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New Ribonucleic Acid Species Associated with the Formation of the Photosynthetic Apparatus in *Euglena gracilis**

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The conversion of dark-grown *Euglena gracilis* cells, which lack the photosynthetic apparatus, into green organisms under the influence of light is accompanied by a massive synthesis of plastid proteins and a considerable rise in the RNA content of several subcellular fractions. The nucleotide composition of the RNA of the various subcellular fractions was determined, and substantial differences between the ribonucleic acids of homologous fractions from colorless and green cells were recorded. These findings suggest that specific RNA species are produced during the development of chloroplasts.

We have shown in a previous paper (Brawerman and Chargaff, 1959) that the nucleotide composition of the total ribonucleic acid of green *Euglena gracilis* differed significantly from that of the colorless organisms in which chloroplast formation was prevented by the absence of light. In another recent publication (Brawerman *et al.*, in press) we have discussed the evidence that the process through which light induces the formation of chlorophyll-containing plastids in many ways resembles the induced formation of enzymes in micro-organisms. The massive character of the reorganization of the synthetic abilities of the cell leading to the development of chloroplasts made it appear of interest to examine the composition of the ribonucleic acids of various subcellular fractions of *Euglena* cells engaged in what could be considered as a pronounced example of cell differentiation. A brief account of some of the findings has appeared (Brawerman *et al.*, 1961).

EXPERIMENTAL

Cultures.—*Euglena gracilis*, strain z, was grown in the complex medium used before (Brawerman and Chargaff, 1959). Cultures in the stationary phase of growth served for the experiments.

Cell Fractionation.—The washed cell samples were stored at −15° for 2–3 days and then subjected to the fractionation procedure described in a preceding publication (Brawerman *et al.*, in press). The plastid fractions were always collected by centrifugation after removal of the unbroken cells at low speed.

Nucleotide Analysis.—The analytical procedures used have been described before (Brawerman and Chargaff, 1959). The hydrolysis with NaOH was usually performed for 2 days to ensure complete cleavage to nucleotides. Longer incubation at 30° resulted in the extensive deamination of cytidylic acid. The chromatographic separations were all carried out in the customary ammonium isobutyrate solvent. The minor nucleotide fraction moving more slowly than guanylic and cytidylic acids could, in the case of the hydrolysates of the subcellular RNA fractions discussed here, be separated satisfactorily from the other nucleotide components, in contrast to the behavior of the total RNA studied before (Brawerman and Chargaff, 1959). The preparations from the green plastid specimens gave poor chromatograms; but this could be improved by preliminary paper chromatography of the hydrolysate for one day in 1-butanol–0.5 N ammonia (6:1), in which the nucleotides do not move. The preparations from the colorless plastid fractions were, for the sake of uniformity, treated similarly.

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TABLE I

NUCLEOTIDE COMPOSITION OF RNA FROM SUBCELLULAR FRACTIONS OF COLORLESS AND GREEN CELLS[a]

Fraction	Nucleotide	Colorless N	Colorless n	Colorless Composition	Colorless σ	Green N	Green n	Green Composition	Green σ	t	P
Plastids	A	7	8	*22.0*	0.5	8	12	*24.6*	0.7	9.4	≪0.001
	G	7	8	29.4	0.9	8	12	29.1	1.1		
	C	6	7	*27.1*	0.7	8	12	*24.4*	1.0	6.4	<0.001
	U	6	7	21.5	1.2	8	12	21.9	1.0		
Mitochondria	A	3	6	21.8	0.5	3	6	22.6	1.1	1.6	0.3
	G	3	6	28.2	0.3	3	6	28.0	0.8		
	C	3	6	25.6	0.7	3	6	24.7	0.7	2.3	0.08
	U	3	6	21.7	0.7	3	6	22.3	0.7	1.5	0.3
	Mi	3	6	2.7	0.2	3	6	2.4	0.5		
Microsomes	A	4	9	*21.6*	0.7	4	11	*22.6*	0.3	3.4	0.01
	G	4	9	*28.7*	0.6	4	11	*26.8*	0.3	7.3	<0.001
	C	3	7	24.9	0.5	4	11	25.0	0.4		
	U	3	7	21.1	0.4	4	11	21.6	0.8		
	Mi	4	9	3.7	0.7	4	11	4.0	0.6		
Supernatant	A	4	8	*22.3*	1.1	3	6	*24.2*	0.8	3.5	0.01
	G	4	8	28.2	0.7	3	6	27.8	0.7		
	C	3	6	*26.4*	1.0	3	6	*24.3*	1.0	3.7	0.01
	U	3	6	*19.0*	0.5	3	6	*20.8*	0.7	5.2	0.005
	Mi	4	8	4.1	0.7	3	6	2.9	0.2		

[a] Abbreviations: A, adenylic acid; G, guanylic acid; C, cytidylic acid; U, uridylic acid; Mi, minor, slow-moving nucleotide fraction described before (see text for discussion). N, number of preparations; n, total number of analyses (every analysis consisting of 6 chromatographic determinations); *t*, value obtained by the *t* test (the mean values for each preparation were used in this test); P, probability that the means are not different; σ, standard deviation. Significantly different values ($P \leq 0.01$) are set in italics.

RESULTS

Ribonucleic Acids of the Subcellular Fractions of the Colorless Cells.—A comparison of the values in Table I shows the ribonucleic acids of the various fractions of the colorless cells to vary somewhat with respect to their cytidylic acid content. This nucleotide is present in lower amounts in the mitochondrial and microsomal fractions; it is highest in the plastid fraction. Another characteristic of the latter fraction is the absence of the unidentified RNA component found previously in whole-cell RNA (Brawerman and Chargaff, 1959). This material, originally designated "Compound VI," is present in greatest amounts in the microsomal fraction. In the supernatant, the slow component, as isolated by paper chromatography, also contained a relatively large amount of pseudouridylic acid. This is indicated by the considerable bathochromic shift in the UV spectrum in alkali (Davis and Allen, 1957). No such shift was observed with "Compound VI" obtained from the mitochondrial and microsomal ribonucleic acids. In Table I, the minor fraction moving more slowly in the isobutyrate solvent than uridylic and guanylic acids is, in all cases, referred to as Mi.

The supernatant RNA also contains considerably less uridylic acid. This fraction must include the soluble RNA component that acts as acceptor for the activated amino acids (Hoagland, 1960). Isolated soluble RNA from *Euglena*, which will be discussed separately in a different context, showed, however, a nucleotide composition very different from that of the supernatant nucleic acid preparation described here. It is, therefore, likely that the supernatant fraction comprises more than one type of RNA. The mitochondrial and microsomal RNA preparations are very similar in composition, except perhaps for a lower amount of "Compound VI" in the mitochondria. It is possible that part of the RNA of the latter fraction derives from adsorbed microsomes and that "Compound VI" is present only in microsomal RNA.

The 6-amino to 6-keto ratio (Elson and Chargaff, 1955) is close to unity in the plastid fraction. It cannot easily be determined in the other fractions because of the presence of the minor components. In the mitochondrial and microsomal fractions, the ratio would be unity if "Compound VI" were to be included with the 6-amino components. In the supernatant fraction, the pseudouridylic acid which accompanies "Compound VI" should probably be added to the 6-keto components, but since the amount present is as yet unknown, the actual ratio cannot be computed.

Ribonucleic Acids of the Subcellular Fractions of the Green Cells.—The various subcellular fractions of the green cells, except for the mitochondria, have an RNA nucleotide composition significantly different from that of the corresponding fractions from the colorless organisms (see Table I). Only those differences with a P value not higher than 0.01 (*t* test according to Goulden, 1952) are considered significant. At this level of significance, differences in nucleotide content of 1 mole % are barely detectable with the analytical method used here. Owing to the relatively small spread of values for the adenylic acid content of the microsomal RNA (Table I), the observed difference of 1 mole % still appears to be significant.

When the changes in nucleotide composition, summarized in Figure 1, are surveyed, there appears to be a striking similarity between the shifts in the plastid and those in the supernatant fractions. Both show an increase in adenylic acid and a drop in cytidylic acid. The uridylic acid, however, increases only in the supernatant fraction. The RNA of the microsomes changes in a rather different manner. The major shift is in the guanylic acid, which remains unchanged in the three other fractions.

Validity of Data on RNA Composition in Subcellular Fractions.—The composition of the total

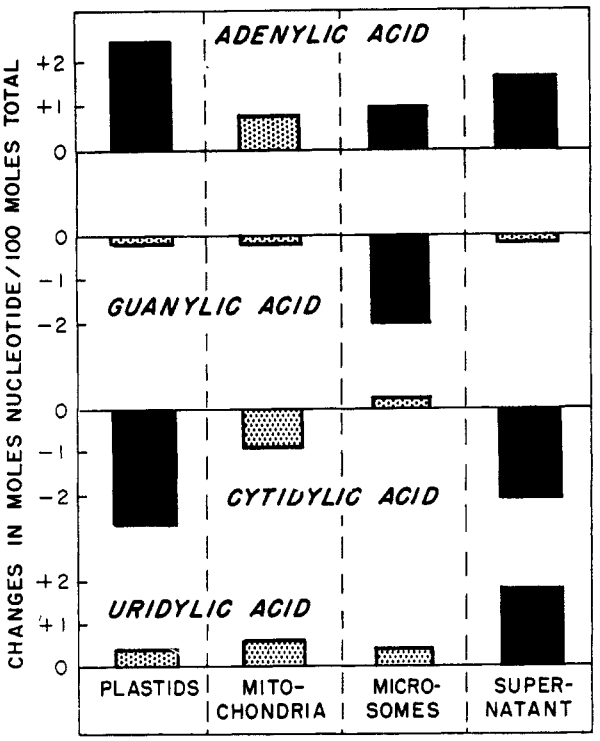

FIG. 1.—Shifts in the nucleotide composition of the ribonucleic acid fractions of *Euglena* accompanying the appearance of the photosynthetic apparatus. The bars represent the changes observed when the RNA of green cells is compared with that of the colorless cells. The black bars indicate differences that are statistically significant ($P \leq 0.01$).

RNA of both green and colorless *Euglena* cells has been reported previously (Brawerman and Chargaff, 1959). From the findings presented in this and a preceding paper (Brawerman *et al.*, in press), the approximate composition of the global RNA of *Euglena* could be reconstructed and compared with that found experimentally before (Table II). The agreement is quite good, despite the considerable uncertainty applying to such a comparison; only the values for cytidylic acid diverge, though even here the trends of shifts in composition are similar.

DISCUSSION

When two cellular high polymers—in the present case the proteins and the ribonucleic acids—undergo important chemical changes accompanying a shift in the physiologic condition of the cell, it is difficult to decide whether chemical changes and biological switch are related as are cause and effect or whether they all are brought about by, and symptomatic of, an as yet unrecognized more comprehensive occurrence. When colorless *Euglena* cells are exposed to light, new synthetic activities appear, aiming at the production of chloroplast constituents. In the absence of cell multiplication, the plastid-bound protein formed preferentially corresponds to approximately 40% of the total protein of the colorless cell (Brawerman *et al.*, in press). A profound reorganization of the functions concerned with protein synthesis must have taken place under these circumstances, and it would be tempting—though perhaps premature—to see a connection with the rise in the RNA content of both the plastid and the microsome fractions that precedes the formation of plastid proteins. As we have shown here, this newly formed RNA also is novel with respect to composition; a fact that obviates the necessity of limiting oneself to the indirect study of transient anabolic shifts.

One of the obstacles to a better understanding of the chemistry of the nucleic acids is the lack of suitable methods for the separation of the high polymers that differ in the relative proportions or in the sequential arrangement of their monomeric nucleotide constituents. This obstacle is quite apart from the fact that even the conceptual definition of what constitutes a nucleic acid molecule encounters great difficulties. It is, for this reason, not yet possible to define the molecular basis of the changes described in this paper. Although there exist indications that both supernatant and ribosomal specimens of ribonucleic acid can be fractionated, we are very far from being able to estimate the number of different RNA species present in a cell. The experiments reported in this paper provide little information on the nature of the novel RNA species associated with chloroplast development. The RNA of the plastid fraction appears to be bound to particles, as it is still sedimentable after the chloroplasts have been disrupted. It is also possible that RNA originating from damaged plastids contributes to the changes in the microsome fraction. The shifts in nucleotide composition in the supernatant fraction, analogous in some respect to the changes in the plastids, suggest the presence of nonparticulate RNA species related to those appearing in the plastids.

The presence of novel RNA species both in soluble and in particulate form in green *Euglena* cells is perhaps reminiscent of the events taking place in *Escherichia coli* during bacteriophage infection. In the latter process a new evanescent RNA species appears which has been identified with the "messenger RNA," part of it bound to the ribosomes and the rest free in the supernatant (Nomura *et al.*, 1960; Brenner *et al.*, 1961; Gros *et al.*, 1961). There can be little doubt, however, that a shift in the small population of short-lived "messengers" cannot be a sufficient explanation for the massive changes observed by us here in the RNA of several

TABLE II

NUCLEOTIDE COMPOSITION OF WHOLE CELL RNA DETERMINED EXPERIMENTALLY AND COMPUTED FROM THE VALUES FOR THE FRACTIONS

	Determined Experimentally[a]			Reconstructed[b]		
	Colorless	Green	Shifts	Colorless	Green	Shifts
A	21.9	23.2	+1.3	21.9	23.2	+1.3
G	28.6	26.5	−2.1	28.6	27.5	−1.1
C	27.8	26.9	−0.9	25.5	24.7	−0.8
U	19.2	20.8	+1.6	20.7	21.5	+0.8
Compound VI	2.5	2.6	+0.1	3.3	3.1	−0.2

[a] Values obtained previously from KOH hydrolysates of whole-cell preparations, chromatographed with the ammonium isobutyrate solvent (Brawerman and Chargaff, 1959). [b] Based on previous findings on the RNA content of the various cellular fractions (Brawerman *et al.*, in press), and on the composition studies presented in Table I.

subcellular fractions as well as previously in the global RNA of green *Euglena* as compared with the colorless variety (Brawerman and Chargaff, 1959).

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Studies on Polynucleotides. XV.* Enzymic Degradation. The Mode of Action of Pancreatic Deoxyribonuclease on Thymidine, Deoxycytidine, and Deoxyadenosine Polynucleotides†

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The degradation by crystalline pancreatic deoxyribonuclease of synthetic homologous thymidine, deoxycytidine, and deoxyadenosine polynucleotides and of thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-deoxycytidine (de-TpApC) has been studied. Divalent ion activation, which was necessary, was provided by manganous ions, the manganous ions being about twice as effective as magnesium ions, in agreement with previous results. Under the standard conditions used (a large amount of enzyme was employed), all of the tetranucleotides and higher homologues bearing 5′-phosphomonoester end-groups were attacked. The di- and trinucleotides bearing 5′-phosphomonoester groups and de-TpApC were resistant under the conditions used. The mode of action was identical in all the series bearing 5′-phosphomonoester end-groups. Cleavage occurred mostly at internal bonds; thus, the tetranucleotides gave mainly dinucleotides; pentanucleotides gave mainly di- and trinucleotides; hexanucleotides gave initially di-, tri-, and tetranucleotides. With the oligonucleotide de-TpTpTpTpTp, which bears a 3′-phosphomonoester end-group, cleavage occurred, in addition, at the terminal linkage so as to release de-pTp and de-TpTpTpT, indicating that the 3′-phosphomonoester group simulates a phosphodiester bond for the enzymic action. That attack occurs equally well at multiple points was shown by studying the substrates de-pTpTpTpTpC and de-pT[pT]$_3$pT. The former gave de-pTpT and de-pTpTpC as well as de-pTpTpT and de-pTpC, while the latter after partial degradation gave all the possible lower homologues. In each series of homologous compounds, the rate of degradation increased with increase in chain length. Of the three analogous pentanucleotides, de-pTpTpTpTpT, de-pCpCpCpCpC, and de-pApApApApA, the last was attacked faster than the pyrimidine analogues. The mode of action of the enzyme on polynucleotides and on DNA is discussed in the light of the present findings.

The action of crystalline pancreatic deoxyribonuclease on deoxyribonucleates (DNA) has been studied by a large number of investigators. Both the kinetics of its action and the composition of the ultimate digestion products have received attention (for a recent review see Laskowski, 1961). Detailed analyses of the products have shown them to consist of about 1% mononucleotides (nucleoside-5′-phosphates), 13–18% dinucleotides, and the remainder a complex mixture of higher oligonucleotides (Sinsheimer, 1954, 1955). The main clearly established feature of the action of the enzyme is that all the products bear 5′-phosphomonoester end-groups. Any further questions regarding the mode of action of the enzyme have remained unanswered, however, although certain interpretations concerning preferential specificity toward purine or pyrimidine bonds have been advanced (Laskowski, 1961; Vanecko and Laskowski, 1961). These conclusions have been derived from studies of (a) the composition of the dinucleotide mixture

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