Rates of Aminoacyl-Transfer-Ribonucleic Acid Synthesis *in vivo* and *in vitro* by Bean Leaves

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1. A procedure for measuring rates of aminoacyl-tRNA synthesis *in vitro* and in intact leaves is presented. 2. Leaf discs showed rates close to those of intact leaves. 3. Cell-free preparations showed similar rates when assayed by pyrophosphate exchange, but actual aminoacyl-tRNA formation rates appeared to be much lower. Evidence is presented that dilution of supplied labelled amino acids was a major factor causing the low apparent rates. 4. Attempts to strip endogenous amino acids from plant tRNA resulted in low acceptor capability of the tRNA.

Aminoacyl-tRNA synthetases isolated from plant tissues yield activities of the order of nmol of amino acid exchanged/min per mg of protein when assayed by the pyrophosphate-exchange reaction of Stulberg & Novelli (1962). However, the activity of aminoacyl-tRNA fractions synthesized by the same enzyme preparations indicated low rates measurable in pmol of amino acid acylated/min per mg of protein (Moustafa & Lyttleton, 1963).

Since the activation of amino acids and their transfer to tRNA by cell-free fractions is a prerequisite to unequivocal demonstration of amino acid incorporation into protein it is important to obtain high rates of aminoacyl-tRNA synthesis. Loftfield & Eigner (1968) indicated that both the hydroxamate assay for amino acid activation (Hoagland, 1955) and the pyrophosphate-exchange assay differ from aminoacyl-tRNA formation with regard to the rate-determining step. Therefore in the establishment of cell-free protein-synthesizing systems it is essential that direct confirmation of the ability of the system to form aminoacyl-tRNA be obtained.

As a basis for determining what rates of aminoacyl-tRNA formation *in vitro* can reasonably be expected, we wished to determine the rates *in vivo*. Since the rates measured *in vivo* and *in vitro* apparently differ by five orders of magnitude, we also sought the causes contributing to low rates of aminoacyl-tRNA formation by plant cell-free systems.

EXPERIMENTAL

Materials. For most of the experiments expanding bean (*Phaseolus vulgaris* L. var. Tendergreen) leaves were used. The plants were grown under normal greenhouse conditions. All chemicals were of reagent grade. Hepes [1-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid],

dithiothreitol and Aquacide were products of Calbiochem, Los Angeles, Calif., U.S.A. Polyvinylpyrrolidone was purchased as the white insoluble form Polyclar AT from General Aniline and Film Corp., Grasselli, N.J., U.S.A.

Preparation of pH5 enzyme and RNA fractions. The protocol followed for the pH5 fraction is shown in Scheme 1. RNA was extracted essentially as described by Li & Weiser (1969) with the modifications noted in Scheme 1. Scheme 1 also shows the procedure followed for the extraction of aminoacyl-tRNA, which is similar to that of Shearn & Horowitz (1969).

Pyrophosphate-exchange assay. The procedure of Stulberg & Novelli (1962) was followed with the modifications described by Attwood & Cocking (1965).

Calculation of aminoacyl-tRNA synthesis rates. The typical system for cell-free aminoacylation contained: MgCl_2 , 40 μmol ; ATP, 10 μmol ; KCl, 10 μmol ; pH5 synthetase-enzyme fraction; RNA, 200 μg ; radioactive amino acid mixture, 10^6 d.p.m.; in a final volume of 1 ml buffered at pH 7.4 with 0.1 M-Hepes. Addition of CTP (Francki, Boardman & Wildman, 1965; Wong & Mustard, 1969) did not generally give stimulation in our system, and was therefore usually omitted. After reaction at 30°C the RNA, which contained newly aminoacylated tRNA, was precipitated (Scheme 1), and the amount of amino acid incorporated during the experiment calculated from the known specific radioactivity of the supplied amino acid (Scheme 2). For experiments *in vivo* this procedure could not be used, and calculation of the amount of amino acid incorporated into the RNA followed from determination of the specific radioactivity of amino acid hydrolysed from the aminoacyl-tRNA after adjustment of the pH to 8.9 with 1 M-KOH and saponification at 38°C for 2 h. The radioactivity released from 1 mg on hydrolysis is proportional to the amount of amino acid attached to tRNA during the experiment, and can therefore be quantified as outlined in Scheme 2.

Specific radioactivity measurement. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), and amino N by the procedure of Yemm & Cocking (1955). RNA was determined by the orcinol method (Slater, 1958), or by u.v. absorption, assuming

(a) pH5 enzyme

Young bean leaves (10g) ground under N₂ in an extraction medium (30 ml) containing: Hepes (50 mM, adjusted to pH 7.6 with KOH), dithiothreitol (4 mM), polyvinylpyrrolidone (4.5 g). The grinding flask was surrounded by crushed ice, and maceration was complete within 2 min by grinding with short bursts at 45 000 rev./min with a Virtis 45 homogenizer

Dark-green brei strained through acetate cloth and centrifuged
(10 000g_{av} for 30 min) at 4°C

Supernatant

Slowly adjusted to pH 5 at 4°C
by the addition of acetic acid

Kept for 20 min at 4°C, then centrifuged
(10 000g_{av} for 30 min)

Supernatant (discarded)

Sediment (discarded)

Sediment

Resuspended in Hepes
(pH 7.6, 50 mM, 2 ml), containing
dithiothreitol (4 mM)

(b) RNA

Young leaves (10g) ground, in a similar manner to the above, into an extraction medium containing a 30 ml portion of: (i) an aqueous solution (48 ml) of Hepes (pH 7.4, 10 mM), KCl (60 mM), MgCl₂ (10 mM); (ii) 50 ml of liquid phenol; (iii) 2 ml of sodium dodecyl sulphate (25%, w/v, in water)

Extract centrifuged (20 000g_{av} for 10 min)

Upper layer (aqueous)

Decanted, liquid phenol (20 ml)
added. Centrifugation repeated and
final aqueous layer dialysed with stirring (12 h).
The dialysis buffer was changed three times and contained:
tris (pH 8.3, 0.1 M), KCl (60 mM), MgCl₂ (10 mM)

Dialysed solution concentrated to 6 mg/ml by Aquacide II, or by precipitation with ethanolic potassium acetate and solution in
Hepes (pH 7.6, 50 mM)-dithiothreitol (4 mM) buffer

Lower layer (discarded)

(c) Aminoacyl-tRNA

After incubation the reaction was stopped with phenol (1 ml). The mixture was centrifuged, and the upper layer re-extracted with phenol (2 ml)

Upper (aqueous) layer treated with 2 vol. of ethanol (100%) and 0.1 vol. of potassium
acetate (pH 5, 20%). Kept at 4°C for 10 min, centrifuged (1200g_{av} for 10 min)

Supernatant (discarded)

Sediment

RNA containing radioactive aminoacyl-tRNA.
Dissolved in a small volume of water for measurement of
radioactivity and quantitative determination.

Scheme 1. Preparations of enzyme and RNA fractions from bean leaves.

E_{260} 25 for a solution containing 1 mg of RNA/ml. The radioactivity of samples was counted directly in a dioxan scintillator solution, or on glass-fibre filter paper (Davies & Hall, 1969).

RESULTS

Pyrophosphate exchange was readily demonstrated by pH5 enzyme fractions from expanding bean leaves. The rate was proportional to the amount of enzyme protein, and was linear over a 30 min period. Specific activity varied somewhat from preparation to preparation, but 57.4 nmol/min per mg of enzyme (at 10 mM-Mg²⁺; Table 1) was a typical rate and is similar to that determined by Anderson & Fowden (1969). The reaction was stimulated by each of the amino acids commonly found in proteins, although the stimulation by a

mixture was less than the amount calculated by summing the stimulation observed by individual amino acids present in the mixture.

The reaction was dependent on added ATP, and was increased by additional ATP until a plateau at 10 mM-ATP was reached. Negligible effect of K⁺, NH₄⁺, Ca²⁺ or Mn²⁺ at concentrations of 2.5, 5.0 and 10.0 mM was observed. However, Mg²⁺ gave a linear stimulation up to 12.5 mM, and high rates were observed (Table 1) at concentrations at which Mg²⁺ fully chelates with the ATP and precipitates the pyrophosphate. Fowden & Frankton (1968) also observed stimulation by high Mg²⁺ concentrations. The reported influence of Mg²⁺ on the pyruvate kinase reaction (Holmsen & Storm, 1969) indicates that the stimulation may not wholly reflect increased aminoacylation, although subtraction of exchange

(a) *Direct method (cell-free experiments)*

Specific radioactivity of supplied ^{14}C -labelled amino acid mixture = 54 mCi/mg-atom of C
 = 54 mCi/14 mg of C

Assuming 50% of the weight of the amino acid mixture is C, then the specific radioactivity = 54 mCi/28 mg of amino acid

i.e. 54/28 mCi/mg of amino acid = $(54/28) \times 2.2 \times 10^9$ d.p.m./mg of amino acid
 = 4.2×10^6 d.p.m./ μg of amino acid

$\therefore$ 4.2 d.p.m. in the isolated aminoacyl-tRNA $\equiv$ 1 μg of amino acid incorporated into tRNA during the experiment

(b) *Hydrolysis method (tissue and cell-free experiments)*

The difference in the specific radioactivity of isolated RNA before and after mild alkaline hydrolysis is assumed to represent the loss of labelled amino acid from tRNA. On a per milligram basis the difference gives the total d.p.m. of amino acid lost. Calculation of the specific radioactivity of the hydrolysed fraction as d.p.m./ μg of amino acid is made after radioactivity determination and ninhydrin reaction.

(i) The difference in the specific radioactivity of the supplied amino acids (cell-free experiment) and that calculated for the hydrolysed amino acid fraction represented dilution of the labelled amino acids by unlabelled amino acids attached to tRNA. An estimation of the amount of endogenous aminoacyl-tRNA can therefore be made.

(ii) If it is assumed that the loss of amino acids from aminoacyl-tRNA on hydrolysis is equivalent to the amount incorporated during the experiment, then calculation of the amount of aminoacylation during the experiment is possible:

n d.p.m. = 1 μg of amino N (where n = observed d.p.m./ μg of amino N from above)

Assume each amino acid contains 1 α -amino N.

$\therefore n$ d.p.m. = $M/14 \mu\text{g}$ of α -amino N (where M = mol.wt. of amino acid)

$\therefore 14n$ d.p.m. = $M \mu\text{g}$ of amino acid = 1 μmol of amino acid.

Hence, given the mol.wt. of the amino acid, the d.p.m. equivalent to the wt. of amino acid incorporated into tRNA during the experiment can be determined.

Scheme 2. Calculation of rate of aminoacyl-tRNA formation.

Table 1. *Effect of Mg^{2+} on the rate of pyrophosphate exchange*

The system contained, in a final volume of 1 ml: ATP, 10 μmol ; [^{32}P]pyrophosphate, 10 μmol ; mixture of 20 amino acids, 0.75 mmol (total); tris, pH 8.3, 50 μmol ; bean-leaf enzyme, 2.7 mg of protein; MgCl_2 , as stated. Incubation was at 30°C for 45 min.

MgCl_2	(μmol):	0	10	12.5	15	20	25	50	100	150	200	250
Pyrophosphate exchanged	(μmol):	3.31	6.99	8.00	8.09	5.81	9.30	6.27	3.06	2.44	2.32	3.88

rates in the absence of added amino acid is an essential control.

Conditions yielding high rates of pyrophosphate exchange did not give reproducibly positive results in a reaction system *in vitro* similar to that of Shearn & Horowitz (1969). To measure the rate of aminoacyl-tRNA formation *in vivo* the system described in Table 2 was used. Approach to the cell-free system from the intact system *in vivo* was made by using leaf-disc systems as intermediate stages. Table 3 presents the results for detached leaf discs in radioactive carbon dioxide or bicarbonate. Since amino acids are used more commonly than bicarbonate as substrates in studies on protein synthesis, the substitution of radioactive glycine for bicarbonate in the leaf-disc system (Table 4) permitted a more valid comparison between rates

observed for leaf discs and rates obtained in cell-free experiments. Positive evidence for aminoacyl-tRNA formation *in vitro* was obtained by the system shown in Table 5 with pH 5-enzyme fractions from *Escherichia coli* supernatant, bean pod and bean leaf. A summary of the rates of aminoacyl-tRNA formation calculated from the data of Tables 2–5 is presented as Table 6. It appears that leaf detachment (cutting discs) decreased the rate by rather less than one order of magnitude, and that the preparation of cell-free extracts further decreased the rate by about four orders.

A comparison of the apparent rate of aminoacyl-tRNA formation when calculated by each of the methods described above is made in Table 7. The conjecture that endogenous non-labelled aminoacyl-tRNA greatly dilutes the radioactivity when the

Table 2. *Aminoacylation of tRNA (bean leaf) in vivo*

Individual leaves, still attached to the plant, were enclosed in a Plexiglas box (15 cm × 10 cm × 2 cm) into which 7.12×10^7 d.p.m. of $^{14}\text{CO}_2$ (generated from $\text{Ba}^{14}\text{CO}_3$) was introduced. At the end of the incubation the leaves were detached and RNA was rapidly extracted. The molar amounts of amino acids attached per unit of RNA were calculated by the method presented in Scheme 2(b). Count rates are corrected for efficiency.

Incubation time ...	Incubation in light				Incubation in dark			
	10 min	20 min	30 min	60 min	10 min	20 min	30 min	60 min
Sp. radioactivity of RNA before hydrolysis (d.p.m./mg)	110000	78000	74800	68000	5670	7830	6320	9120
Sp. radioactivity of RNA after hydrolysis (d.p.m./mg)	50200	28000	32000	34600	4000	4840	2930	1180
Radioactivity lost from 1 mg of RNA on hydrolysis (d.p.m.)	59800	50000	42800	33400	1670	2990	3390	7940
Sp. radioactivity of amino acids released (d.p.m./μg of N)	18300	17600	13200	12400	2640	2000	1600	1640
Hence d.p.m. $\times 10^{-3} \equiv 1 \mu\text{mol}$ of glycine equivalent*	256.2	246.4	184.8	173.6	37.0	28.0	22.4	23.0
Hence amount of amino acid attached to RNA (nmol/mg)	234	203	232	192	45	107	151	346

* $14 \times$ Sp. radioactivity of amino acids released, since $1 \mu\text{mol}$ of amino acid = $1/14 \mu\text{g}$ of N; value for amino acids with a single α -amino N ('glycine equivalent').

Table 3. *Aminoacylation of tRNA (bean-leaf discs) in vivo*

Discs (23, 18 mm diam.) were punched from bean leaves and incubated for 30 min in Plexiglas boxes under fluorescent light (2000 ft.-candles) with: (1) $^{14}\text{CO}_2$ (7.12×10^7 d.p.m.) from $\text{Ba}^{14}\text{CO}_3$; (2) $^{14}\text{CO}_2$ (5.25×10^7 d.p.m.) from $\text{NaH}^{14}\text{CO}_3$; (3) $\text{H}^{14}\text{CO}_3^-$ (5.25×10^7 d.p.m.) as $\text{NaH}^{14}\text{CO}_3$. Count rates are corrected for efficiency.

Source of ^{14}C ...	(1)	(2)	(3)
Sp. radioactivity of RNA before hydrolysis (d.p.m./mg)	472000	239000	395000
Sp. radioactivity of RNA after hydrolysis (d.p.m./mg)	188000	110000	49200
Radioactivity lost from 1 mg of RNA on hydrolysis (d.p.m.)	284000	129800	345800
Sp. radioactivity of amino acids released (d.p.m./μg of N)	128000	66500	103000
Hence d.p.m. ($\times 10^{-3}$) $\equiv 1 \mu\text{mol}$ of glycine equivalent*	1792	932	1442
Hence amount of amino acid attached to RNA (nmol/mg)	158	139	240

* $14 \times$ Sp. radioactivity of amino acids released, since $1 \mu\text{mol}$ of amino acid = $1/14 \mu\text{g}$ of N; value for amino acids with a single α -amino N ('glycine equivalent').

Table 4. *Aminoacylation of tRNA by glycine (bean-leaf discs) in vivo*

Discs (28, 18 mm diam.) were punched from bean leaves and incubated in Plexiglas boxes under fluorescent light (2000 ft.-candles) with a solution of $[^3\text{H}]$ glycine (3.52×10^8 d.p.m.). Count rates are corrected for efficiency.

Incubation time ...	15 min	30 min	50 min
Sp. radioactivity of RNA before hydrolysis (d.p.m./mg)	16000	18000	13400
Sp. radioactivity of RNA after hydrolysis (d.p.m./mg)	13400	7340	5220
Radioactivity lost from 1 mg of RNA on hydrolysis (d.p.m.)	2600	10660	8180
Sp. radioactivity of amino acids released (d.p.m./μg of N)	4100	3600	3430
Hence d.p.m. ($\times 10^{-3}$) $\equiv 1 \mu\text{mol}$	57.4	50.5	48.0
Hence amount of glycine attached to RNA (nmol/mg)	45.3	211.1	170.3
Dilution of supplied amino acids ($\times 10^{-5}$)*	3.4	3.9	4.1

* Calc. from sp. radioactivity of amino acid released, since sp. radioactivity of the glycine supplied was 885 mCi/mmol, which is equivalent to 1.947×10^9 d.p.m./μmol.

Table 5. *Aminoacyl-tRNA synthesis by plant and bacterial enzymes in vitro*

The cell-free system was prepared and incubated as described in the Experimental section. The ^{14}C -labelled amino acid mixture contained: alanine (10%), arginine, (6.5%) aspartate (9.0%), glutamate (12.5%), glycine (5%), leucine (12%), isoleucine (5%), lysine (5.5%), phenylalanine (7%), proline (6%), serine (5%), threonine (6%), tyrosine (3.5%), valine (7%). Total radioactivity of supplied phenylalanine was 1.1×10^7 d.p.m.

Enzyme ...	Bean pod pH 5		Bean leaf pH 5		<i>E. coli</i> pH 5	
Substrate ...	Amino acid mixture	Phenylalanine	Amino acid mixture	Phenylalanine	Amino acid mixture	Phenylalanine
Radioactivity in tRNA after 30 min incubation (d.p.m.)	1200	590	7520	4990	7080	7330
Enzyme protein (mg)	2.5	2.8	4	4	1.1	1.1
Amino acid incorporated into 0.2 mg of RNA (pg/min per mg of enzyme protein)*	3.2	0.7	12.5	4.2	42.7	22.2

* Calculated from specific radioactivity of amino acid(s) supplied: amino acid mixture = 54 mCi/mg-atom of C; 1 pg of amino acid = 5 d.p.m.; [^{14}C]phenylalanine 750 mCi/mmol, 1 pg of amino acid = 10 d.p.m..

Table 6. *Rate of aminoacyl-tRNA formation by systems from bean leaves in vivo and in vitro*

System	Substrate	Source of data		Rate (nmol of amino acid incorporated/min per mg of RNA)
		Table	Column	
Intact leaves	$^{14}\text{CO}_2$	2	1	23.4
Leaf discs	$^{14}\text{CO}_2$	3	1	5.1
Leaf discs	$\text{NaH}^{14}\text{CO}_3$	3	3	8.0
Leaf discs	[^{14}C]Glycine	4	1	3.0
Leaf, pH 5 enzyme	^{14}C -labelled amino acid mixture	5	3	0.0006
Leaf, pH 5 enzyme	[^{14}C]Phenylalanine	5	4	0.0001

calculation is based on the specific radioactivity of the supplied amino acid tracer is supported by the determination of the specific radioactivity of amino acids released from aminoacyl-tRNA on mild alkaline hydrolysis.

DISCUSSION

If pyrophosphate exchange is a valid basis for the measurement of the activity of aminoacyl-tRNA synthetases, the rates presented here and for several studies (Moustafa & Lyttleton, 1963; Hall & Cocking, 1966; Anderson & Fowden, 1969) with plant systems would suggest that approx. 50–100 nmol of amino acid should be adenylated/min by the equivalent of 1 mg of pH 5 enzyme. If all this amino acid were accepted by tRNA and transferred to growing polypeptides and proteins at the same rate, then we would expect a synthesis of approx. 7 μg of protein/min per original mg of protein, assuming mol.wt. 110 for the amino acids. Rates *in vivo* of 66 μg (bean leaf; Hall, 1968) and 40 μg (tomato leaf; Hall & Cocking, 1966) of protein synthesized/min

per original mg of protein are less than an order different from the estimate above. Amino acid incorporation into protein by higher-plant cell-free systems occurs at much lower rates, although demonstration of tobacco-mosaic-virus coat-protein synthesis by a tobacco-leaf ribosome system (Sela & Kaesberg, 1969) suggests that apparent rates of amino acid incorporation calculated from the specific radioactivity of supplied amino acids may represent only a fraction of the true activity of the system.

Aminoacylation of tRNA could be a rate-limiting step for amino acid-incorporation systems. The formation of aminoacyl-tRNA shown in Table 2 indicates that no blockage at this step occurs *in vivo*. Cutting leaf discs decreased the efficiency of this step only slightly, as the results of $^{14}\text{CO}_2$, $\text{H}^{14}\text{CO}_3^-$ and [^3H]glycine assimilation presented in Tables 3 and 4 show. To follow the rates *in vivo* as described in the Experimental section and Scheme 2 it is necessary to assume that the radioactivity released on alkaline hydrolysis of aminoacyl-tRNA represents the loss of amino acid from the tRNA and,

Table 7. *Rate of aminoacyl-tRNA synthesis by bean leaf and E. coli pH5-enzyme preparations*

The cell-free system was prepared as described in the Experimental section. The synthetase-enzyme fraction contained 8 mg (bean leaf) or 1.1 mg (*E. coli*) of protein/ml final volume. After reaction a portion of the aminoacyl-tRNA was taken for measurement of radioactivity and the remainder was hydrolysed, so that calculation of the rate of aminoacyl-tRNA formation by both methods given in Scheme 2 could be made. The rates calculated by the two procedures differ by a factor of $(219/0.045) = 4.8 \times 10^3$ for bean leaf, and $(1430/0.563) = 2.5 \times 10^3$ for *E. coli*. The difference is apparently due to dilution of the supplied amino acids by endogenous amino acids since:

sp. radioactivity of supplied ^{14}C -labelled amino acid mixture = 5 d.p.m./pg. Assuming average mol.wt. 110,

1 pmol of amino acid = 5.5×10^2 d.p.m. (110×5)

$\therefore$ 1 μmol of amino acid = 5.5×10^8 d.p.m.

From the hydrolysed amino acids the specific radioactivity was:

1 μmol of amino acid = 0.4×10^5 d.p.m., i.e. a dilution of 1.4×10^4 , for bean leaf.

1 μmol of amino acid = 1.7×10^5 d.p.m., i.e. a dilution of 3.2×10^3 , for *E. coli*.

Rate calculated from apparent uptake			Rate calculated from hydrolysis procedure		
	Bean	<i>E. coli</i>		Bean	<i>E. coli</i>
Radioactivity in RNA after 20 min incubation (d.p.m.)	3974	6200	Sp. radioactivity of RNA before hydrolysis after 20 min incubation (d.p.m./mg)	2100	5640
Sp. radioactivity of ^{14}C -labelled amino acid mixture was 54 mCi/mg-atom of C (5 d.p.m./pg), hence pg of amino acid incorporated/min per mg of enzyme	4.96	56.3	Sp. radioactivity of RNA after hydrolysis (d.p.m./mg)	694	800
Assuming average mol.wt. 110 for the amino acids, then pmol of amino acid incorporated/min per mg of enzyme	0.045	0.563	Hence amino acid released/mg of RNA (d.p.m.)	1406	4840
			Sp. radioactivity of amino acid released (d.p.m./ μg of N)	2870	12100
			Hence d.p.m. $\equiv$ 1 μmol of amino acid	40000	169500
			Hence pmol of amino acid incorporated/min per mg of enzyme	219	1430

further, that the amount of this radioactivity reflects the amount of amino acid attached to tRNA over the experimental period. Non-specifically absorbed amino acids were expected to be removed during isolation of the RNA, which gave typical u.v. spectra with E_{260}/E_{280} ratios of 1.9:1. The non-hydrolysed RNA was ninhydrin-positive; after hydrolysis the reprecipitated RNA gave no colour with ninhydrin, but the supernatant gave positive results when tested for amino acids. Measurement of radioactivity and amino N permits calculation of the specific radioactivity of released amino acids. The total radioactivity of this fraction can then be divided by the specific radioactivity to determine the amount of amino acids released on hydrolysis.

Since the specific radioactivity of the amino acid supplied as a tracer was known, calculation of specific radioactivity after release from tRNA as described above permitted an estimation of the dilution occurring in the reaction. Table 4 indicates that this dilution was of the order of 10^5 for glycine supplied to leaf discs. A comparison of the rates obtained for the various systems *in vitro* and *in vivo* is presented in Table 6. Table 7 compares rates

of aminoacyl-tRNA formation by systems *in vitro* obtained by using two methods of calculation, and the dilution of specific radioactivity of the supplied amino acid was greater than 1000-fold. It seems possible that this dilution could account for the low apparent rates of aminoacylation of tRNA.

The results from the studies *in vivo* indicate that the tRNA is rapidly saturated with labelled amino acid in the light (Table 2), since the amount of amino acid attached to tRNA remained fairly constant after 10 min. In the dark aminoacylation took place at the rate of some 4.5 nmol of amino acid/min per mg of RNA compared with 23.4 nmol in the light over the first 10 min. However, the attachment appeared to continue at the same rate over 60 min of administration of labelled substrate in the dark, finally resulting in a higher specific radioactivity than was found for the uptake in light. This may represent an accumulation of aminoacylated tRNA due to cessation of amino acid transfer from tRNA to protein in the dark. The amount of amino acid attached to RNA decreased when leaf discs were incubated in the light for periods exceeding 30 min (Table 4). Lengthy incubations

in vitro gives similar decreases. Possibly ribonucleases become particularly active in such situations (Shearn & Horowitz, 1969), although the reason is not clear, nor has this been quantitatively checked in our system.

An examination of the rates of aminoacyl-tRNA synthesis indicates that cell-free systems from plants may be quite active, but that endogenous concentrations of amino acids dilute supplied tracer amino acids. The pH 5 fraction probably contributes to this dilution, but we have found that RNA isolated from plant tissues frequently has appreciable amounts of amino acid attached (approx. 1 μ g of amino N/mg of RNA), presumably to the tRNA. Mild alkaline hydrolysis and reprecipitation of the RNA yielded material that gave no significant reaction with ninhydrin, but had very low amino acid-acceptor activity. Further experimentation is required to determine if additional precautions against ribonuclease degradation during mild alkaline hydrolysis, or alternative methods of stripping amino acids from tRNA, will yield cell-free preparations capable of aminoacylation at rates approaching those of the intact cell.

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