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The Turnover of Nucleic Acids in *Lemna minor*

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ABSTRACT

A method is described for measuring the rate constants of both synthesis and degradation of nucleic acids in sterile growing cultures of *Lemna minor* which avoids the difficulties of environmental changes in isotope uptake and precursor pool size. In fast growing cultures the half-life of ribosomal RNA has been estimated to be between 5 and 8 days.

This half-life has been shown to consist of two components, cytoplasmic ribosomal RNA with a half-life of about 4 days and chloroplast ribosomal RNA with a half-life of about 15 days. The possible interference of recycling has been checked, and the evidence indicates its likely insignificance. "Heavy" labeling of *Lemna* with D_2O and $^{15}NO^-_3$ has provided evidence for the conservation of ribosomal RNA in fast growing cultures and has also provided an alternative assessment of recycling. When *Lemna* is placed on water, the rate of degradation of ribosomal RNA is increased and that of synthesis is decreased. Under partial "step down" conditions it has been found that omission of either nitrate or phosphate, or calcium or magnesium leads to an increase in the rate of degradation of ribosomal RNA. In *Lemna* grown on water, benzyladenine increases both the synthetic and degradative rates of nucleic acid metabolism. Abscisic acid, on the other hand, markedly reduces the rate of synthesis of ribosomal RNA but leaves the degradative rate unaltered.

Turnover in nucleic acids in animal systems is a well established phenomenon and has been so for many years (8). In contrast, evidence concerning the stability of nucleic acids in plant systems is infrequent. Ingle and Key (3) and Loening (4) have demonstrated rapid turnover of certain nucleic acid fractions of probable nuclear origin. In other cases, excised plant tissues frequently exhibit losses of RNA (13). These data cannot be construed, however, as evidence for degradation of nucleic acids in growing plants since bacteria exhibit nucleic acid stability under conditions of normal growth but readily exhibit degradation when grown under step down conditions (8). The stability of the major species of nucleic acids in growing plants is therefore largely unknown. The work described in this paper was instituted to determine the stability of the major species of nucleic acids, in particular ribosomal RNA, in plant tissues. Some of the factors which control either degradative or synthetic rates of nucleic acids in plants are considered in this paper.

MATERIALS

Plant Material. Single fronds of *Lemna minor* were obtained from local sources and sterilized by treatments of about 30 sec with 6% sodium hypochlorite. *L. minor* was grown under sterile conditions on the following medium: sucrose, 10 mM; $Ca(NO_3)_2 \cdot 4H_2O$, 5 mM; KNO_3, 5 mM; KH_2PO_4, 2 mM; $MgSO_4 \cdot 7H_2O$, 2 mM; H_3BO_3, 0.05 mM; $MnCl_2 \cdot 4H_2O$, 0.01 mM; $CuSO_4 \cdot 5H_2O$, 0.03 μM; $FeSO_4$, 0.05 mM; EDTA Na_2, 0.05 mM; and pH adusted to 5.0.

Cultures were grown under a constant light intensity of 10^3 ft-c ($\mp$ 10%) obtained from mixtures of warm white and daylight fluorescent light tubes at 25 C. Cultures were checked for microbial contamination at each transfer by placing several fronds from each culture on 2% sucrose–nutrient broth (Oxoid, Ltd.) and incubating at 30 C for 7 days. Absence of cloudiness was accepted as evidence for sterility. In cases of uncertainty, portions of the nutrient broth were streaked on 1½% nutrient agar and, after further incubation, examined for colonies. All nonsterile experiments were discarded.

CsCl and Cs_2SO_4. Reagent grade chemicals were obtained, boiled with acid-washed charcoal, and recrystallized from hot water after filtration. The A_{260} of a saturated solution of CsCl was 0.028 and of Cs_2SO_4 0.06.

Radiochemicals. ^{3}H-2,8-Adenine (3 c/mmole) and ^{14}C-8-adenine (35 mc/mmole) were obtained from the Radiochemical Centre, Amersham. Before use the ^{3}H-adenine was purified by one-dimensional chromatography on Whatman No. 4 paper with isopropanol–H_2O–concentrated hydrochloric acid (130:37:33). In the same system the ^{14}C-adenine had a purity of 99.7% and was therefore used without chromatographic purification. $H^{15}NO_3$, 96.5% was obtained from Office National Industrial de l'Azote, France. $Ca(^{15}NO_3)_2$ and $K^{15}NO_3$ were prepared from aliquots of the nitric acid by neutralization with $CaCO_3$ or KOH.

Abscisic acid was the kind gift of Dr. R. W. A. Leach of the Woodstock Agricultural Research Centre, Sittingbourne, Kent.

METHODS

Isolation and Separation of Nucleic Acids. The fresh tissue was ground in a pestle and mortar with equal volumes of 1% sodium lauryl sulfate and 80% phenol–10% *m*-cresol–0.01% 8-hydroxyquinoline at room temperature (6). After stirring for 10 min the aqueous phase was made 3% with respect to NaCl and stirring continued for another 5 min. After centrifugation, the upper phase was removed and precipitated with 2 volumes of ethanol. The precipitate was washed with 80% ethanol, dissolved in freshly prepared tris, 0.08 M; sodium acetate, 0.04 M; sodium EDTA, 4 mM, pH 7.8, containing 0.2% sodium lauryl sulfate, and reprecipitated with ethanol.

Subsequent purification was carried out by dissolving in tris-acetate-EDTA and adding equal volumes of 2.5 M potassium phosphate (pH 8.0) and methoxyethanol (9). After mixing and centrifugation, the upper phase was removed and mixed with 3 volumes of tris–acetate–EDTA containing 0.1% cetyltrimethylammonium bromide. After standing at 0 C for 15 min, the precipitate was spun down and washed twice with 70% ethanol containing 0.1 M potassium acetate (pH 5.0) (9).

The product obtained at this stage was of high spectral purity with an $A_{260}/_{230}$ of 2.35 to 2.45 and an $A_{260}/_{280}$ of 2.2 to 2.24. The $E^{1\%}_{260}$ was 230.

Further separation was carried out by dissolving the pellet in tris–acetate–EDTA and adding solid NaCl to a final concentration of 2 M. After standing at 0 C for several hours, the precipitate was spun down and washed with 2 M NaCl. The pellet is referred to as the salt-insoluble fraction and the supernatant and washing as the salt-soluble fraction. When nucleic acids were dissolved in buffer not containing detergent, the buffer was pretreated with an equal volume of 1% macaloid, (11) and the macaloid was removed by centrifugation.

Aqueous radioactive nucleic acid samples were counted in duplicate as previously described (12).

Acrylamide gel separations were carried out as described by Loening (5) and scanned at 265 nm in a Joyce-Loebl chromoscan. After freezing, radioactive gels were sliced automatically into 0.5-mm sections, and the discs were dried onto glass fibre paper discs and counted in a Packard Tricarb scintillation counter.

Isopycnic Centrifugation. Isopycnic banding of salt-insoluble RNA was carried out as described by Lozeron and Szybalski (7), in a mixed gradient of CsCl and Cs_2SO_4 containing 1% HCHO. All solutions were dissolved in tris–acetate–EDTA, pH 7.8. The RNA solution (0.25 ml) was pretreated with 0.25 ml of 5% HCHO for 10 min. One milliliter of saturated Cs_2SO_4 (saturated at 25 C) was added dropwise with stirring, followed by 0.105 ml of 37% HCHO and subsequently 3.5 ml of saturated CsCl also with stirring. The RNA was banded in the Spinco 65 angle head for 3 days at 33,000 rpm at 15 C. The tube was filled with liquid paraffin to prevent collapse during centrifugation. The gradient was fractionated into 20-drop fractions with the aid of a capillary inserted to the bottom of the gradient and a peristaltic pump. Refractive indices of several fractions were taken, and densities were determined pycnometrically on a blank gradient. Optical densities of fractions were determined after dilution with the aid of micro cells. Fractions were counted after dilution to 5 ml as previously described (12).

Experimental design. Unless stated otherwise, the following experimental design has been used. A single inoculum of 60 fronds was transferred to 100 ml of sucrose-mineral salts and grown for 6 days, in which time it had grown and divided to about 400 to 450 fronds. The whole culture was then transferred to fresh medium containing 1 μc of ^{14}C-adenine for 4 hr and then to unlabled medium before division into inocula of 60 fronds into separate flasks on unlabeled medium. The flasks were then sampled on successive days, and the nucleic acids were prepared.

In experiments in which a step down onto water or partly deficient nutrient conditions has been performed, the initial transfer after labeling was onto the appropriate one of these media.

Heavy Labeling of Lemna. *L. minor* was heavy labeled on 50% deuterium oxide, calcium nitrate-^{15}N (5 mM), potassium nitrate-^{15}N (5 mM), and kinetin (10^{-6} M) (1). The remaining constituents of the medium and the growth conditions were as described under "Materials" except for the omission of calcium and potassium nitrates-^{14}N. *L. minor* grew apparently normally on this medium with the exceptions of reduced rate of growth and shortened roots.

Measurement of Rates of Synthesis and Degradation of Nucleic Acids in Growing Cultures of Lemna. The method consists of simplifying the metabolic conversion of labeled adenine to polymerized nucleic acid. The following scheme may be drawn

$$X \rightarrow A \overset{V_1}{=} B \qquad A \downarrow V_2 \; Y \qquad (1)$$

where X = labeled adenine on outside of cell; B = nucleic acid; A = labeled acid-soluble precursors; Y = alternative products of acid-soluble material; V_1 = rate of synthesis of nucleic acid; and V_2 = rate of degradation of nucleic acid.

Following the procedure of Reiner (10), if A and B are labeled, they are related by the equation

$$B\frac{dS_b}{dT} = (S_a - S_b)\,V_1 \qquad (2)$$

where B = total amount of B; S_b = specific radioactivity of B; S_a = specific radioactivity of A; and V_1 = rate of synthesis as shown in scheme 1. In a growing system B will be a variable and

$$\frac{dB}{dT} = V_1 - V_2 \qquad (3)$$

To simplify equation 2, experimental conditions are set such that during a considerable portion of the experimental period growth takes place from unlabeled sources; *i.e.*, X is zero and S_a at or near zero. The assumption that under the experimental conditions used, S_a is at or near zero has not been accepted dogmatically, but evidence has been provided for this contention later in the paper. Thus, equation 2 is now

$$\frac{dS_b}{dT} = -S_b\frac{V_1}{B} \qquad (4)$$

Both V_1 and B will not be constant in growing cultures of *Lemna*. However, if the composition of the culture remains uniform, then the rates of synthesis and degradation as well as the levels of nucleic acid will be proportional to the amount of tissue. Only in this way will a steady state of growth be maintained. Thus the ratio of V_1/B will remaint constant. Equation 4 may then be rewritten as a simple first order equation.

$$\frac{dS_b}{dT} = -K_1S_b$$

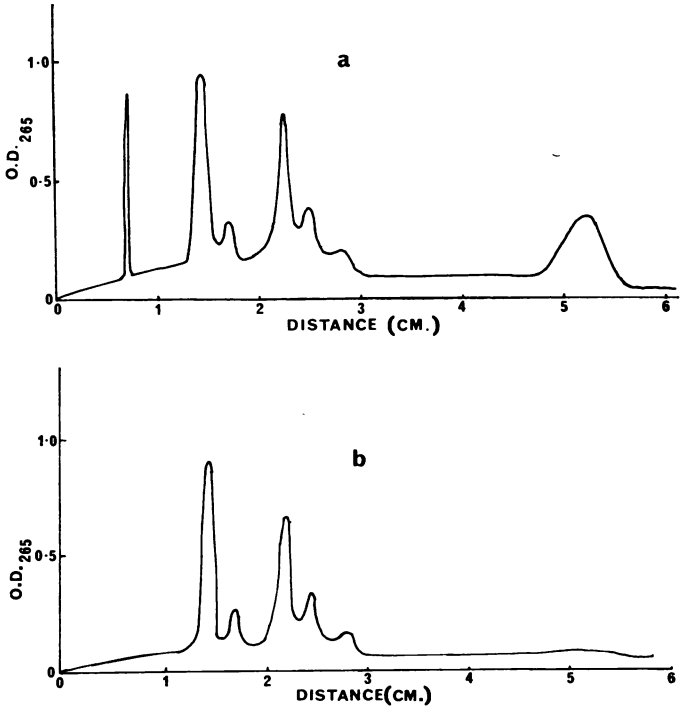

FIG. 1. Acrylamide gel electrophoresis of total nucleic acid and salt-insoluble nucleic acid from *L. minor*. The nucleic acids of *L. minor* were prepared and separated on 10-cm gels at 5 ma for 2 hr. Gels were scanned at 265 nm in a Joyce-Loebl chromoscan with the instrument set to expand the scanned length of the gel by the factor 3. a: Total nucleic acid in *L. minor*; b: the 2 M sodium chloride-insoluble nucleic acids in *L. minor*

where

$$K_1 = \frac{V_1}{B} \quad (5)$$

and

$$S_b = e^{-K_1 t} + C$$

Therefore, a graph of $\ln S_b$ plotted against time should yield a straight line (see Fig. 3) with a slope $K_1 = V_1/B$, thus

$$V_1 = K_1 B \quad (6)$$

Since B varies continuously in a growing culture, V_1 can only be determined absolutely with respect to a particular value of B at a particular time. It is thus easier to refer to V_1 by the rate constant of synthesis K_1. From scheme 1, $V_2 = K_2 B$ where K_2 = rate constant of degradation. Thus from equations 3 and 6

$$\frac{dB}{dT} = K_1 B - K_2 B = B(K_1 - K_2) \quad (7)$$

A graph of $\ln B$ plotted against time should yield a straight line (see Fig. 3) with a slope $= K_1 - K_2$. Since K_1 has already been determined, K_2, the rate constant of degradation, can be calculated. To determine V_1 and V_2, two graphs are needed: (*a*) a graph of $\ln S_b$ with time; (*b*) a graph of $\ln B$ with time. From the slope of the first graph the rate constant of synthesis is determined, and from the second the difference between the slope and K_1 represents the rate constant of degradation. The doubling time (for synthesis) and the half-life (for degradation) can be determined from the equation $\ln 2$/rate constant.

All slopes of lines have been determined by the method of least squares, and the standard error of this regression coefficient has been determined in the customary way. In this paper, all graphs have been constructed using $\log_{10}$. The rate constants have been determined by multiplying the slopes of these graphs by $\log_e 10$.

RESULTS

Figure 1 shows acrylamide gel separations of both the total nucleic acids of *L. minor* (a) and the 2 M NaCl-insoluble nucleic acid (b). By analogy with other published data on the nucleic acids of green plants (6) the peaks may be identified from left to right as peak 1, DNA; peaks 2 through 6 as ribosomal RNA; and peak 7 as soluble RNA. Salt precipitation results in almost tota elimination of the DNA (confirmed with the diphenylamine re

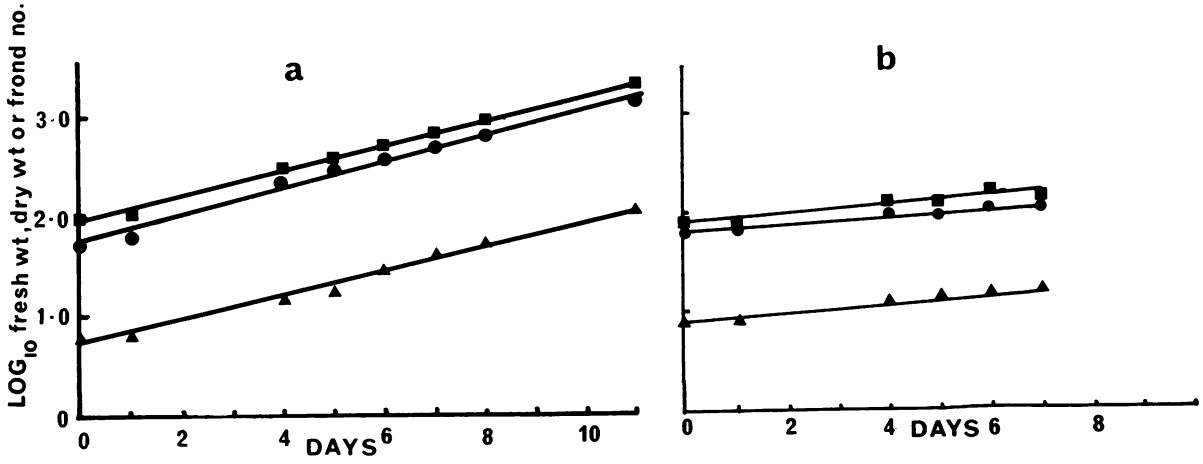

FIG. 2. The effect of different growth conditions on the increases in fresh weight, dry weight, and frond number of *L. minor*. Inocula of 60 fronds were placed on 100 ml of medium and grown as described under "Methods." At the time intervals shown, the number of fronds were counted and the fresh and dry weights were determined in mg. a: Plants grown on sucrose-mineral salts; b: plants grown on water; ●: frond number; ■: fresh weight; ▲: dry weight.

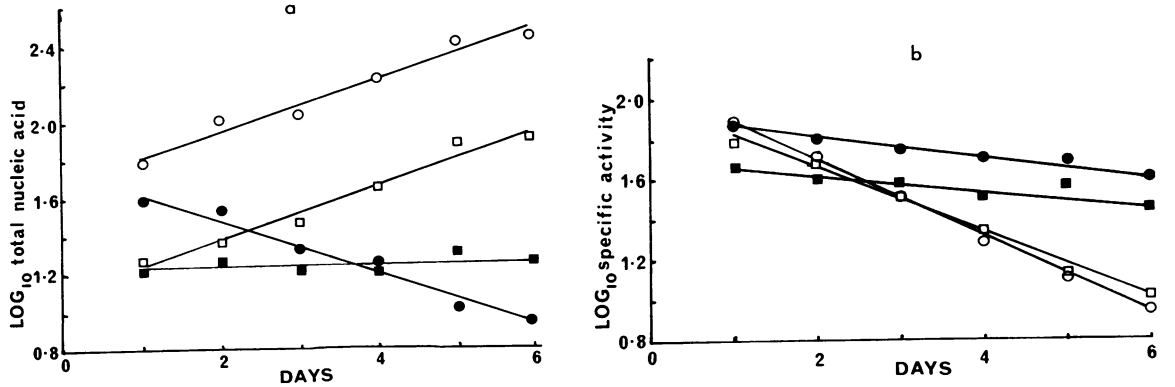

FIG. 3. Variation of level and specific activity of ribosomal RNA and salt-soluble nucleic acid of *Lemna* under high and low growth rate conditions. Cultures of *Lemna* were labeled with 1 μ C of ^{14}C-adenine for 4 hours and then divided into batches of 60 fronds and placed either on sucrose-mineral salts (high growth conditions) or on water (low growth conditions.) Samples were taken for the 6 succeeding days, and the levels (μg) and specific radioactivity (cpm/μg) of ribosomal RNA and salt-soluble nucleic acid were determined. All lines have been fitted by the method of least squares. Open symbols: grown on sucrose-mineral salts; closed symbols: grown on water; ○: ribosomal RNA; □: salt-soluble nucleic acid.

action) and soluble RNA. The salt-insoluble nucleic acids have subsequently been referred to as ribosomal RNA. It can be seen that the ribosomal RNA of *Lemna* is separable into five components. With pea stem tissue RNA as a standard (6), the S values of these five components may be calculated to be 25, 23, 18, 16 and 13.7. The 13.7 S component is probably a degradation product of the 23 S (6), but attempts to eliminate it by modifying the preparative procedure of the nucleic acids have not so far been successful.

Turnover Rates of Ribosomal and Salt-soluble Nucleic Acid. Figures 2 and 3 show an experiment in which determinations of growth characteristics and rate constants of nucleic acid metabolism have been made under two different environmental conditions, high growth rates on sucrose-mineral salts and low growth rates on water. Figure 2 shows the effect of the two environmental conditions on three growth parameters, frond number, fresh weight, and dry weight. Rate constants for each of these parameters have been calculated from the slopes of the appropriate lines and are shown in Table I. Growth on water results in a reduction in all three parameters to about the same degree. More important is the demonstration in Figure 2a that growth on sucrose-mineral salts is apparently constant for at least 11 days. Labeling experiments have been conducted only on the first 6 of these 11 days. In Figure 3, a and b, *Lemna* has been labeled for 4 hr with ^{14}C-adenine, and then samples of the labeled tissue were placed on either unlabeled sucrose-mineral salts or water. Figure 3b shows the resultant changes in the specific activity of ribosomal RNA and salt-soluble nucleic acid, and Figure 3a, changes in the levels of ribosomal RNA and salt-soluble nucleic acid under both environmental conditions. The data in the two figures support the predictions of equations 5 and 7 that the variation of log S_b and log B with time are linear. Rate constants have been determined from these figures and are shown in Table II together with calculations of doubling time and half-life.

Under conditions of high growth rate both ribosomal RNA and the salt-soluble nucleic acid exhibit measurable turnover, half-lives of 5.85 and 17.3 days, respectively. Subsequent experiments shown in this paper indicate that values of the half-life of ribosomal RNA may vary between this value and 8 days in exponential growth. As is to be expected under these growth conditions, the rate of synthesis of ribosomal RNA is very much higher than the rate of degradation, and this leads to an accumulation of ribosomal RNA. When *Lemna* is placed on water and the growth rate decreases, the degradative rate of ribosomal RNA is increased 3½-fold, while that of synthesis is strongly reduced. Under these conditions there is a net loss of ribosomal RNA. The salt-soluble nucleic acid does not behave identically. While the rate of synthesis is reduced about 4-fold (a similar size of decrease to that observed for ribosomal RNA), the rate of degradation is only increased 1.7-fold, and this leads to the level of salt-soluble nucleic acid remaining effectively constant or slightly increasing during incubation on water.

Table I. *Effect of Different Growth Conditions on the Rate Constants of Frond Number Fresh and Dry Weight Increase*

Data obtained from Figure 2. Rate constants were determined from the slopes of the lines and multiplied by $\log_e 10$.

Growth Medium	Rate Constants		
	Frond No.	Fresh weight	Dry weight
	day^{-1}		
Sucrose-mineral salts	0.28	0.30	0.26
Water	0.10	0.08	0.11

Sources of Error

Possible Nonlinearity of Extraction. Several objections may be posed to the evidence of turnover of nucleic acids in the cultures with high growth rates. First, in this paper the phenol technique has been used as a quantitative method for assessing the levels of nucleic acids in different amounts of tissue. Since the phenol method is rarely if ever used for this purpose, a demonstration that it yields a representative level of nucleic acid and thus adequate comparison to be made between tissue batches from 100 to 800 mg fresh weight is necessary. Figure 4 shows the results of an experiment in which 5 g of *Lemna* were grown and the nucleic acid was isolated from different initial fresh weights. The data indicate that a representative level of nucleic acid is obtained from a wide range of initial tissue weight and also indicate the size of the error (∓ 10%) to be expected with this technique.

Cell and Frond Death. A second objection to the phenomenon of turnover demonstrated here is the possibility of frond or cell death. Under the growth conditions used for *Lemna* in these experiments, dead fronds have only rarely, if ever, been observed. The criticism that cell death might account for turnover is a more difficult problem, but cell death should result in uniform turnover constants for all fractions of nucleic acid.

Recycling. A third objection to the measured rates of turnover is the possibility of recycling of labeled nucleic acid degradation products. Considerable recycling would imply that the specific radioactivity of the precursor (S_a in equation 2) was not zero as was assumed. It might be expected in this case, however, that a graph of log S_b with time would not be linear. In fact, as Figure 3 shows, log S_b is linear with time, indicating the likely insignifi-

Table II. *Effect of High and Low Growth Rate Conditions on the Rate Constants of Synthesis and Degradation on Nucleic Acids*

Rate constants were calculated from the data in Figure 3. The doubling time and the half-life were determined by dividing ln2 by the rate constant of synthesis or degradation. Values represent the slope determined by the method of least squares plus or minus the standard error of the slope. Errors for the rate constant of degradation were calculated as the square root of the sums of the variances of the rate constant of synthesis and rate constant of total nucleic acid.

Growth Medium	Rate Constants			Doubling Time	$t_{\frac{1}{2}}$
	Synthesis	Total	Degradation		
	day^{-1}			*days*	*days*
Ribosomal RNA					
Sucrose-mineral salts	0.44 ∓ 0.02	+0.32 ∓ 0.03	0.12 ∓ 0.036	1.58 ∓ 0.08	5.85 ∓ 1.4
Water	0.11 ∓ 0.01	−0.31 ∓ 0.03	0.42 ∓ 0.031	6.30 ∓ 0.07	1.65 ∓ 0.15
Salt-soluble nucleic acid					
Sucrose-mineral salts	0.38 ∓ 0.02	+0.34 ∓ 0.03	0.04 ∓ 0.036	1.82 + 0.08	17.3 ∓ 8.0
Water	0.09 ∓ 0.01	+0.02 ∓ 0.02	0.07 ∓ 0.023	8.0 ∓ 0.70	10.1 ∓ 2.6

cance of the precursor specific radioactivity. If recycling does occur, its effect would be to reduce the apparent rate constants of degradation and synthesis. The values quoted in this paper would therefore represent minimal values.

The second reason for considering recycling as insignificant is the data shown in Tables III and IV. In the experiment described in Table III, *Lemna* was labeled and then placed on sucrose-mineral salts in the presence or absence of 10^{-4} M adenine, adenosine, or guanosine. The table shows the rate constants of synthesis and degradation for ribosomal RNA under these four different conditions. There is little difference between the values whatever the condition of growth. In Table IV, label chase conditions have been established in a step down experiment of the kind shown in Figure 3. Again under high growth rate conditions the rate constants of ribosomal RNA are little altered by the presence of 10^{-4} M adenine. Under the low growth rate conditions adenine increases the rate constant of synthesis and reduces the rate constant of degradation compared to fronds incubated on water. While this effect of adenine may be due to lowered recycling, the rate of loss of ribosomal RNA is reduced by adenine, which suggests that the rate constants may, in part or whole, be genuine. In the salt-soluble nucleic acid, adenine has a slight effect on the rate constant of degradation under high growth rate conditions, and thus some recycling may occur in this fraction.

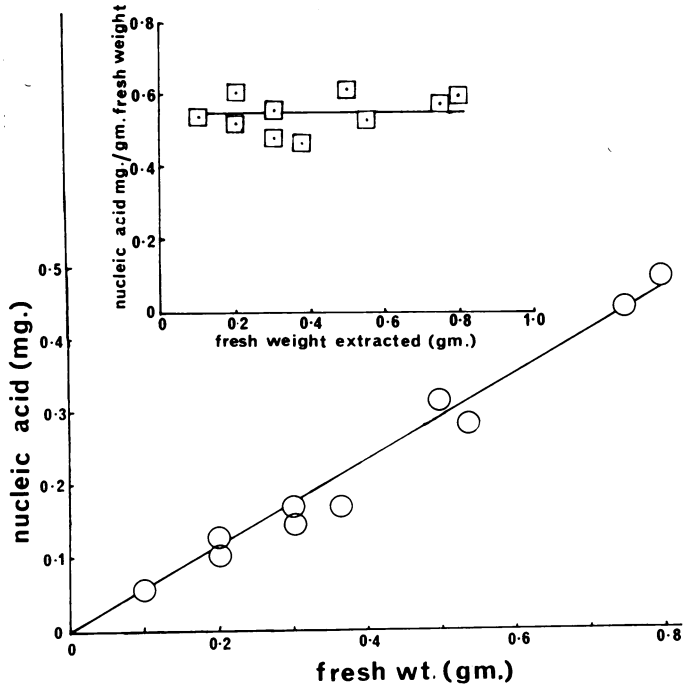

FIG. 4. Recovery of nucleic acid from different fresh weight batches of *L. minor*. Five grams fresh weight *L. minor* were grown, and the nucleic acid was extracted from the amounts shown in the figure. The A_{260} of each nucleic acid sample was determined and converted into μg using the factor $E^{1\%}_{260} = 230$ (see "Methods"). The mean nucleic acid mg/g fresh weight *Lemna* was 0.55 ± 0.057.

Evidence for Conservation of Ribosomal RNA during Growth of Lemna. The third piece of evidence on recycling concerns the data

Table III. *Effect of Label Chase Conditions on the Rate Constants of Synthesis and Degradation of Ribosomal RNA*

Four batches of *Lemma* were labeled in the customary manner and were then divided onto sucrose-mineral salts in the presence or absence of 10^{-4} M adenine, adenosine, or guanine. Samples were taken for the succeeding 6 days, and the rate constants were determined as described for Table II.

Incubation Medium	Rate Constants			
	Synthesis	Total	Degradation	Fresh weight
		day^{-1}		
Sucrose-mineral salts	0.40 ∓ 0.02	+0.28 ∓ 0.02	0.12 ∓ 0.028	0.32
Sucrose-mineral salts + adenine (10^{-4} M)	0.44 ∓ 0.03	+0.33 ∓ 0.04	0.11 ∓ 0.05	0.35
Sucrose-mineral salts + adenosine (10^{-4} M)	0.41 ∓ 0.02	+0.31 ∓ 0.05	0.10 ∓ 0.053	0.36
Sucrose-mineral salts + guanine (10^{-4} M)	0.41 ∓ 0.02	+0.29 ∓ 0.03	0.12 ∓ 0.036	0.34

Table IV. *Effect of Label Chase Conditions on the Synthetic and Degradative Rate Constant of Nucleic Acids under High and Low Growth Conditions*

Batches of *Lemna* were labeled in the customary manner and then distributed onto sucrose-mineral salts plus or minus 10^{-4} M adenine or water plus or minus 10^{-4} M adenine. Samples were taken for the succeeding 6 days and the rate constants determined as described for Table II.

Incubation Conditions	Rate Constants			
	Synthesis	Total	Degradation	Fresh weight
		day^{-1}		
Ribosomal RNA				
Sucrose-mineral salts	0.43 ∓ 0.02	+0.32 ∓ 0.04	0.11 ∓ 0.045	0.37
Sucrose-mineral salts + adenine (10^{-4} M)	0.44 ∓ 0.02	+0.32 ∓ 0.04	0.12 ∓ 0.045	0.35
Water	0.06 ∓ 0.043	−0.46 ∓ 0.045	0.52 ∓ 0.057	0.03
Water + adenine (10^{-4} M)	0.22 ∓ 0.024	−0.14 ∓ 0.04	0.36 ∓ 0.045	0.147
Salt-soluble nucleic acid				
Sucrose-mineral salts	0.38 ∓ 0.03	+0.33 ∓ 0.004	0.05 ∓ 0.032	
Sucrose-mineral salts + adenine (10^{-4} M)	0.41 ∓ 0.03	+0.33 ∓ 0.04	0.08 ∓ 0.05	
Water	0.09 ∓ 0.04	+0.03 ∓ 0.03	0.062 ∓ 0.050	
Water + adenine (10^{-4} M)	0.20 ∓ 0.04	+0.05 ∓ 0.03	0.15 ∓ 0.049	

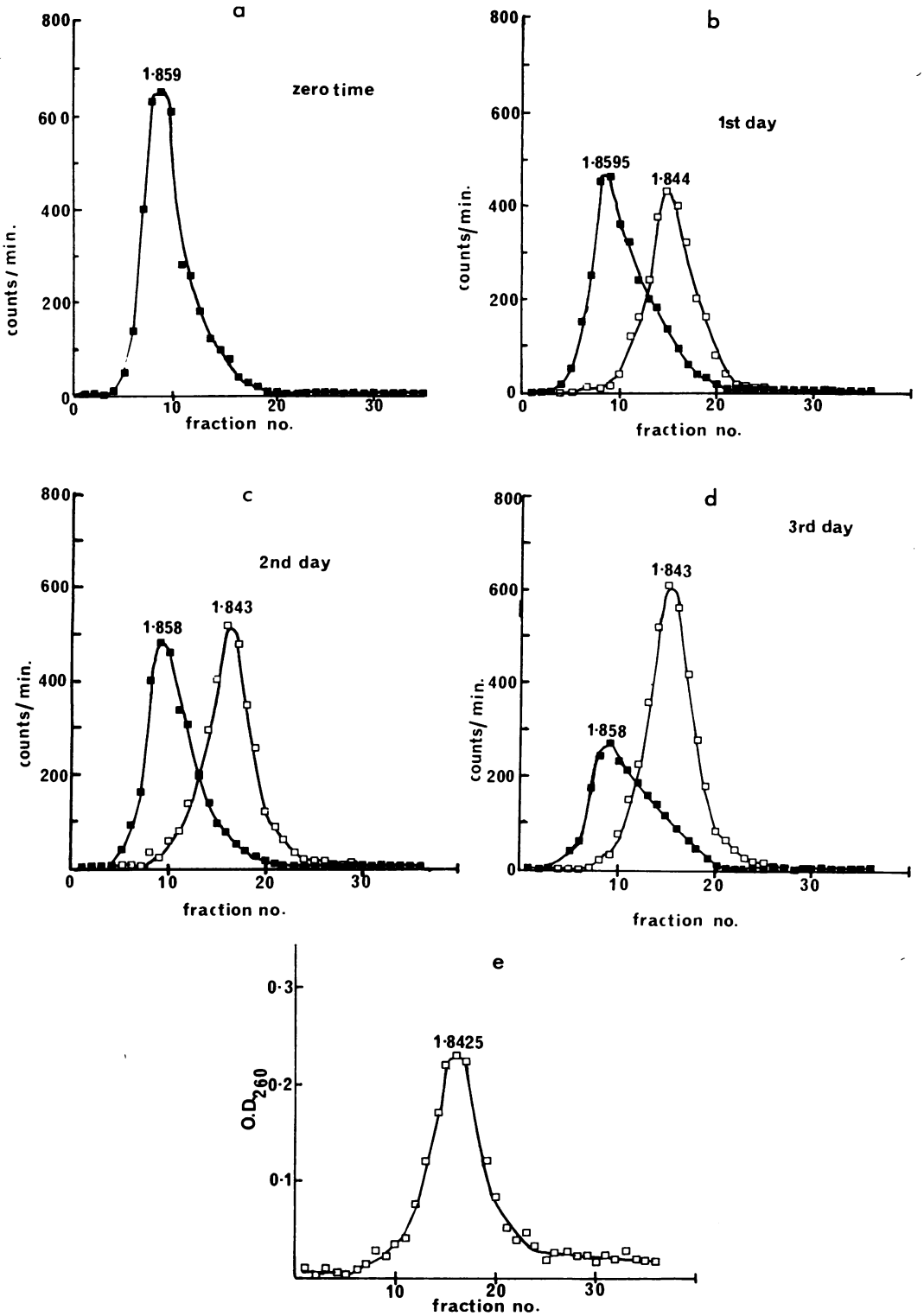

FIG. 5. Density distributions of ribosomal RNA produced 0 to 3 days after transfer of *Lemna* growing in D, ^{15}N, ^{14}C medium to H, ^{14}N, ^{3}H medium. *Lemna* was grown for eight generations in heavy medium and then labeled with ^{14}C-adenine for 24 hr. The label was then removed, and the cultures were grown for a further 16 hr in heavy medium before transfer to light medium containing ^{3}H-adenine. Samples were taken 0 to 3 days after transfer, and the ribosomal RNA was prepared and banded isopycnically on mixed $CsCl/Cs_2SO_4$ (a–d). After fractionation, the density of several samples was determined with a refractometer, and the ^{14}C and ^{3}H was determined in each fraction. The density of each peak was determined by interpolation, and the values are shown above each peak. e: Banding of ribosomal RNA from *Lemna* grown continuously on light medium. ○: ^{14}C-cpm; □: ^{3}H-cpm.

shown in Figure 5. The reasoning behind the experiment was as follows. *Lemna* was grown on 50% D_2O-^{15}-nitrate and labeled with ^{14}C-adenine. After transfer onto ordinary sucrose-mineral salts the "old" heavy RNA should be separable from the new light RNA by isopycnic centrifugation. Extensive recycling would then be revealed as the incorporation of ^{14}C-adenine into the new "light" RNA. Data from this type of experiment can also be used to determine whether the replication of ribosomal RNA in *Lemna* is conservative or semiconservative.

An original inoculum of several fronds of *Lemna* was grown on heavy medium with several subculturings until eight complete generations had passed (1). After a lag period of several days, the plants grew apparently normally except for shortened roots and with a frond rate constant of 0.207 day^{-1} (compared to 0.274 day^{-1} for the original inoculum).

The heavy fronds were labeled with 1 μc of ^{14}C-adenine for 24 hr in heavy medium and then transferred to unlabeled heavy medium for a further 16 hr to allow a decline in the radioactivity of the precursor pools. The fronds were then divided into a number of flasks containing normal light medium together with 1 μc of ^{3}H-adenine. Samples were taken 0 to 3 days after transfer, and the ribosomal RNA was prepared and banded isopycnically. Fractions of 20 drops were taken from the gradients, and the density of eight fractions was determined. The fractions were counted for for both ^{14}C and ^{3}H, and the densities of each peak were determined by interpolation. The results of the experiment are shown in Figure 5, a to d. Ribosomal RNA was isolated from plants grown continuously on ordinary sucrose-mineral salts and banded isopycnically. The results of this banding are shown in Figure 5e. Preliminary experiments indicated the necessity of allowing a 16-hr break after labeling with ^{14}C-adenine. If this period was not used and the transfer to light medium was allowed to take place immediately after labeling, considerable levels of ^{14}C were found at an intermediate density to both the heavy and the light RNA. This is presumably the result of very rapid exchange of D_2O after transfer, resulting in the formation of labeled precursor fragments of intermediate density. Figure 6 shows the rate of synthesis of ribosomal RNA after transfer and the specific radioactivity using the levels of ^{14}C-adenine present in each peak from Figure 5, a to d. The rate constants for synthesis after transfer are 0.49 and degradation 0.152 day^{-1}.

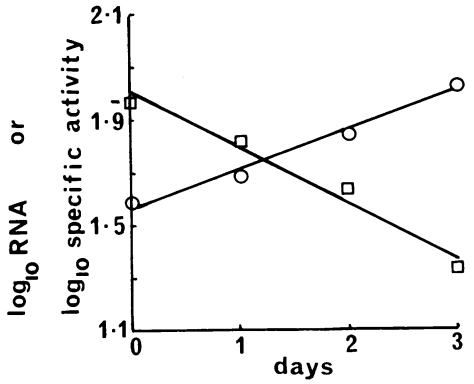

FIG. 6. Variation of specific radioactivity and total ribosomal RNA from *Lemna* transferred at day 0 from heavy (D, ^{15}N, ^{14}C) medium to light (H, ^{14}N, ^{3}H) medium. Specific radioactivity was determined with ^{14}C only. ○: Total ribosomal RNA; □: specific radioactivity, cpm/μg.

Several features are noticeable from Figure 5. First, the densities of the peak fractions remain virtually identical within the experimental error of determining density by refractive index. This provides evidence for the notion that the replication of ribosomal RNA in *Lemna* is conservative. Second, there is no appearance of ^{14}C material under the tritium peak apart from the normal crossover between the two peaks. This would again indicate the lack of significance of recycling in this system. The pronounced assymetry of the heavy peak noticeable even in the zero samples is probably the result of unequal expansion of the gradient as described by Flamm *et al.* (2). If the ribosomal RNA is banded higher in the gradient by, for example, using Cs_2SO_4 as described by Lozeron and Szybalski (7), the assymetry disappears. The mixed CsCl and Cs_2SO_4 have been used in preference since they provided superior separative power.

Turnover of Individual Nucleic Acid Fractions. One further objection to the turnover data shown in Table II is the possibility that the short labeling period used (4 hr) was selecting out a group of highly labile ribosomes while the majority were stable. This possibility may be tested by increasing the labeling time and redetermining rate constants. In the experiment shown in Table V *Lemna* was labeled for 6 days with ^{14}C-adenine, and the rate constants were determined in the usual manner. The data show that under these conditions the measured degradation rate constant is reduced but still measurable. These data can be interpreted as suggesting that ribosomes are heterogeneous with respect to their turnover rates. Measurements of the turnover constants of both the ribosomal RNA and salt-soluble nucleic acid are heterogeneous. The latter fraction, for example, contains both 4 and 5 S soluble RNA (14) and DNA. The rate constants for the individual fractions of nucleic acid have been determined by labeling cultures of *Lemna* for 4 hr, subculturing onto unlabeled medium, preparing the nucleic acids 3 to 6 days later, and then subjecting them to polyacrylamide gel separation. After scanning at 265 nm the first 60 mm of the gel were sliced into 0.5-mm sections, and the sections were counted for radioactivity. Specific radioactivities were determined from the areas under the individual peaks and the associated radioactivity. All samples were run in duplicate,

Table V. *Effect of Long Term Labeling on the Rate Constants of Synthesis and Degradation of Ribosomal RNA*

Batches of *Lemna* were labeled for 6 days with 1 μc of ^{14}C-adenine before distribution onto sucrose-mineral salts plus or minus 10^{-4} M adenine or water plus or minus 10^{-4} M adenine. Samples were taken for the succeeding 6 days, and the rate constants were determined as described for Table II.

Incubation Conditions	Rate Constants			
	Synthesis	Total	Degradation	Fresh weight
		day^{-1}		
Sucrose-mineral salts	0.45 ∓ 0.03	+0.39 ∓ 0.03	0.06 ∓ 0.043	0.33
Sucrose-mineral salts + adenine (10^{-4} M)	0.47 ∓ 0.02	+0.39 ∓ 0.02	0.08 ∓ 0.028	0.34
Water	0.21 ∓ 0.032	−0.07 ∓ 0.048	0.28 ∓ 0.058	0.16
Water + adenine (10^{-4} M)	0.29 ∓ 0.02	−0.02 ∓ 0.02	0.31 ∓ 0.028	0.194

Table VI. *Rate Constants of Synthesis and Degradation of Individual Nucleic Acid Components in Lemna under High Growth Rate Conditions*

Cultures of *Lemna* were labeled for 4 hr with 1 μc of ^{14}C-adenine as previously described and then placed on unlabeled medium. Three to 6 days after the transfer, the nucleic acids were prepared and separated on 2½% polyacrylamide gels for 2¼ hr at 5 ma per gel. After scanning at 265 nm, the gels were frozen and cut into ½-mm sections, and the sections were counted. All nucleic acid components were counted to an accuracy greater than ±4% of the count. Specific radioactivities were determined from the area under each peak and its associated radioactivity. Rate constants determined as described for Table II.

Nucleic Acid Component	Rate Constants			
	Synthesis	Total	Degradation	$t_{\frac{1}{2}}$
	day^{-1}			*days*
Cytoplasmic ribosomal RNA				
25 S	0.51 ∓ 0.016	0.36 ∓ 0.011	0.15 ∓ 0.019	4.3
18 S	0.52 ∓ 0.007	0.35 ∓ 0.016	0.17 ∓ 0.017	
Chloroplast ribosomal RNA				
23 + 13.7 S	0.38 ∓ 0.012	0.32 ∓ 0.016	0.06 ∓ 0.020	15.5
16 S	0.37 ∓ 0.012	0.34 ∓ 0.021	0.03 ∓ 0.024	
Soluble RNA				
4 + 5 S	0.47 ∓ 0.025	0.34 ∓ 0.016	0.13 ∓ 0.030	5.3
DNA	0.31 ∓ 0.018	0.32 ∓ 0.02	...	...

Table VII. *Effect of Partial Nutrient Deficiency on the Rate Constants of Synthesis and Degradation of Nucleic Acids*

Batches of *Lemna* were labeled for 4 hr as previously described and then transferred onto sucrose-mineral salts or one of the following media: (*a*) Nitrate-deficient. Calcium nitrate and potassium nitrate were replaced by the appropriate levels of potassium and calcium chlorides. (*b*) Phosphate-deficient were produced by replacing potassium hydrogen phosphate with potassium chloride. (*c*) Calcium-deficient were produced by replacing calcium nitrate with potassium nitrate. (*d*) Magnesium-deficient were produced by replacing magnesium sulphate with potassium sulphate. (*e*) Sucrose-deficient were produced by omitting sucrose from the medium. Samples were taken for each of 6 successive days, and the rate constants were determined as described for Table II.

Incubation Conditions	Rate Constants			
	Synthesis	Total	Degradation	Fresh weight
	day^{-1}			
Ribosomal RNA				
Full medium	0.35 ∓ 0.02	+0.25 ∓ 0.02	0.10 ∓ 0.028	0.314
Nitrate-deficient	0.17 ∓ 0.02	−0.17 ∓ 0.04	0.34 ∓ 0.045	0.099
Phosphate-deficient	0.33 ∓ 0.02	+0.10 ∓ 0.02	0.23 ∓ 0.028	0.30
Calcium-deficient	0.22 ∓ 0.01	−0.12 ∓ 0.03	0.34 ∓ 0.03	0.138
Magnesium-deficient	0.27 ∓ 0.02	+0.10 ∓ 0.04	0.17 ∓ 0.045	0.33
Sucrose-deficient	0.30 ∓ 0.03	+0.25 ∓ 0.03	0.05 ∓ 0.042	0.323
Salt-soluble nucleic acid				
Full medium	0.28 ∓ 0.007	+0.21 ∓ 0.015	0.07 ∓ 0.012	
Nitrate-deficient	0.12 ∓ 0.04	−0.04 ∓ 0.01	0.16 ∓ 0.041	
Phosphate-deficient	0.25 ∓ 0.02	+0.12 ∓ 0.03	0.13 ∓ 0.036	
Calcium-deficient	0.17 ∓ 0.01	+0.06 ∓ 0.02	0.11 ∓ 0.022	
Magnesium-deficient	0.22 ∓ 0.02	+0.12 ∓ 0.03	0.10 ∓ 0.036	
Sucrose-deficient	0.27 ∓ 0.03	+0.23 ∓ 0.03	0.04 ∓ 0.042	

and the values shown in Table VI represent the average of the duplicate gels.

Several features are apparent. Within experimental error DNA shows no detectable turnover. However, the method used for extraction in this paper seems to result in poor yields compared to other plant tissues (6), and thus labile DNA species may be lost. There is a very distinct difference between the turnover of the cytoplasmic ribosomal RNA and the chloroplast ribosomal RNA: the average half-life of the chloroplast ribosomal RNA is 15 days and that of the cytoplasmic ribosomal RNA is just over 4 days. Since the 13.7 S component is considered to be a degradation product of the 23 S, the values for these two fractions have been included together. The different turnover constants for the cytoplasmic and chloroplast ribosomal RNAs suggest a degree of autonomy for the chloroplast in the cell, a notion supported by other evidence (6), and perhaps the result of having separate ribosomal RNA. The turnover values for soluble RNA are still heterogeneous since complete separation of the 4 and 5 S cannot be obtained on 2½% polyacrylamide gels.

Effects of Partially Deficient Nutrient Conditions on Turnover Rates. In Figure 3 it was shown that when *Lemna* was placed on water there was a considerable increase in the rate of degradation of RNA. It is of interest to examine whether this is the result of depletion of any of the particular nutrients on which *Lemna* is grown or a general response to poor growth conditions. Accordingly, the rate constants of degradation and synthesis of the nucleic acids in *L. minor* grown under partially deficient nutrient conditions have been determined. Media deficient in sucrose, nitrate, phosphate, magnesium or calcium have been examined. The results are shown in Tables VII and VIII.

Omission of calcium, nitrate, phosphate, or magnesium leads to increase in the rate of degradation of both the ribosomal and salt-soluble fraction. The increases are more marked in the ribosomal RNA fraction. The largest increases in degradative rates of ribosomal RNA are associated with the omissions of calcium and nitrate, and in both these cases net loss of ribosomal RNA is observed. The effects on the synthetic rates are less dramatic. Again omission of calcium and nitrate leads to the biggest reductions, while omission of phosphate has a barely perceptible effect. Cultures grown in the immediate absence of sucrose show little change in the rate constants, a slight decrease in the degradative rates being discernible. However, if cultures are grown continuously in the absence of sucrose, there is a more marked effect. In the experiment shown in Table VIII, cultures of *Lemna* were grown either in the presence or in the absence of sucrose for 4 weeks before measurement of the rate constants. It can be seen that the long term omission of sucrose results in a considerable drop in the rate constant of synthesis, but that of degradation is little altered.

Effect of Hormones on Turnover Rates. It has been claimed that the inclusion of benzyladenine or abscisic acid in the culture medium alters the rate of synthesis of nucleic acids in *L. minor* (14). Since these studies failed to take into account the possible effect of both these hormones on the uptake of the label for measuring the rate of synthesis, it was decided, first, to check the claim that benzyladenine and abscisic acid alter the rates of synthesis of the nucleic acids, using the method described in this paper, which will not be affected by alterations in uptake of precursors; second, to examine the effects of both hormones on the degradative rates of the nucleic acids. The effects of benzyladenine have been examined on *Lemna* grown both on sucrose-mineral salts and on water and abscisic acid on *Lemna* grown on sucrose-mineral salts. The results of the experiment are shown in Table IX.

Under high growth rate conditions the effects of benzyladenine are barely perceptible. These results are contrary to those of Van Overbeek *et al.* (14), 3- to 5-fold increases in the rates of incorporation of $^{32}P_i$ were observed. Since these authors failed to examine the effect of benzyladenine on the uptake of the label, the results of Table IX suggest that the main effect of benzyladenine is on the uptake of the label rather than on the rate of synthesis of the nucleic acids. Benzyladenine has a much more marked effect on *Lemna* grown on water, but the rates of both synthesis and degradation have been increased. Inclusion of abscisic acid leads to a very marked reduction in the rate of synthesis of nucleic acids. It leaves the rate of degradation of ribosomal RNA unaltered but appears to stabilize completely the salt-soluble nucleic acid.

Table VIII. *Effects of Long Term Omission of Sucrose on the Rate Constants of Synthesis and Degradation of Ribosomal RNA*

Cultures of *Lemna* were grown for 4 weeks on mineral salts in the presence or absence of sucrose before labeling and estimation of rate constants.

Growth Medium	Rate Constants			
	Synthesis	Total	Degradation	Fresh weight
	day^{-1}			
Sucrose-mineral salts	0.46 ∓ 0.03	0.37 ∓ 0.03	0.09 ∓ 0.042	0.31
Mineral salts	0.25 ∓ 0.02	0.13 ∓ 0.03	0.12 ∓ 0.036	0.23

DISCUSSION

This paper provides evidence for the turnover of ribosomal RNA during the growth of a higher plant. Evidence from heavy labeling indicates that the replication of ribosomal RNA is conservative and that the turnover therefore is degradation to nucleotides or small polynucleotides which are not reincorporated into ribosomal RNA. Other experiments in this paper indicate that the rate of turnover of ribosomal RNA is very dependant on the nutritional status of the plant. Inclusion of benzyladenine or omission of particular nutrients alters the degradative rate of ribosomal RNA. Many plant tissues lose substantial levels of nucleic acids when incubated on water, and it has been shown that this also happens with *Lemna*. It has been demonstrated here that this loss is occasioned not only by a reduction in the rate of synthesis but by an increase (2- to 4-fold) in the rate of degradation. In this respect *Lemna* is behaving in an identical manner to micro-

Table IX. *Effect of Benzyladenine and Abscisic Acid on the Rate Constants of Synthesis and Degradation of Nucleic Acids*

Batches of *Lemna* were labeled in the customary manner and distributed onto the media below. Samples were taken on each of the successive 6 days, and the rate constants were determined as described for Table II.

Growth Medium	Rate Constants			
	Synthesis	Total	Degradation	Fresh weight
		day^{-1}		
Ribosomal RNA				
Sucrose-mineral salts	0.39 ∓ 0.011	+0.31 ∓ 0.01	0.08 ∓ 0.014	0.33
Sucrose-mineral salts + benzyladenine (10^{-6} M)	0.42 ∓ 0.013	+0.31 ∓ 0.01	0.11 ∓ 0.014	0.38
Sucrose-mineral salts + abscisic acid (5×10^{-6} M)	0.14 ∓ 0.015	+0.05 ∓ 0.012	0.09 ∓ 0.014	0.065
Water	0.08 ∓ 0.007	−0.09 ∓ 0.01	0.17 ∓ 0.012	0.085
Water + benzyladenine (10^{-6} M)	0.19 ∓ 0.02	−0.09 ∓ 0.03	0.28 ∓ 0.036	0.13
Salt-soluble nucleic acid				
Sucrose-mineral salts	0.29 ∓ 0.005	+0.26 ∓ 0.01	0.03 ∓ 0.011	
Sucrose-mineral salts + benzyladenine (10^{-6} M)	0.30 ∓ 0.006	+0.27 ∓ 0.022	0.03 ∓ 0.02	
Sucrose-mineral salts + abscisic acid (5×10^{-6} M)	0.06 ∓ 0.01	+0.07 ∓ 0.01	0	
Water	0.04 ∓ 0.02	−0.02 ∓ 0.02	0.06 ∓ 0.028	
Water + benzyladenine (10^{-6} M)	0.10 ∓ 0.01	+0.01 ∓ 0.01	0.09 ∓ 0.014	

organisms which also show an enhanced degradative rate of RNA under poor growth conditions (8). In microorganisms it has been suggested that the stability of the ribosome may be an important factor in RNA degradation, and perhaps similar conclusions may be drawn in the case of plants. Conditions which alter the stability of the ribosome (for example, omission of magnesium) lead to an increased rate of degradation.

The rate of turnover of cytoplasmic ribosomal RNA in this tissue is surprisingly high. The half-life of both components 25 S and 18 S appears to be identical within experimental error and suggests that we are observing the turnover of a single entity, *i.e.*, the ribosome rather than the individual RNA components. The average half-life of these two components, 4.3 days, may be compared with the generation time of *Lemna* (time required to produce two new fronds) a value of 3.5 days in this experiment. Thus a very considerable proportion of the cytoplasmic ribosomes is lost during the production of two new fronds. The much greater stability of the chloroplast ribosomal RNA is indicative of separate mechanisms operating to degrade cytoplasmic and chloroplast ribosomal RNA and indicates a degree of autonomy for the chloroplast.

The experiments quoted in this paper indicate the very considerable value of *Lemna* as a test organism. There are few other plants which can be grown reproducibly in a steady state of growth, on D_2O and ^{15}N, under rigorously sterile conditions, under both darkness and light with all cultures derived from a single frond. It would have been difficult to make many of the measurements quoted in this paper without an organism such as *Lemna*. It is hoped that the data in this paper will point the way to a much greater use of this organism in plant biochemistry.

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LITERATURE CITED

1. Cope, B. T., S. Bose, H. L. Crespi, and J. J. Katz. 1965. Growth of Lemna in H_2O/D_2O mixtures: enhancement by kinetin. Bot. Gaz. 126: 214–221.
2. Flamm, W. G., H. E. Bond, and H. E. Burr. 1966. Density gradient centrifugation of DNA in a fixed angle rotor. Biochim. Biophys. Acta 129: 310–319.
3. Ingle, J., J. L. Key, and R. E. Holm. 1965. Demonstration and characterization of a DNA-like RNA in excised plant tissue. J. Mol. Biol. 11: 730–746.
4. Loening, U. E. 1965. The synthesis of messenger and ribosomal RNA in pea seedlings as detected by electrophoresis. Proc. Roy. Soc. Ser. B. 162: 121–136.
5. Loening, U. E. 1967. The fractionation of high molecular weight ribonucleic acid by polyacrylamide gel electrophoresis. Biochem. J. 102: 251–257.
6. Loening, U. E. and J. Ingle. 1967. Diversity of RNA components in green plant tissues. Nature 215: 363–367.
7. Lozeron, H. A. and W. Zybalski. 1966. Suppression of RNA precipitation during Cs_2SO_4 density gradient centrifugation. Biochem. Biophys. Res. Commun. 23: 612–618.
8. Mandelstam, J. 1960. The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation. Bacteriol. Rev. 24: 289–308.
9. Ralph, R. K. and A. R. Bellamy. 1964. Isolation and purification of undegraded nucleic acids. Biochim. Biophys. Acta 87: 9–16.
10. Reiner, J. M. 1953. The study of metabolic turnover rates by means of isotopic tracers. Arch. Biochem. Biophys. 46: 53–79.
11. Stanley, W. M. and R. M. Bock. 1965. Isolation and physical properties of the ribosomal ribonucleic acid of *Escherichia coli*. Biochemistry 4: 1302–1311.
12. Trewavas, A. J. 1967. A new method for counting labelled nucleic acids by liquid scintillation. Anal. Biochem. 21: 324–328.
13. Trewavas, A. J. 1968. Relationship between plant growth hormones and nucleic acid metabolism. Progr. Phytochem. 1: 113–160.
14. Van Overbeek, J., J. E. Loeffler and M. Iona R. Mason. 1967. Dormin, inhibitor of plant DNA synthesis. Science 156: 1497–1499.