than protein). Figure 3 and Table III show that the 2.3-fold change in the rate of leucine incorporation into protein was not due to an equal difference in the rate of protein synthesis, but rather to a 2.3-fold increase in the specific radioactivity of the soluble leucine pool. Furthermore, only 40% of the radioactivity in the total soluble pool was even found in leucine. Figure 3 also shows that the tritium in the total pool can increase while that in the particular soluble amino acid (leucine) remains unchanged. We believe it is very important to examine the individual amino acid in the pool and the same amino acid in protein.

As a final example, there are reports that do consider the individual amino acid pools but which equate them to the exogenous pool. This is proper only if equilibrium has occurred. In the present case, the endogenous leucine pool is large and has not equilibrated with the low concentration of highly labeled exogenous leucine. As a consequence, there are almost four orders of magnitude difference in specific radioactivity between the two. The effects on the calculation of a rate of synthesis are obvious.

We calculated the rate of protein synthesis for cultured tobacco cells using eq 2 and the specific radioactivity of leucine in protein, the specific radioactivity of the soluble leucine pool, and the changes with time of these quantities. This calculation was greatly simplified by the observation that SA pool was unchanged for the duration of the labeling period, and that SA protein increased linearly during this period. Thus the use of a simple equation (eq 2) is valid. The equation is a simplification of that reported by other investigators (Fry and Gross, 1970), but without the added complication of a changing SA pool.

Turnover was estimated as the difference between the measured rate of protein synthesis and the measured rate of protein accumulation (Figure 1). Both total protein and soluble protein accumulated at the same rate; and this rate was equal to the growth rate as measured by fresh weight accumulation (see Figure 1). Thus protein degradation in either fraction will equal 10.9 mf of protein degraded $\times$ g of protein^{-1} $\times$ hr^{-1} or a turnover rate of 1.1%/hr. Protein turnover in cultured mammalian cell was reported as 0.7–1% for nongrowing cultures and estimated at the same rate for growing cultures (Eagle *et al.*, 1957). The same range (0.2–2%) has also been reported for cultured plant cells (Bidwell *et al.*, 1964) and echinoderms (Fry and Gross, 1970).

It is generally believed that carbon for protein synthesis enters through the free amino acids within the cell. Several investigators (Bidwell *et al.*, 1964; Berg, 1968; Wright and Anderson, 1960; Steward and Bidwell, 1966) have suggested that only a small part of the total free amino acid pool makes up the biosynthetic precursor pool for protein synthesis. Bidwell *et al.* (1964) has extended the two-pool concept, at least for cultured carrot cells, to include the relative rates at which carbon from exogenous sugar or amino acids enters protein and the soluble amino acid pool. These investigators report that carbon from exogenous, labeled sugar entered protein more rapidly than the soluble amino acid pools; while carbon from exogenous amino acids entered the soluble amino acid pool more rapidly than protein. Their findings necessarily predict a change in the apparent rate of protein synthesis (as calculated by eq 2) when different exogenous sources are supplied. This is not the case with cultured tobacco cells. When the specific radioactivity of leucine in the soluble amino acid pool and in the protein were measured and a rate of synthesis calculated, it made little difference whether labeled sucrose or labeled leucine were exogenously supplied. Pulse-chase experiments where radioactivity was rapidly chased from the soluble leucine pool into protein, with no loss of total radioactivity, strongly suggests that the soluble leucine pool is a precursor in protein synthesis. During these experiments the specific radioactivity of the soluble leucine pool approached that of protein instead of zero suggesting that equilibrium exists between the two due to turnover. For both the 2- and 7-hr pulses the corrected specific radioactivity of the soluble pool appears to asymptote about 30% above the estimated asymptote for the protein. The difference between the pool asymptote and that for protein represents 3% of the radioactivity in the soluble leucine pool after 2-hr labeling and 9% after 7-hr labeling. Since equilibrium between only 90% of the pool and protein occurs, it is very possible that a small amount of a radioactive compound which appears on the amino acid analyzer to be part of the leucine pool has accumulated. Such a compound must be neither available to protein synthesis nor in equilibrium with the soluble leucine pool. A most likely candidate for such a compound is D-leucine. A contamination of the exogenous L-leucine with less than 1% D-leucine would explain these results if D-leucine is taken up but not metabolized by this tissue. In any case, a second pool (3% of the synthetic pool when tissues were labeled 2 hr), be it D- or L-leucine, would not greatly affect the calculation of synthetic rates.

Investigators working with slime mold (Wright and Anderson, 1960), in contrast to our conclusions, have reported that measured protein synthesis varied with the concentration of exogenous amino acid. They concluded that only part of the soluble amino acid pool was used for protein synthesis. Berg (1968) has presented similar data from sea urchin embryos and concluded that the intracellular soluble pool could be used partially if the uptake system of the cells was unsaturated (low exogenous amino acid concentrations); but that exogenous amino acids were used exclusively when the uptake system was saturated (high exogenous amino acid concentrations). In contrast, Fry and Gross (1970) recently demonstrated that, indeed, protein synthesis in sea urchin embryos does occur at the expense of the soluble amino acid pool no matter what the exogenous amino acid concentration. Similar conclusions can be drawn from the work of Smith

(1968) who reports that protein synthesis in *Rana pipiens* is unaffected by variations in pool size. Our preliminary experiments suggest that exogenous leucine has little effect on the measured rate of synthesis in tobacco callus.

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Thymidylate Synthetase from Amethopterin-Resistant *Lactobacillus casei**

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ABSTRACT: Thymidylate synthetase has been isolated from sonically disrupted cells of amethopterin-resistant *Lactobacillus casei* using a procedure that involves fractionation with ammonium sulfate, filtration through Sephadex G-100, and successive chromatography on CM-Sephadex, hydroxylapatite, and DEAE-Sephadex. The enzyme was crystallized by dialysis against ammonium sulfate. Homogeneity of the enzyme was established by disc electrophoresis on polyacrylamide gel and by ultracentrifugal analysis. The molecular weight of the protein (approximately 70,000) was obtained by polyacrylamide electrophoresis and gel filtration. Electrophoresis on sodium dodecyl sulfate–polyacrylamide revealed that the protein contains two subunits, each having a molecular weight of approximately 35,000. In Tris-acetate buffers, the enzyme displays a broad pH optimum between 6.5 and 6.8 when dUMP is the substrate (K_m = 5.1 × 10^{-6} M and 6.8 × 10^{-7} M, respectively, in the presence and absence of Mg^{2+}). The K_m value for *dl*,L-methylenetetrahydrofolate (in the presence of Mg^{2+}) is 3.2 × 10^{-5} M. The enzyme also utilizes UMP as a substrate, although at a reduced rate (*ca.* 40%) compared to dUMP. The UMP-dependent reaction also has a different pH optimum (5.9) and is not stimulated by Mg^{2+}. The catalytic activity of the enzyme, at all stages of purification, is increased severalfold by inclusion of thiols in the assay system. The enzyme is inhibited by *p*-mercuribenzoate, iodoacetate, *N*-ethylmaleimide, cyanide, H_2O_2, tetrathionate, 2-hydroxyethyl disulfide, and 5,5′-dithiobis(2-nitrobenzoic acid). All catalytic activity is lost when one of the four accessible sulfhydryl groups on the protein has reacted with *p*-mercuribenzoate.

Thymidylate synthetase catalyzes a novel reaction (eq 1) in which a methylene group undergoes a reduction while being transferred from tetrahydrofolate to C-5 of deoxyuridylate. Reduction of the one-carbon group is accommodated by oxidation of the tetrahydrofolate to dihydrofolate (Humphreys and Greenberg, 1958; Friedkin, 1959; McDougall and Blakley, 1960). During this oxidoreduction reaction, the hydrogen from C-6 of tetrahydrofolate is transferred without dilution to the methyl group of thymidylate (Friedkin, 1959;

5,10-methylenetetrahydrofolate + deoxyuridylate ⟶ dihydrofolate + thymidylate (1)

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