Studies on Independent Synthesis of Cytoplasmic Ribonucleic Acids in *Acetabularia mediterranea*

HIROTO NAORA, HATSUKO NAORA, and JEAN BRACHET

From Laboratoire de Morphologie Animale, Université Libre de Bruxelles, Bruxelles, Belgique

ABSTRACT 1. The RNA content of anucleate and nucleate fragments of *Acetabularia* has been measured. It was found that there is a net synthesis of RNA in nucleate fragments. On the other hand, the RNA content of anucleate fragments did not change significantly after enucleation.

2. Anucleate fragments, however, can readily incorporate ^{14}C–labeled adenine, orotic acid, and carbon dioxide into their cytoplasmic RNA.

3. The results of experiments on $^{14}CO_2$ incorporation into the RNA of anucleate and nucleate fragments suggest that there is a mechanism for *de novo* synthesis of RNA in anucleate cytoplasm.

4. In *Acetabularia*, 81 per cent of the cytoplasmic RNA is bound to a large granule fraction, consisting mainly of chloroplasts. Even after removal of the nucleus, *RNA is synthesized in this "chloroplast" fraction.* The chloroplasts are thus a major site of RNA synthesis in the cytoplasm of these algae. Synthesis of "chloroplastic" RNA, in anucleate fragments, possibly occurs at the expense of the RNA present in other fractions (microsomes and supernatant).

5. 8-Azaguanine stimulates regeneration and cap formation in anucleate fragments and does not inhibit RNA synthesis in these fragments.

Following the pioneer work of Hämmerling (1) on the regeneration of nucleate and anucleate fragments of the unicellular alga, *Acetabularia*, biochemical investigations of this phenomenon have been carried out in both Hämmerling's and our laboratories (2–5). The main purpose of these investigations has been to gain a clearer understanding of nucleocytoplasmic interactions in the cell.

One of the main results of our previous investigations was the demonstration of a net synthesis of ribonucleic acid (RNA) and protein in the anucleate cytoplasm of *Acetabularia mediterranea* after removal of the nucleus (3, 6).

Dr. Hiroto Naora was a Fellow of The Rockefeller Foundation, 1957–1958. He is on leave from the Laboratory of Cell Research, Cancer Institute, Tokyo, Japan.

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The conclusion which was drawn was that the nucleus does not directly control the cytoplasmic RNA and protein synthesis, but that there is some remote nuclear involvement.

It has been demonstrated by Richter, however, that the RNA content of anucleate fragments of this alga remains constant for 28 days after enucleation; but he confirmed that there is a net synthesis of protein in these fragments (2, 7, 8). Two of us (H. N. and H. N.) were also unable to observe an increase of the RNA content (determined by both ^{14}C-adenine and guanine isotope dilution methods) in the anucleate fragments (9).

These findings, however, do not preclude the possibility of independent RNA synthesis in the cytoplasm. In order to obtain more information about this possibility, further work on anucleate and nucleate fragments appeared necessary.

The present report deals mainly with the determination of the RNA content of various cell fractions in both anucleate and nucleate fragments, and with the ability of these fragments to incorporate several radioactive precursors into their RNA.

The results obtained indicate the existence of an independent synthesis of cytoplasmic RNA in the course of regeneration, although there is no significant increase of the total RNA content of anucleate fragments after removal of the nucleus.

EXPERIMENTAL

Materials

The *Acetabularia mediterranea* used in these experiments were cultured in phosphate and nitrate containing artificial sea water at 22°C. The algae were illuminated 14 hours per day by Philips' fluorescent tube. Some of the cultures were maintained in natural sea water containing *Erdschreiberlösung* (3).

Nucleate and anucleate fragments were usually prepared by cutting the algae one-third of the way up to the stalk from the rhizoids. The smaller fragment contained the nucleus. In some experiments, the nucleus-containing rhizoid was removed and the remaining cytoplasm was then employed as the anucleate fragment.

Determination of RNA Content

Before extraction, the nucleate fragments were fixed in ice cold 80 per cent alcohol for a short time (approximately 15 minutes) and the rhizoid part was then removed. Cytoplasms of both anucleate and nucleate fragments were homogenized in the glass homogenizer designed by Vanderhaeghe and Chantrenne (10).

RNA fractions were obtained by the procedure of either Ogur and Rosen (11) or of Richter (7). RNA was extracted with 0.6 to 1.2 ml. of N perchloric acid (PCA) for 17 hours at 3–4°C. The RNA content was determined by measurement of the

ultraviolet absorption at 260 mμ in a quartz microcuvette in the Beckman model DU spectrophotometer.

Fractionation of Subcellular Components

The procedure used in the present experiments was a modification of that described by Vanderhaeghe and Brachet (6, 12). All operations were carried out at 2–5°C.

Twenty to thirty anucleate, nucleate fragments, or whole algae were washed with 0.65 M KCl to remove the culture medium. The rhizoid parts were carefully cut off. The cytoplasms of the algae were very gently homogenized by hand in the glass homogenizer in 0.4 to 0.5 ml. of ice cold 0.65 M KCl. The resulting homogenate was gently centrifuged (at approximately 60 R.P.M. for 1 minute). The green supernatant was withdrawn with a micropipette, the tip of which was 0.1 to 0.2 mm. in inner diameter. The sediment was resuspended in 0.4 to 0.5 ml. of 0.65 M KCl and was homogenized and centrifuged until the sediment became white and the supernatant water-clear. Usually this procedure was repeated 4 or 5 times. The supernatants were pooled for subsequent fractionation. The pooled supernatants (usually 2 ml.) were centrifuged at 9,000 *g* for 10 minutes. Although the sediment fraction possibly contained large particles and mitochondria in addition to chloroplasts, it can be regarded as essentially a "chloroplast fraction." The resulting supernatant was again centrifuged at 80,000 *g* for 100 minutes. A slightly yellowish pellet and a clear supernatant were obtained; they served as the microsomal and supernatant fraction, respectively. For fractionation of the microsomal fraction, the volume of the original homogenate was adjusted to less than 1 ml. The obtained microsomal fraction was almost free of chlorophyll.

Procedure for $^{14}CO_2$ Incorporation into Acid-Soluble and RNA Adenine and Guanine

One hundred to two hundred and fifty anucleate and nucleate fragments, taken 1, 2, 10, and 20 days after operation, were incubated at 22°C., and were illuminated in the way described above for 24 hours in the presence of isotopically labeled carbon dioxide. Labeled sodium bicarbonate (specific activity, 6 μc./μM) was dissolved in the phosphate- and nitrate-containing artificial sea water which was carefully adjusted to pH 7.0 with 0.3 N KOH. Experiments were carried out with 0.9 to 1.2 μc./ml. as the final radioactivity. After incubation with the labeled sea water, the fragments were washed several times in non-labeled sea water. The rhizoid part of the nucleate fragments was cut off before homogenization. Nucleate and anucleate cytoplasms were homogenized with the glass homogenizer. Three ml. of ice cold 10 per cent trichloroacetic acid (TCA) were added and the acid-soluble fraction was extracted for 20 minutes. The residue was washed with another 3 ml. of ice cold TCA and then centrifuged. The wash fluid was combined with the first extract. The residue was washed twice with alcohol; lipid was then extracted twice with hot ether-alcohol (1:3). The lipid-free residue was washed with an ice cold 0.2 N PCA. One ml. of N PCA was added and the RNA was extracted for 17 hours at 3–4°C.

Hydrolysis of the acid-soluble fraction was accomplished by boiling for 30 minutes. TCA was then removed by several treatments with ether. A one-third volume of 25 per cent NH_3 and a one-half volume of 17 per cent $AgNO_3$ were added to the hydrolysate and the mixtures were stored overnight in a cool room (about 10°C.). The liberated purines were precipitated as purine-silver salts (9, 13, 14). The precipitate was washed three times with distilled water. The washed precipitate was finally suspended in 0.03 ml. of N HCl and boiled for 5 minutes. The centrifuged supernatant was placed directly on a Whatman No. 4 filter paper and chromatographed in methanol-concentrated HCl–H_2O (70:20:10) for 16 to 18 hours at room temperature (9, 15). After drying the paper, the ultraviolet-absorbing spots were spotted in the light of a mineralight lamp provided with a short wave ultraviolet filter model SL 2537 and the *Rf*'s of the adenine and guanine bases were compared with those of known standards. Each spot was cut out and the adenine and guanine were eluted from the chromatograms by treatment with 1.0 ml. of 0.1 N HCl overnight. Spectrophotometric blanks were simultaneously obtained from the control part of the same strip. It was observed that the absorption spectra of the eluted adenine and guanine were quite similar to those obtained from purified standards.

The RNA fraction was hydrolyzed with N PCA at 100°C. for 30 or 60 minutes. One volume of 25 per cent NH_3 and one-fourth volume of 17 per cent $AgNO_3$ were added to precipitate the liberated purines. Adenine and guanine were isolated by the procedure described above.

Procedure for ^{14}C-Adenine Incorporation into RNA

In studying the ^{14}C-adenine incorporation into cytoplasmic and "chloroplastic" RNA's, nucleate and anucleate fragments of algae taken 10 days after operation were employed.[1] Before incubation with the labeled sea water, the fragments were incubated in the presence of 10 mg. of streptomycin per 100 ml. sea water for 1 to 2 days. After this preincubation period, the fragments were washed thoroughly with sea water; ^{14}C-adenine incorporation into cytoplasmic RNA was carried out in an incubation mixture containing 0.4 μM ^{14}C-8-adenine (1 μc./μM) and 4 ml. of nitrate and phosphate containing artificial sea water. The fragments were placed in the incubation mixture in 20 ml. cylindrical flasks and illuminated with fluorescent lamps for 5, 14.5, and 24 hours.

After incubation, cell walls, "chloroplasts," and supernatant fractions were obtained by homogenization and centrifugation as above. RNA was extracted by the procedure of Ogur and Rosen (11) and the ultraviolet absorption of the RNA fraction was measured in the usual way. An aliquot of the RNA-containing fraction was directly deposited on a small ground glass planchet and dried. During evaporation of the extracts, no loss of radioactivity was observed. The radioactivity was then measured.

[1] As explained on page 1085 the terms "chloroplast" and "chloroplastic" as used throughout this paper indicate a fraction possibly containing large particles and mitochondria in addition to chloroplasts.

Procedure for ^{14}C–Orotic Acid Incorporation into RNA

Twenty to thirty nucleate and anucleate fragments, taken 2 and 10 days after operation, were employed in each experiment. Preincubation in the presence of streptomycin was carried out as described above. The washed fragments were transferred in 20 ml. cylindrical flasks with 4 ml. of the artificial sea water containing 0.2 μM ^{14}C–6-orotic acid (2 μc./μM). The fragments were incubated under full illumination for 24 hours in the same way as for ^{14}C-adenine incorporation.

At the end of the incubation, the fragments were washed with 0.65 M KCl, homogenized in ice cold 0.65 M KCl, and the cell walls were removed according to the method described previously. The pooled green supernatants were exactly divided into two portions. An aliquot of this supernatant was transferred to a conical centrifuge tube. RNA was extracted by the procedure of Ogur and Rosen (11) and the amount of RNA was determined as usual. Another portion of the supernatant was twice extracted by treatment with 1.0 ml. of 10 per cent TCA at 90°C. for 15 minutes following removal of the acid-soluble and lipid fractions. To remove the TCA, the RNA fraction was shaken 7 or 8 times with 3 to 4 ml. of ether. The RNA fraction was then quantitatively deposited on a ground glass planchet and dried. The radioactivity of each sample was counted.

^{14}C–orotic acid incorporation was expressed in terms of specific activity as given by the formula:

$$\text{Specific activity} = \frac{\text{Total radioactivity of RNA fraction extracted with hot TCA}}{\text{Total RNA content of RNA fraction extracted with cold PCA}}$$

Measurement of the Specific Activity

Samples were plated in infinitely thin layers on the ground glass planchets and counted with a Geiger-Müller counter (type Pn 123/C). The specific activity was expressed in C.P.M./μM of the bases or C.P.M./μg. RNA.

RESULTS

Absorption Spectra of RNA Fraction

Absorption spectra of the RNA fractions are shown in Fig. 1. The ratios of E_{260}/E_{230} are approximately between 1.7:1 and 2.1:1 instead of more than 2.5:1 obtained for the RNA fraction from *Euglena* and plant tissues (16, 17). It can be concluded, therefore, that the original Ogur and Rosen method (11) or the modified method of Richter (7) is adequate, but not absolutely perfect, to determine the RNA content of *Acetabularia*. No difference was observed between the results obtained by the Ogur and Rosen method and by that of Richter. If, however, cells were not homogenized before extraction (8), the ultraviolet absorption spectra of the RNA so obtained were not satisfactory for the determination of the RNA content.

The absorption spectra of the RNA extract of each of the subcellular fractions obtained by centrifugation were compared. It was noted, as seen in Fig. 1, that the absorption spectra of the PCA extract of chloroplasts were better than those of whole cytoplasm. It is unfortunate, however, that the absorption spectra obtained from the other fractions were not suitable for RNA determination because of the presence of an ultraviolet-absorbing contaminant. It is conceivable, therefore, that the decreased ratio E_{260}/E_{230} of the extract obtained from the whole cytoplasm may be mainly due to a contamination of the ultraviolet-absorbing materials in the microsomal and/or supernatant fraction (but not to the contaminant derived from cell walls).

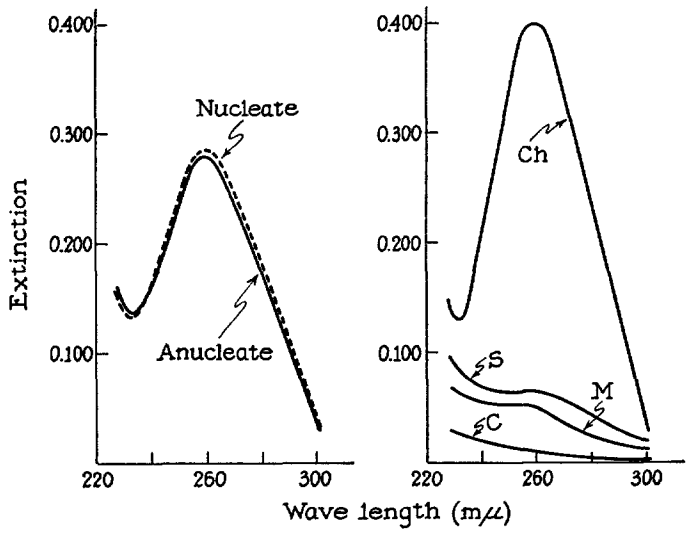

FIGURE 1. Ultraviolet absorption spectra of the RNA fraction from nucleate (– – –) and anucleate fragments (——) taken 4 days after operation (left figure) and different subcellular components of normal *Acetabularia* (right figure). *Ch*, chloroplasts, *S*, supernatant fraction, *M*, microsomes, and *C*, cell walls.

The absorption spectra shown in Fig. 1 clearly indicate that the microsomal and supernatant fractions do not contain such a large quantity of RNA as do those of animal cells. A large part of the cytoplasmic RNA of *Acetabularia* was found in the chloroplast fraction and there is probably no RNA in the cell wall fraction.

RNA Content of Whole Cytoplasm in Anucleate and Nucleate Fragments during Regeneration

The results obtained are summarized in Table I. In every experiment, regeneration had progressed well in both nucleate and anucleate fragments.

The length of the nucleate fragments, 12 days after operation, was more than twice that of the original, and these fragments had begun to form their own caps. Likewise, more than 50 per cent of the anucleate fragments had formed caps after 14 days (see Fig. 3).

TABLE I

RNA CONTENT (MICROGRAMS)/FRAGMENT OF *ACETABULARIA* AFTER OPERATION

Experiment No.	Procedure	Fragment*	Time after operation, *days* 0–1	4–6	10–12	20–25
I	Ogur and Rosen	N	0.80	1.28	1.58	1.80
		A	1.31	1.25	1.33	1.27
II	Ogur and Rosen	N	0.71	—	0.86	1.10
		A	1.39	—	1.42	1.60
III	Richter	N	0.64	—	0.73	—
		A	1.36	—	1.43	—
IV	Richter	N	0.86	—	—	1.65
		A	1.57	—	—	1.57
V	Ogur and Rosen	A	0.39	0.41	0.41	—
VI	Ogur and Rosen	A	1.11	—	0.92	—
VII	Ogur and Rosen		(0)‡	(3)	(7)	(14)
		A	1.01	1.10	0.94	0.88

* N and A indicate the nucleate and anucleate fragments respectively.

‡ In Experiment VII, determination of the RNA content of anucleate fragments was carried out 0, 3, 7, and 14 days after enucleation.

TABLE II

DISTRIBUTION OF CHLOROPLASTIC RNA IN *ACETABULARIA**

Time after operation	Normal cells	Regenerating cells: Nucleate fragments	Regenerating cells: Anucleate fragments
days			
0	81 ± 1.6‡ (5)	—	—
6	—	94 (1)	110 (1)
10–11	—	95 ± 5.2 (4)	105 ± 5.2 (4)
34	—	93 (1)	103 (1)

* The values are expressed as follows:—

$$\frac{\text{RNA content of the isolated chloroplast/fragment}}{\text{Total RNA content/fragment}} \times 100.$$

‡ The values represent averages. The figures in parentheses give the number of experiments.

The results obtained by the techniques of ultraviolet absorption indicate that there is a net synthesis of RNA in nucleate fragments; although the increment of the RNA increase in nucleate fragments differed in each experiment, the RNA content of the nucleate fragments, 3 weeks after enucleation, was approximately twice (maximum increase: 125 per cent, minimum: 55 per cent) that of the original content. In contrast, the RNA content of

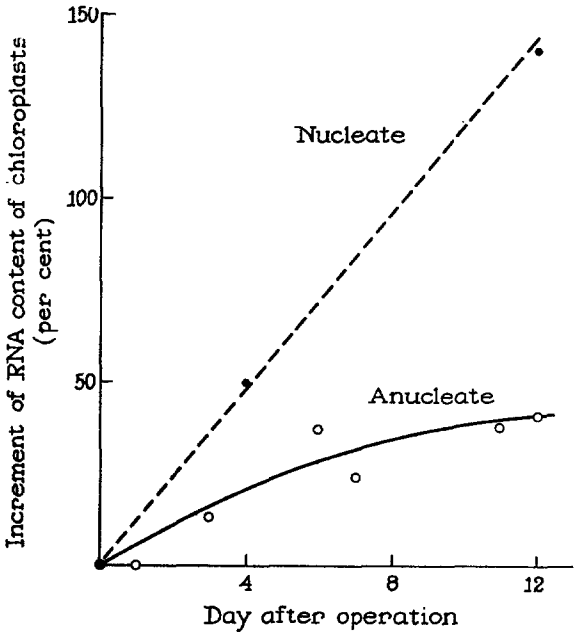

FIGURE 2. Increase of the RNA content of chloroplasts in the anucleate and nucleate fragments of *Acetabularia* after operation. The values of the ordinate are expressed as:

$$\frac{M_t - M_0}{M_0} \times 100$$

where M_0 and M_t represent the RNA content of chloroplasts per fragment at 0 or 1 day and t days after operation respectively.

anucleate fragments did not change remarkably after enucleation, although such fragments were very capable of regeneration, including the formation of a large sized cap. The results summarized in Table I suggest that the physiological conditions might be very important in this experiment: In Experiment II, the RNA content of the anucleate fragments increased (15 per cent); but in Experiments I, III, IV, and V, there was no change; furthermore in Experiments VI and VII, a drop in the RNA content was found (13 to 17 per cent).

Synthesis of Chloroplastic RNA in Anucleate and Nucleate Fragments

As previously described in this paper, chloroplasts (plus large particles) can easily be separated by centrifugation and their RNA content can also be easily measured. It was observed, as can be seen in Table II, that 81 ± 1.6 per cent of the RNA content of the whole cytoplasm is concentrated in the chloroplasts of normal *Acetabularia*. It is assumed therefore that less than 20

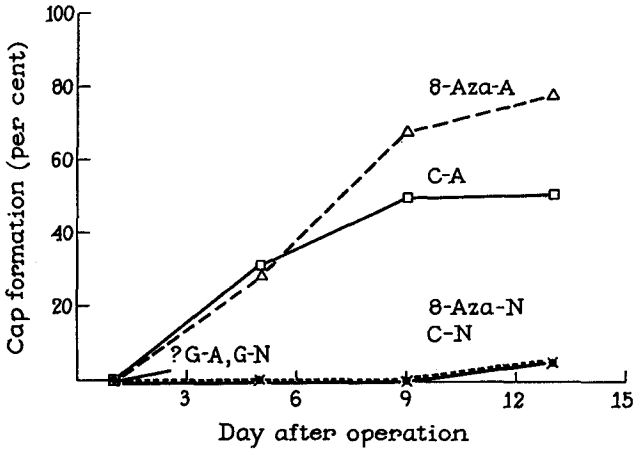

FIGURE 3. Effect of 8-azaguanine and guanine on cap formation of anucleate and nucleate fragments of *Acetabularia*. One day after operation, the fragments were cultivated in the presence of 8-azaguanine, guanine, or in the absence of these compounds. The values of the ordinate are expressed as:

$$\frac{\text{No. of fragments having a cap at } t \text{ days after operation}}{\text{No. of fragments at } t \text{ days after operation}} \times 100$$

C-A and *C-N*, anucleate and nucleate fragments of control groups; 8-Aza-*A* and 8-Aza-*N*; anucleate and nucleate fragments in 8-azaguanine-containing medium; *G-A* and *G-N*, anucleate and nucleate fragments in guanine-containing medium.

per cent of the total cytoplasmic RNA is present in the other fractions (the microsomal and supernatant fractions).

It should be stated again that the chloroplast fraction referred to in this paper was simply fractionated by centrifugation and that it might have been contaminated with mitochondria and other large particles. It has been reported that the RNA of the chloroplasts, in *Euglena*, represents only a small fraction (11 per cent) of the total RNA of the cell and that its RNA content is almost the same as that of the mitochondria (18). It is possible, therefore, that the measured content of chloroplastic RNA in these experiments represents the RNA content of other large particles as well as that of chloroplasts;

the RNA content of the mitochondria and other large particles of *Acetabularia* has not yet been measured.

An increase in the chloroplastic RNA of anucleate and nucleate fragments, following enucleation, is demonstrated in Fig. 2. Table II shows the changes in chloroplastic RNA during regeneration. Some of the values given as expressing the percentage of the total RNA which is chloroplastic RNA are a little greater than 100 per cent. Such high values might be due to experimental errors, sampling errors, or to an ultraviolet-absorbing contaminant which has ac-

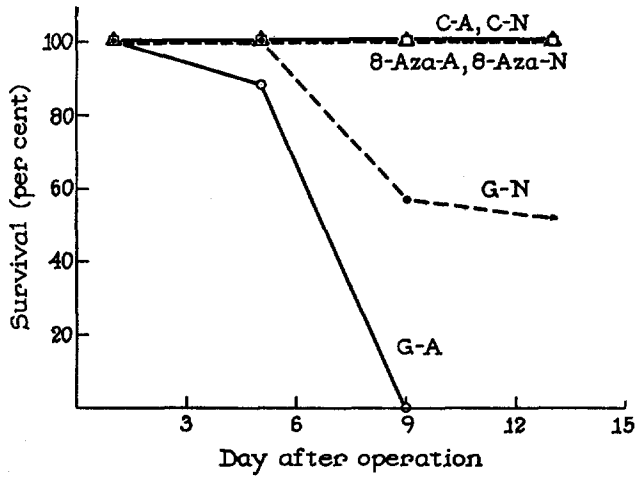

FIGURE 4. Effect of 8-azaguanine on the survival of the anucleate and nucleate fragments of *Acetabularia*. The experimental conditions are the same as in the experiment of Fig. 3. The values of the ordinate are expressed as:

$$\frac{\text{No. of the living fragments at } t \text{ days after operation}}{\text{No. of fragments at 1 day after operation}} \times 100$$

C-A and *C-N*, anucleate and nucleate fragments of control groups; 8-Aza-*A* and 8-Aza-*N*, anucleate and nucleate fragments in 8-azaguanine–containing medium; *G-A* and *G-N*, anucleate and nucleate fragments in guanine-containing medium.

cumulated in anucleate fragments. The differences between each group (normal cells, anucleate, and nucleate fragments) are statistically significant.

It was also observed that, after enucleation, the RNA content of the chloroplasts of the anucleate fragments immediately (during the 1st week) increases, and that finally all the cytoplasmic RNA is distributed in chloroplasts. This finding clearly suggests that the RNA of the microsomal and supernatant fractions disappears shortly after enucleation; however, as previously observed, determination of the RNA content of the microsomal and supernatant fractions was unsatisfactory.

On the other hand, the RNA content of the chloroplasts of the nucleate

fragments extensively increased after operation. Twelve days after the operation, chloroplastic RNA of the nucleate fragments had increased to 140 per cent of its initial value. A slightly changed distribution of chloroplastic RNA was observed in nucleate fragments after operation (see Table II). It is probable, however, that RNA remains in the microsomal and supernatant fractions of the nucleate fragments and that their RNA content also increases after operation.

Thus it seems likely that, during regeneration of both nucleate and anucleate fragments, a net synthesis of RNA occurs in chloroplasts, but the microsomal and supernatant RNA's are synthesized and maintained in the presence of the nucleus only.

TABLE III

EFFECT OF 8-AZAGUANINE ON THE RNA CONTENT OF ANUCLEATE AND NUCLEATE FRAGMENTS OF *ACETABULARIA* DURING REGENERATION*

	Nucleate fragments			Anucleate fragments		
	Control	Guanine	8-azaguanine	Control	Guanine	8-azaguanine
Regeneration.....	Very good	Died	Very good	Very good	Died	Very good
RNA content (μg.)/Fragment	1.33	—	1.40	0.83	—	0.97
Percentage of increase over control.....	0	—	+5	0	—	+17

* One day after enucleation, 18 to 21 fragments were incubated in natural sea water (control), or in the presence of guanine or 8-azaguanine for 26 days.

Effect of 8-Azaguanine on Regeneration and RNA Synthesis of Nucleate and Anucleate Fragments

One of us has already reported that chemical analogues are, as a rule, less inhibitory and toxic for the regeneration and growth of nucleate and anucleate fragments than the normal nucleic acid bases or the amino acids (19, 20). In the present investigation, the effects of 8-azaguanine have been compared with those of guanine on the regeneration and RNA synthesis of nucleate and anucleate fragments.

Saturated solutions of guanine and 8-azaguanine in natural sea water were employed. The results are summarized in Figs. 3 and 4 and Table III.

It was confirmed that guanine is extremely toxic for regenerating anucleate and nucleate fragments; 9 days after incubation in the presence of guanine, all the anucleate and 57 per cent of the nucleate fragments had died. In the

case of 8-azaguanine, however, there was no difference in the survival and in the RNA content between the nucleate fragments of the control and experimental groups. Regeneration of the anucleate fragments was still active in the presence of 8-azaguanine for 26 days after enucleation and the RNA content of these anucleate fragments was greater than that of the controls (16 per cent increase). It could be clearly shown, moreover, that 8-azaguanine actually promotes regeneration and cap formation in anucleate fragments (Fig. 3). This result is in agreement with the previous observations made by one of us (19, 20).

It can be concluded that cytoplasmic RNA in both nucleate and anucleate fragments can be synthesized or maintained in the presence of 8-azaguanine; however, it has not been determined so far whether 8-azaguanine is incorporated into the RNA or not.

^{14}C-Adenine Incorporation into the RNA of Chloroplasts and Whole Cytoplasm of Nucleate and Anucleate Fragments

As previously described in this paper, chloroplasts seem to be capable of synthesizing RNA in the absence of the nucleus. However, the possibility remains that, after enucleation, the microsomal and supernatant RNA's might simply penetrate into the chloroplasts. Biochemical experiments on the incorporation of precursors into the RNA of chloroplasts and whole cytoplasm were therefore performed in an attempt to provide an answer to the question of independent synthesis of RNA in the cytoplasm.

^{14}C-adenine incorporation into the RNA of chloroplasts was compared with that of the whole cytoplasm of nucleate and anucleate fragments. The results are summarized in Fig. 5 and Table IV.

As can be seen in Fig. 5, incorporation into chloroplastic RNA of nucleate and anucleate fragments increases linearly with the incubation time; nucleate fragments incorporate radioactive adenine into the RNA of their chloroplasts more rapidly than do anucleate fragments. The average ratio of the specific activity in the two types of fragments (nucleate/anucleate) was 1.35. This value is almost the same as that obtained in previous experiments of Brachet and Szafarz on ^{14}C–orotic acid incorporation (21).

It should be added that the chloroplasts might well be the main site of incorporation of ^{14}C-adenine in the cytoplasm during regeneration. This is suggested by Fig. 5. Significant differences in the time course of labeling bebetween chloroplastic and whole cytoplasmic RNA were found neither in nucleate nor anucleate fragments. It was observed, furthermore, that the total radioactivity of the RNA extracts from the microsomal and supernatant fractions is negligibly small when compared with that of the chloroplasts in nucleate fragments; furthermore no radioactivity at all could be detected in

the RNA of these fractions in anucleate fragments. It can therefore be concluded that ^{14}C-adenine incorporation into RNA of anucleate fragments essentially takes place in chloroplasts.

Vanderhaeghe has reported that ^{14}C-adenine is more rapidly incorporated into the RNA of the nucleolus of *Acetabularia* than into the RNA of cytoplasmic elements (22). Stich and Hämmerling have also observed a rapid incorporation of ^{32}P into the RNA of the nucleolus of *Acetabularia* (23, 24). A number of other recent investigations suggest that part of the nuclear RNA can move into the cytoplasm. It is possible, therefore, that some of the label-

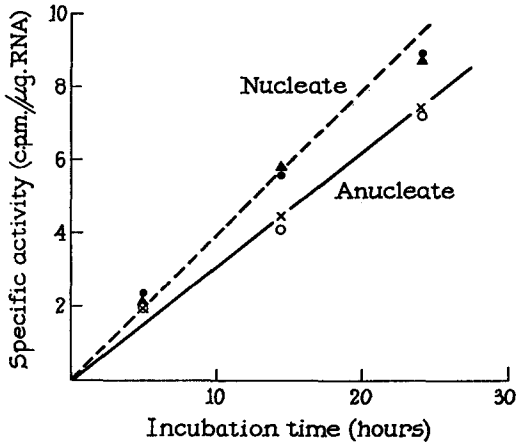

FIGURE 5. ^{14}C-8-adenine incorporation into the whole cytoplasmic and the chloroplastic RNA of anucleate and nucleate fragments of *Acetabularia*. Ten days after operation, both anucleate and nucleate fragments were incubated in presence of ^{14}C-8-adenine for 5, 14.5, and 24 hours under illumination. ▲---▲, whole cytoplasmic RNA of nucleate fragments; ●---●, chloroplastic RNA of nucleate fragments; ×——×, whole cytoplasmic RNA of anucleate fragments, and ○——○, chloroplastic RNA of anucleate fragments.

ing of the cytoplasmic RNA in nucleate fragments might be due to a transfer of RNA from the nucleus; however, such a labeling seems to be quantitatively small in *Acetabularia*.

^{14}C–Orotic Acid Incorporation into Cytoplasmic RNA of Nucleate and Anucleate Fragments

Table V shows clearly that nucleate and anucleate fragments rapidly incorporate ^{14}C–orotic acid into their cytoplasmic RNA. Two days after enucleation, the RNA of anucleate fragments is as strongly labeled as is that of nucleate fragments. Ten days after operation, however, the nucleate fragments incorporate ^{14}C–orotic acid into RNA more rapidly than the anucleate

fragments. A ratio of the specific activities (nucleate/anucleate) of 1.33 was found 10 days after enucleation. This value agrees well with the value previously obtained by Brachet and Szafarz (21).

It is thus confirmed that the cytoplasm is capable of incorporating labeled orotic acid into RNA in the absence of the nucleus.

^{14}C-Carbon Dioxide Incorporation into Acid-Soluble and RNA Purines of Nucleate and Anucleate Fragments

The purpose of these experiments was to determine, first, whether a *de novo* synthesis of RNA occurs in the cytoplasm and, second, if such a synthesis exists, whether such a process is controlled by the nucleus.

TABLE IV

^{14}C-8-ADENINE INCORPORATION INTO CYTOPLASMIC AND CHLOROPLASTIC RNA OF ANUCLEATE AND NUCLEATE FRAGMENTS OF *ACETABULARIA**

Experiment No.	Fraction	Nucleate fragments	Anucleate fragments	Ratio (N/A)
I	Chloroplastic	5.34	3.70	1.44
II	Chloroplastic	8.03	5.62	1.43
III	Chloroplastic	8.92	7.24	1.23
	Whole cytoplasmic	8.77	7.36	1.19
				Average ratio 1.32

* Ten days after enucleation, the fragments were incubated in presence of the labeled adenine for 24 hours under illumination (see text). The values are expressed as the specific activity (C.P.M./μg. of RNA).

Two separate experiments, using cells which had been cultured under different culture conditions (see *Materials*), were carried out.

The results are shown in Table VI. In Experiment I, ^{14}C incorporation into acid-soluble purines did not considerably change in anucleate and nucleate fragments after operation; however, in anucleate fragments, the values of the relative specific activity for guanine were slightly higher than were those for adenine. But, the figures obtained in Experiment II are somewhat different from those of Experiment I: anucleate fragments incorporated ^{14}C into their purines more slowly than did nucleate fragments 1 day after operation. Twenty days after enucleation, a decreased incorporation was found in both kinds of fragments; but there was still no difference in ^{14}C incorporation between the nucleate and anucleate fragments. In general, ^{14}C was more rapidly incorporated into guanine than adenine in the anucleate

fragments. $^{14}CO_2$ incorporation into RNA, as can be seen in Table VI, could always be observed in both anucleate and nucleate fragments.

In the 2 day estimations of Experiment I, a somewhat more rapid incorporation into the RNA of the anucleate fragments than into that of the nucleate fragments was found. Twenty days after operation, the incorpora-

TABLE V

^{14}C-6-OROTIC ACID INCORPORATION INTO CYTOPLASMIC RNA OF *ACETABULARIA**

Experiment No.	Time after operation	Nucleate fragments	Anucleate fragments	Ratio (N/A)
	days			
I	2	6.71	6.69	1.00
II	2	2.40	2.47	0.97
	10	2.68	2.02	1.33

* The fragments were incubated in presence of the labeled orotic acid for 24 hours under illumination (see text). The values are expressed as the specific activity (C.P.M./μg. of RNA).

TABLE VI

^{14}C–CARBON DIOXIDE INCORPORATION INTO ACID-SOLUBLE PURINE COMPOUNDS AND RNA-PURINES OF ANUCLEATE AND NUCLEATE FRAGMENTS OF *ACETABULARIA**

Experiment No.	Time after operation	Nucleate fragments				Anucleate fragments			
		Adenine		Guanine		Adenine		Guanine	
		Acid-soluble	RNA	Acid-soluble	RNA	Acid-soluble	RNA	Acid-soluble	RNA
	days								
I-*a*	1	1883	—	—	—	1540	87.3	—	—
I-*b*	2	1408	77.3	1720	35.4	1288	89.0	1747	45.5
	10	2025	—	—	—	1553	—	—	—
	20	1836	219	1862	110	1674	92.4	2240	62.4
II	1	1420	102	1240	88.5	607	63.4	913	35.7
	20	489	121	642	116	482	65.9	658	51.1

* The fragments were incubated in the presence of ^{14}C-labeled carbon dioxide for 24 hours under illumination (see text) The values are expressed as the relative specific activity:
(C.P.M./μM adenine or guanine/C.P.M./ml. of sea water) $\times 10^4$

tion into the individual purines of RNA in the nucleate fragments was approximately twice that found in anucleate fragments. It was observed in Experiment II, however, that ^{14}C was more rapidly incorporated into the RNA purines of nucleate fragments than into those of anucleate fragments even 1 day after operation. Twenty days after operation, the values obtained for nucleate fragments were twice those found for the anucleate fragments, as also found in Experiment I.

But, because of the lack of uniformity in the sensitivity of the organisms to injury after the operation, in recovery from such injury, and in the following regeneration, no direct comparison of the figures obtained in the two experiments is feasible.

One can conclude, however, from the results obtained in Experiments I and II, that a process of *de novo* synthesis of RNA takes place in the cytoplasm after removal of the nucleus.

Calculations of the specific activity ratio, RNA purines/acid-soluble purines, were also made (Table VII). The absolute values showed considerable variation between the two different experiments; but, with the exception of

TABLE VII

COMPARISON OF ^{14}C-CARBON DIOXIDE INCORPORATION INTO RNA-PURINES BY ANUCLEATE AND NUCLEATE FRAGMENTS OF *ACETABULARIA**

Experiment No.	Time after operation	Nucleate fragments		Anucleate fragments	
		RNA-adenine	RNA-guanine	RNA-adenine	RNA-guanine
	days				
I-*a*	1	—	—	5.67	—
I-*b*	2	5.49	2.06	6.91	2.60
	20	11.9	5.91	5.52	2.79
II	1	7.18	7.14	10.4	3.91
	20	24.7	18.1	13.7	7.77

* The values are expressed as follows:—

$$\frac{\text{C.P.M.}/\mu\text{M adenine or guanine of RNA}}{\text{C.P.M.}/\mu\text{M adenine or guanine of acid-soluble fraction}} \times 10^2$$

the guanine determination in nucleate fragments at 1 day after operation, the over-all results can be stated as follows: incorporation into RNA adenine is higher (approximately twice) than into RNA guanine in both anucleate and nucleate fragments. It was also observed that, immediately after enucleation, anucleate fragments are slightly more capable of incorporating ^{14}C into RNA adenine and guanine than are nucleate fragments. On the other hand, significantly higher specific activity ratios of purines were observed in nucleate fragments at 20 days after operation than in the anucleate fragments.

In conclusion, these experiments confirm the existence of a probably significant *de novo* synthesis of RNA in anucleate fragments; but the synthesis of RNA is stronger in the regenerating nucleate fragments.

DISCUSSION

As shown above, the absorption spectrum of the chloroplastic RNA fraction shows a ratio E_{260}/E_{230} greater than 2.8. Absorption spectra of nucleic acids

are dependent upon their purine and pyrimidine contents and may therefore vary from tissue to tissue, and possibly from one subcellular fraction to another within a cell. We believe, however, that the absorption spectra of the chloroplastic RNA fraction are a satisfactory index of the RNA content of this fraction in *Acetabularia*, since they are very similar to the spectra of pure ribonucleotide solutions in a molar ratio given by Volkin and Carter (25). One can thus conclude that the chloroplastic RNA fraction contains only negligible amounts of ultraviolet-absorbing contaminants.

We shall now discuss the work which has been done on RNA synthesis in anucleate cytoplasm. One of us and his colleagues have previously concluded, from determinations of the RNA content of anucleate and nucleate *Acetabularia* fragments by the ^{14}C-adenine isotope dilution method, that anucleate fragments are capable of synthesizing RNA, and that this RNA synthesis is faster in anucleate than in nucleate fragments during the 1st week after operation (3). With the ultraviolet absorption procedure used here in order to estimate the RNA content, no significant change in the total RNA content of anucleate fragments could be found after removal of the nucleus. This discrepancy in the results is probably not due to the different procedures which were employed in order to measure the RNA content in the respective experiments (9) and the reason for it is not yet clear. It is likely that biological differences (culture conditions, strains, etc.) are mainly responsible for these discrepancies: in the experiments reported in the present paper, a slight increase (in Experiment II, Table I), a decrease (in Experiments VI and VII, Table I), or no change (in Experiments I, III, IV, and V, Table I) in the RNA content of the anucleate fragments during regeneration was observed. The factors responsible for such a fluctuation in the results are probably the different physiological conditions characteristic of the various batches of the material used in each experiment: even though each batch of *Acetabularia* was cultivated and maintained under apparently constant and well defined conditions, the amount of regeneration after enucleation differed from one batch to another.

Even though a net increase in the total RNA content of anucleate fragments could not be detected, the possibility of independent RNA synthesis in the cytoplasm still remains. There are two reasons for this conclusion: first, anucleate fragments can incorporate radioactive precursors into their RNA as well as nucleate ones can; second, a net synthesis of chloroplastic RNA was found in anucleate fragments.

We shall now discuss the results of our experiments on the incorporation of radioactive adenine, orotic acid, and CO_2 into RNA.

Our experimental results show that anucleate fragments of *Acetabularia* can readily incorporate ^{14}C-adenine and orotic acid into their RNA. Immediately after operation, no significant difference in the labeling between the nucleate

and anucleate fragments can be found. Furthermore, 10 days after operation, the differences which are found are still slight. These findings clearly indicate that anucleate cytoplasm is still capable of incorporating ^{14}C-adenine and orotic acid into its RNA, and that removal of the nucleus does not necessarily lead to a rapid block of the mechanisms for RNA synthesis.

Furthermore, our experiment on $^{14}CO_2$ incorporation shows that labeling of acid-soluble adenine and guanine is as efficient in anucleate as in nucleate fragments (with the exception of the result obtained for 1 day in Experiment II, Table VI). Under our experimental conditions, rapid labeling of the purines of the cytoplasmic RNA occurred in both nucleate and anucleate fragments. It is possible that this labeling of acid-soluble and RNA purines corresponds to their *de novo* synthesis rather than to partial exchanges of ^{14}C in the molecule.

The results obtained in the present experiments therefore strongly suggest that a process of *de novo* synthesis of RNA still takes place in the cytoplasm after removal of the nucleus. The first stage of the process, from $^{14}CO_2$ to ^{14}C-labeled acid-soluble purine compounds, is not significantly controlled by the nucleus. The second part of the process; *i.e.*, the incorporation of acid-soluble purine compounds into RNA, is remotely, but not directly, controlled by the nucleus.

Let us turn now to the second type of evidence supporting the hypothesis of independent RNA synthesis in the cytoplasm.

The results obtained in the present experiment show that the large granules (mainly chloroplasts) contain approximately 80 per cent of the cytoplasmic RNA in normal *Acetabularia*, and that the remainder is associated with small particles and the supernatant. It was recently reported by Brawerman *et al.* (18) that chloroplasts of *Euglena gracilis* contain only a small fraction of the total RNA of the cell and that more than half of the total RNA is associated with small particles and the supernatant. The distribution of RNA in the chloroplast fraction of *Acetabularia* is thus very different from that which was found in *Euglena*. But RNA determinations on the chloroplast fraction of anucleate fragments of *Acetabularia* provide interesting results bearing on the problem of independent synthesis of cytoplasmic RNA: an immediate increase of the chloroplastic RNA content and a concomitant disappearance of microsomal and supernatant RNA after removal of the nucleus are found. It is thus possible that, even though no significant increase in the total RNA content of anucleate fragments after enucleation can be observed, chloroplasts (and perhaps other large granules) autonomously synthesize RNA without the intervention of the nucleus. *The microsomal and soluble RNA's might thus be more dependent on nuclear function* than chloroplastic RNA. Furthermore, biochemical experiments on the incorporation into the RNA of chloroplasts and of the whole cytoplasm suggest that there is no penetration of microsomal and

soluble RNA into chloroplastic RNA after enucleation. It can thus be concluded that, in *Acetabularia, chloroplastic RNA can be synthesized in the absence of the nucleus.* Such a synthesis takes place quite normally in the presence of 8-azaguanine.

It is conceivable, of course, that the observed synthesis of chloroplastic RNA in the absence of the nucleus takes place at the expense of microsomal and soluble RNA's.

The results of the present investigation suggest that net RNA synthesis takes place in chloroplasts in the absence of the nucleus, perhaps at the expense of microsomal RNA; it is therefore not surprising that a net synthesis of RNA has not been found to occur in the cytoplasm of cells where the RNA is mainly associated with small granules (microsomes).

It has been demonstrated several times that a net protein synthesis takes place in the cytoplasm of *Acetabularia* after enucleation (2, 3). This finding strongly suggested that protein synthesis in the cytoplasm is not under the direct control of the nucleus. Recently Clauss measured the protein content of chloroplasts of *Acetabularia* using a different isolation medium and found an increase of chloroplastic proteins after removal of the nucleus, although the rate of synthesis of these chloroplastic proteins was greater in nucleate than in anucleate fragments (26). This result agrees well with the hypothesis of a close link between RNA and protein synthesis in the chloroplasts. In contrast to the decrease of microsomal and soluble RNA's in anucleate *Acetabularia*, however, there is a net increase of soluble (and microsomal) proteins after removal of the nucleus. The experiments of Clauss do not preclude the possibility that some of these proteins were released from the large granules during fractional centrifugation. Further biochemical experiments with soluble proteins are needed to clarify this point.

Some of the results of the *Acetabularia* experiments could be explained by the idea that the large granules in cells are not directly controlled by the nuclear function: it was observed by Clauss recently that the phosphorylase activity of *Acetabularia* increases intensively in the absence of the nucleus. One-third of this enzyme's activity is normally bound to the chloroplasts in *Acetabularia* (27). In contrast, the acid phosphatase activity of the soluble fractions (but not of the chloroplastic fractions) does not increase in the absence of the nucleus. In the small anucleate fragments, it slowly decreases after enucleation (28). Baltus has found increased activity of aldolase in anucleate fragments of *Acetabularia* after enucleation. This enzyme is mainly found in the supernatant and large granule fractions, but not in the microsomal fraction (29). It is possible, however, that, during the fractionation procedure, some of the enzymes found in the supernatant fraction were released from the large granules.

The present situation, regarding the respective role of chloroplasts and other

cell fractions in RNA and protein synthesis by anucleate fragments of *Acetabularia*, thus remains obscure and further experiments are obviously required to reach more definite conclusions.

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