Appearance of a Methylated Ribonucleic Acid on Illumination of *Euglena gracilis* Cells Grown in the Dark

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Investigation of the methylation of nucleic acids by [$Me\text{-}^3H$]methionine after illumination of *Euglena* cells grown in the dark has shown that a high-molecular-weight nucleic acid fraction undergoes methylation after exposure to light for 60–120 min. This methylated nucleic acid fraction was isolated both by sucrose-density-gradient centrifugation and exclusion chromatography on Sephadex G-200. The fraction was shown to consist of a preformed RNA that is present in cells grown in the dark and which on illumination is transmethylated by methionine.

The presence and function of methylated bases found in nucleic acids have been intensively investigated (Baliga, Srinivasan & Borek, 1965; Comb, 1965; Revel & Littauer, 1965) and several review articles have been published (Borek, 1963; Srinivasan & Borek, 1964, 1966; Borek & Srinivasan, 1966).

The possibility that methylated bases are involved in protein synthesis has been examined in studies comparing tRNA poor in methylated bases with normal tRNA (Littauer, Muench, Berk, Gilbert & Spahr, 1963; Neidhart & Eidlic, 1963; Revel & Littauer, 1965). Little attention has been given to the methylation of high-molecular-weight RNA.

Illumination of *Euglena* cells grown in the dark causes a biological differentiation process in which a considerable *de novo* synthesis of nucleic acids and proteins occurs (Pogo, Brawerman & Chargaff, 1961, 1962). In the present paper I report investigations of the problem of the appearance of nucleic acid fractions enriched with methylated bases under such conditions.

MATERIALS AND METHODS

Growth and labelling conditions. *Euglena gracilis* (var. *bacillaris*) cells were grown in 1 litre Erlenmeyer flasks containing Hutner's medium (Hutner, 1956), pH 3.0, kept at 22°C in a rotatory shaker at 80 rev./min under a 300 ft-candle/cm² fluorescent light. Cells grown in darkness were cultivated under identical conditions, but the flasks were wrapped with 2–3 layers of aluminium foil to exclude light. All transfer manipulations of the cells grown in darkness were performed in complete darkness with cell cultures that had been cultivated in the dark for over 1 year. To 100 ml of a *Euglena* cell culture in its logarithmic

phase of growth in the dark (4–5 days of growth, containing 6×10^4 – 8×10^4 cells/ml) 200 μ Ci of [$Me\text{-}^3H$]methionine (specific radioactivity 150 mCi/mmol) was added. One half of the cells was then exposed to light and the other half was kept in darkness. Both groups of cells were then returned to the rotatory shaker.

Cold-hot trichloroacetic acid extraction of labelled nucleic acids. Samples (3 ml) were simultaneously removed at known times from the two groups of cells and the amount of labelled methyl groups incorporated into the nucleic acids was determined. The cells were harvested by centrifugation at 15000g for 10 min and the packed cells were washed twice with the growth medium. The pellets were suspended in 3 ml of ice-cold 5% (w/v) trichloroacetic acid and kept in ice for another 10 min. The cells were re-centrifuged at 15000g for 10 min then the precipitate was resuspended in 1 ml of 5% (w/v) trichloroacetic acid, which was then boiled for 5 min. The supernatant obtained after a third centrifugation at 15000g for 5 min contained the nucleic acid fraction. The supernatant was analysed for nucleic acids by measurement of the E_{260} against 5% (w/v) trichloroacetic acid. Incorporation of 3H was measured by determination of radioactivity in Bray's (1960) solution in a Packard Tri-Carb liquid-scintillation counter.

Isolation of nucleic acids from *Euglena* cells. *Euglena* cells from a 1 litre culture flask were harvested by centrifugation at 3000 rev./min for 5 min at 0–4°C. They were washed with TMA buffer (0.1 M-tris-HCl buffer, pH 7.4, containing 0.001 M-magnesium acetate). They were resuspended in 30 ml of the same buffer. After addition of a sodium dodecyl sulphate solution to a final concentration of 1%, the cells were broken in a French pressure cell at 10000 lb/in² and then transferred directly to 30 ml of ice-cold 70% (w/v) phenol. The mixture was shaken for 1 h at 4°C, after which it was centrifuged at 15000g for 10 min. The nucleic acids were precipitated by adding 2.5 vol. of ice-cold ethanol to the supernatant. The precipitate obtained by centrifugation at 15000g for 10 min, was washed twice with ice-cold 70% (v/v) ethanol

and finally dissolved in 2 ml of TMA buffer. The RNA was treated with purified deoxyribonuclease ($5 \mu\text{g/ml}$) for 15 min at 37°C and reprecipitated by ethanol.

Sucrose-density-gradient analysis. For RNA fractionation 4–20% linear sucrose gradients were prepared in 0.1 M-NaCl. A portion (0.2 ml) of RNA was layered over 4.2 ml of the gradient and was then centrifuged in a Spinco SW39 rotor at 39000 rev./min for 2 h at 4°C . Two-drop fractions, collected from the bottom of the tubes, were diluted with 1 ml of water before measurement of the E_{260} . For determination of incorporation of labelled methyl groups, 0.5 ml of each fraction was mixed with Bray's solution and the radioactivity counted.

Preparation of Sephadex G-200 columns. Columns of Sephadex G-200 ($25 \text{ cm} \times 1.5 \text{ cm}$) were equilibrated overnight with 0.1 M-NaCl. A 1 ml sample of the nucleic acid fraction was placed on the column and was eluted with 0.1 M-NaCl. Fractions (5 ml) were collected. The nucleic acid content was determined by measuring the E_{260} , and incorporation of labelled methyl groups by counting the radioactivity.

RESULTS AND DISCUSSION

Study of the kinetics of methylation of nucleic acids prepared by the cold-hot trichloroacetic acid method.

Examination of the nucleic acid fraction prepared by the cold-hot trichloroacetic acid method shows that illumination of *Euglena* cells grown in the dark in the presence of [$\text{Me-}^3\text{H}$]methionine causes the appearance of a methylated nucleic acid fraction, the amount of which steadily increases during the initial 60 min of exposure to light, but which then decreases (Fig. 1). The maximal rate of incorporation of labelled methyl groups is obtained after about 50 min illumination. Although there is also an incorporation of radioactive methyl groups in cells kept in the dark, the extent of labelling in the illuminated cells is 3 times greater than that of cells kept in the dark.

The results in Table 1 show that, if the incorporation of methionine is assumed to give a measure of methionine uptake, then there is an increase in

Fig. 1. The methylation of the nucleic acid fraction, in *Euglena* cells grown in the dark and during illumination. A dark and an illuminated *Euglena* culture were exposed to equal amounts of [$\text{Me-}^3\text{H}$]methionine. From each culture a 3 ml sample of cells was harvested at the indicated times. The cells were washed with cold 5% (w/v) trichloroacetic acid. The nucleic acids were extracted with hot trichloroacetic acid and examined for radioactivity. ○, Cells grown in the dark; ●, cells grown in the light.

Table 1. Methylation of RNA on illumination of *Euglena* cells grown in the dark

Euglena cells grown in the dark were exposed to light under normal growing conditions. At the indicated times 10 ml samples of cells were transferred to 50 ml flasks and $2 \mu\text{Ci}$ of [$\text{Me-}^{14}\text{C}$]methionine was added. After 20 min of incubation, the cells were centrifuged, washed and subjected to the cold-hot trichloroacetic acid procedure. The nucleic acid fraction (hot trichloroacetic acid supernatant) and the protein fraction (hot trichloroacetic acid precipitate) were counted for ^{14}C radioactivity.

Time after illumination (min)	Radioactivity (d.p.m./ 10^6 cells)		Radioactivity in nucleic acid fraction (% of total radioactivity incorporated)
	Nucleic acid fraction	Protein fraction	
In dark	5100	16200	24
0	8400	14800	37
30	7700	14400	35
60	6200	15000	29
120	5400	13600	28

Fig. 2. Fractionation pattern of RNA methylated by $[Me-^3H]$ methionine in illuminated *Euglena* cells grown in the dark. $[Me-^3H]$ Methionine (200 μCi) was added to 1 litre of *Euglena* culture grown in the dark then the cells were exposed to growth conditions in light. After 50 min the RNA was extracted by treatment with sodium dodecylsulphate, phenol and ethanol. About 5.0 E_{260} units in 0.3 ml were fractionated on a 4–20% linear sucrose gradient (a) and about 30.0 E_{260} units were fractionated on a Sephadex G-200 column (b). ●, Radioactivity; ○, E_{260} .

methylation of RNA during the first hour of illumination.

The decrease of the total incorporation of the labelled material can be explained by the fact that illumination of the cells causes a large free amino acid pool in the cell. Amino acid analysis of equal protein concentrations in the 100 000g supernatants shows that the amount of free amino acids is 2–3-fold higher during the early illumination of cells (up to 2 h) than in the cells grown in the dark.

Fractionation of ^3H -labelled nucleic acids by sucrose-density-gradient centrifugation and exclusion chromatography. The profiles of ^3H -labelled methylated RNA obtained after 50 min of incubation are shown in Fig. 2. The S value of the labelled fraction indicates that this methylation does not occur in the whole rRNA but in a unique high-molecular-weight nucleic acid fraction. Nucleic acids were tested for DNA and RNA content by enzyme treatment and subsequent filtration (Millipore H.A., 0.45 μm). When the labelled nucleic acid fraction was treated with ribonuclease (10 $\mu\text{g}/\text{ml}$ at 37°C for 10 min) and the undigested nucleic acids were precipitated with cold trichloroacetic acid, only 10% of the labelled material remained undigested and was retained by

the filter. After treatment of the same nucleic acid fraction with deoxyribonuclease all of the radioactivity remained in the undigested portion on the filter, clearly establishing that the $Me-^3\text{H}$ -labelled nucleic acid fraction is RNA.

Binding of methionine to RNA or transmethylation to bases in RNA. The question now arises as to whether the $Me-^3\text{H}$ -labelled fraction contains complete methionine bound directly to RNA, or whether only the methyl groups had been transferred by transmethylation from methionine to bases present in the RNA. To answer this question, the extent of incorporation of $[^3\text{S}]$ methionine was compared with the extent of incorporation of $[Me-^3\text{H}]$ -methionine into the labelled fraction of the nucleic acids. *Euglena* cells grown in the dark were incubated during 60 min illumination, simultaneously with 70 μCi of $[^3\text{S}]$ methionine (specific radioactivity 69.8 mCi/mmol) and 150 μCi of $[Me-^3\text{H}]$ -methionine (specific radioactivity 150 mCi/mmol), i.e. 1 μmol of each radioactive compound. The nucleic acid fraction was extracted and isolated as described above and the double labelling was measured after either sucrose-density-gradient or Sephadex G-200 column separation. Fig. 3 (a and b)

Fig. 3. Methylation and methionine incorporation measured with $[Me-^3H]$ methionine and $[^{35}S]$ methionine. To 100 ml of *Euglena* culture grown in the dark 1 μ mol each of $[^{35}S]$ methionine and $[Me-^3H]$ methionine were added. After 50 min of growth in the light the RNA was extracted. (a) About 3000 c.p.m. of the 3H was fractionated on a 4–20% sucrose density gradient. Two-drop fractions were collected in counting vials and counted for 3H (●) and ^{35}S (○) radioactivity. (b) About 3000 c.p.m. of 3H was fractionated on a Sephadex G-200 column and the fractions were counted for 3H (●) and ^{35}S (○) radioactivity.

Fig. 4. Methylation of RNA and nucleic acid synthesis *de novo* after 50 min of illumination of *Euglena* cells grown in the dark. A *Euglena* culture (400 ml) grown in the dark was exposed to light in the presence of a final concentration of 3.0 μ M- $[Me-^3H]$ methionine and 1 mM- $[^{32}P]P_i$. About 3000 c.p.m. of 3H was fractionated on a 4–20% sucrose density gradient (a), and about 25000 c.p.m. were fractionated on a Sephadex G-200 column (b). The fractions were counted for ^{32}P (○) and 3H (●) radioactivity.

shows that only the high-molecular-weight nucleic acid fraction was labelled by ^3H whereas the ^{35}S labelling appeared only in the low-molecular-weight tRNA fraction. After this ^{35}S -labelled tRNA was treated with 0.01 M-hydroxylamine, pH 8.4 at 60°C for 30 min the nucleic acid fraction precipitated by ethanol contained practically no ^{35}S radioactivity. This treatment did not affect the high-molecular-weight RNA labelled by ^3H . These results indicate that a transmethylation reaction occurs in the high-molecular weight RNA and not a methionine incorporation in the nucleic acid during illumination of *Euglena* cells grown in the dark.

Incorporation of [Me- ^3H]methionine into pre-existing and newly synthesized RNA after illumination of Euglena cells grown in the dark. Previous investigations (Peterkofsky, Jesensky, Bank & Mehler, 1964) have shown that methylation of nucleic acids takes place on preformed RNA already present in the cells and not at the level of free nucleotides. The question here, with illuminated *Euglena* cells grown in the dark is similar. Does illumination of *Euglena* cells grown in the dark cause *de novo* synthesis of RNA, which then undergoes methylation, or is pre-existing RNA, already present in cells grown in the dark, methylated on exposure to light? In order to answer this question, the fate of [Me- ^3H]methionine in the presence of ^{32}P -labelled orthophosphate was investigated. A 400 ml sample of *Euglena* cells grown in the dark was incubated in the light with 200 μCi of [Me- ^3H]methionine (specific radioactivity 150 mCi/mmol) and 1 mCi of ^{32}P (since the growth medium contained 3 mM- H_3PO_4 , the specific radioactivity of ^{32}P was 1 mCi/mmol). After illumination for 1 h, the nucleic acids were extracted, separated by sucrose-density-gradient centrifugation or exclusion chromatography and the radioactivity of [Me- ^3H]methionine and [^{32}P]P_i measured as described above. Fig. 4(a and b) shows that the amount of ^{32}P in the ^3H -labelled nucleic acid fraction was very small, indicating that very little RNA synthesis had taken place during the short period of

illumination and that methylation of pre-existing RNA had occurred.

The results show that a methylating process in high-molecular-weight RNA fraction occurs when *Euglena* cells are transferred from a heterotrophic to an autotrophic stage, by illumination. A light-stimulated methylation occurs during the early period of illumination, in a pre-existing RNA molecule. This RNA is not identical with rRNA but may be a part of this fraction.

As the extent of methylation decreases after a short period of illumination it seems that the methylated bases in the high-molecular-weight RNA may be a product of an earlier light activation process, rather than an active factor, as such, in the biological function of the RNA.

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