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Isolation and Identification of Cytokinins from *Euglena gracilis* Transfer Ribonucleic Acid†

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ABSTRACT: Three ribonucleosides responsible for cytokinin activity in *Euglena gracilis* var Bacillaris tRNA have been isolated and identified as 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine, and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. The structures of these compounds were assigned on the basis of their chromatographic properties and ultraviolet and mass spectra which were identical with those of the corresponding synthetic compounds. The elution profiles of cytokinin bioassay activity and of ^{35}S radioactivity suggest the presence of a trace amount of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine.

Cytokinin activity has been reported in tRNA preparations from a wide variety of organisms (Skoog and Armstrong, 1970; Hall, 1973). The cytokinin-active ribonucleosides which have been identified as constituents of tRNA are *c*-io^{6}A,[1] *t*-io^{6}A, i^{6}A, ms^{2}i^{6}A, and ms^{2}io^{6}A. Studies on the distribution of cytokinins in tRNAs from various organisms suggest that i^{6}A and ms^{2}i^{6}A are the two major cytokinins generally found in bacterial tRNAs. However, *t*-io^{6}A has been reported recently in the tRNA of plant pathogen *Agrobacterium tumefaciens* (Chapman et al., 1976). Also ms^{2}io^{6}A has been reported in *Pseudomonas aeruginosa* tRNA preparation (Thimmappaya and Cherayil, 1974). Unlike bacteria, animal tRNAs are reported to contain only i^{6}A (Robins et al., 1967). The predominant cytokinin-active ribonucleoside found in plant tRNA is *c*-io^{6}A. Minor amounts of i^{6}A, ms^{2}io^{6}A, and ms^{2}i^{6}A are also present. Hall et al. (1967) have reported the occurrence of *c*-io^{6}A and i^{6}A in the tRNAs from spinach and peas. The cytokinins responsible for activity in wheat germ tRNA were identified as i^{6}A, io^{6}A, and their thiomethyl derivatives (Burrows et al., 1970). Pea shoot tRNA hydrolysates have been shown to contain i^{6}A, ms^{2}i^{6}A, *c*-io^{6}A, *c*-ms^{2}io^{6}A, and *t*-ms^{2}io^{6}A (Vreman et al., 1972, 1974). Cytokinin-dependent tobacco callus tissue, grown in the presence of synthetic benzylaminopurine (bzl^{6}Ade) to test possible incorporation of this base into tRNA, contained relatively high amounts of *c*-io^{6}A and i^{6}A and a small amount of ms^{2}io^{6}A as well as a trace of bzl^{6}A derived from the exogenous base bzl^{6}Ade (Burrows et al., 1971). Thus the cytokinins in plant tRNAs show greater diversity than those generally found in animal and bacterial tRNAs. The significance of the occurrence of several cytokinins in the tRNAs of a single organism is not known. Since eukaryotes contain organelles such as chloroplasts and mitochondria which have their own transcriptional and transla-

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[1] Abbreviations used: i^{6}A, 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; *c*-io^{6}A, 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine; *t*-io^{6}A, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine; ms^{2}i^{6}A, 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine; ms^{2}io^{6}A, 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine; bzl^{6}A, 6-(benzylamino)-9-β-D-ribofuranosylpurine; bzl^{6}Ade, 6-(benzylamino)purine; CTAB, cetyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetate; KE, kinetin equivalent; Me_3Si, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; UV, ultraviolet; GLC, gas-liquid chromatography.

tional apparatus, it is conceivable that cytokinins found in plant tRNAs might be derived in part from organelle tRNAs. To investigate this possibility *Euglena gracilis* was chosen as the experimental organism. The identification of the cytokinins in its tRNAs is reported in this paper. The subcellar compartmentalization of these cytokinins is reported elsewhere (Swaminathan et al., 1977).

Experimental Section

Growth of Euglena gracilis var Bacillaris. Axenic cultures of *Euglena gracilis* var Bacillaris were obtained from Dr. L. B. Graves of the Botany Department, University of Wisconsin-Madison. Mass cultures were grown in 20-L carboys at 28 °C with constant aeration and at a light intensity of approximately 500 ft-candles. The cells were grown in the medium devised by Cramer and Myers (1952). The cultures were microscopically examined prior to inoculation as well as at harvest. Cells in late log phase at OD_{700} of ~3.0 were harvested in a cooled Sharles centrifuge and immediately frozen and stored at −20 °C.

Isolation of tRNA from Euglena gracilis. The cells were homogenized in a Waring blender in 0.1 M Tris-HCl buffer (pH 7.5) containing 1% sodium dodecyl sulfate and 0.01 M EDTA. The homogenate was extracted with phenol twice and the aqueous phase was precipitated with 2 volumes of ethanol after the addition of one-tenth the volume of 2.5 M potassium acetate. The precipitate was dissolved in 2.5 M potassium acetate (pH 6.0) to extract tRNA. The extract was precipitated with ethanol and redissolved in 0.005 M potassium acetate, pH 6.0. The soluble material was precipitated with 2–3 equiv excess of cetyltrimethylammonium bromide at 4 °C. The CTAB precipitate was solubilized in 0.45 M sodium chloride at 4 °C and tRNA precipitated with ethanol. The CTAB form was converted to sodium form by repeated treatment with 0.4 M sodium acetate, pH 6.0, and ethanol precipitation. The final precipitate was dissolved in 0.1 M Tris-HCl, pH 7.5, containing 0.25 M sodium chloride and 0.01 M magnesium chloride, and loaded on a DEAE column and eluted with the same buffer. The absorbed tRNA was eluted with buffered 1.0 M sodium chloride. The sample eluting with high salt was collected and precipitated with ethanol. Polyacrylamide gel electrophoresis of the preparation showed that it contained mainly 4S RNA. It was free of high molecular weight nucleic acids.

Hydrolysis of tRNA. The tRNA was dialyzed against water for 24 h at 4 °C prior to enzymatic hydrolysis. The hydrolysis was carried out with snake venom and alkaline phosphatase under the conditions described by Hall (1964). After the hydrolysis, the solution was adjusted to pH 7.0 and heated to 60 °C for 30 min. After cooling to room temperature, it was readjusted to pH 6.0 and stored overnight at 4 °C, after which it was centrifuged at 10 000*g* for 60 min. Following centrifugation, the supernatant was evaporated to dryness. The dried material was extracted six times with water-saturated ethyl acetate. The extract was dried and redissolved in 35% ethanol and chromatographed on Sephadex LH-20 column.

Sephadex LH-20 Column Chromatography for the Isolation of Cytokinins. The Sephadex LH-20 column chromatography was performed as described by Armstrong et al. (1969) eluting first with 35% ethanol and subsequently separating the i^6A and ms^2io^6A by rechromatography on a Sephadex LH-20 column with water as eluent. The absorption of these eluates from the column was continuously monitored at 260 nm. After chromatography of the tRNA hydrolysate, about 2–3 OD_{260} units of standard synthetic cytokinin ribonucleosides was chromatographed on the same column to locate their positions in the elution profile. The column fractions were pooled in accordance with the positions of these standards and the UV-absorbing peaks in the elution profile. The pooled fractions were evaporated to dryness and aliquots taken for the detection of cytokinin-active ribonucleosides.

Detection of Cytokinin-Active Fractions by Tobacco Bioassay. The column fractions were hydrolyzed with 0.1 N HCl at 100 °C for 45 min before incorporation into the bioassay medium and were tested in serial dilutions by a procedure described by Linsmaier and Skoog (1965). Kinetin standards were assayed concurrently in a concentration range of 1–15 μg per L. The cultures were grown at 28 °C and ca. 50% humidity for about 5 weeks, after which time tissue fresh weights were determined. Activity of test samples were expressed as KE per 20 OD_{260} units of tRNA. KE is defined as the micrograms of kinetin required to give the same growth response as the test sample.

Chromatography of Cytokinin-Active Fractions. a. Sephadex LH-20 Column Chromatography. Column chromatography was carried out as described by Armstrong et al. (1969). The elution volumes of active materials were compared with those of the authentic cytokinins and were further fractionated accordingly for the isolation and identification of individual active components.

b. Paper Chromatography. The cytokinin-active fractions were purified by paper chromatography on acid-washed Whatman No. 1 paper. The paper chromatograms were developed in an ascending fashion for 30 cm. The UV-absorbing bands were recovered from the chromatograms in small volumes (ca. 0.5 mL) by elution with 95% ethanol. The samples were repeatedly chromatographed on paper until a single pure band was obtained, after which it was eluted and evaporated to dryness under a stream of nitrogen.

c. Thin-Layer Chromatography. To distinguish between the stereoisomers of io^6A, a thin-layer chromatography procedure developed by Playtis and Leonard (1971) was used.

d. Gas–Liquid Chromatography. GLC was carried out in a Hewlett-Packard 7620 research chromatograph equipped with a flame ionization detector. The chromatograph was fitted with a coiled glass column (1.5 m × 1.5 mm) containing 2% DC-11 (Dow Corning, silicon grease) on 80–100 mesh gas Chrom Q. Helium was used as carrier gas. The general operating parameters of the column were: helium, 60 mL/min; hydrogen, 26 mL/min; and air, 400 mL/min. Chromatograms were run isothermally at 250 °C.

The cytokinins were converted to their Me_3Si derivatives prior to injection. About 2–3 OD_{260} units of nucleosides was dissolved in 25 μL of pyridine and mixed with 25 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Silylation was carried out at room temperature for 30 min in a desiccator to preclude moisture.

Mass Spectra of Cytokinins. High resolution mass spectra were obtained in a CEC-21-110 double-focusing mass spectrometer by direct introduction of the sample into the ion source.

Low-resolution mass spectra of the Me_3Si derivatives of ms^2io^6A were obtained with a Du Pont 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph. The GC was fitted with a coiled glass column (180 × 0.2 cm) containing 3% OV-1 on 100–120 mesh Aeropak. Column parameters include the carrier gas at a flow of 30 mL/min; hydrogen, 30 mL/min; and air, 300 mL/min. Chromatographs were run isothermally at 275 °C. The *m/e* values of the fragments were obtained with an AEI-MS-9 mass spectrometer

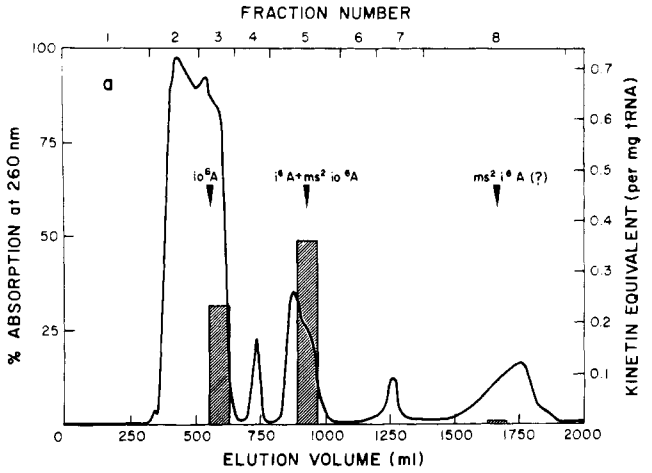

FIGURE 1: Sephadex LH-20 column chromatography of *Euglena* tRNA hydrolysate and distribution of cytokinin activity in eluate fractions. The ribonucleosides from 18 700 OD_{260} units of tRNA were dissolved in 10 mL of 33% aqueous ethanol and applied to a Sephadex LH-20 column (50 × 3.7 cm, 537 mL), equilibrated with the same solvent, and eluted at a flow rate of 51.6 mL/h. The eluate was pooled into eight fractions as indicated. A suitable aliquot from each fraction was bioassayed for cytokinin activity. The cytokinin activities are expressed as KE per mg of tRNA in the starting preparation and are represented by a bar in the middle of each active fraction. Twenty OD_{260} units is assumed to equal 1.0 mg of tRNA.

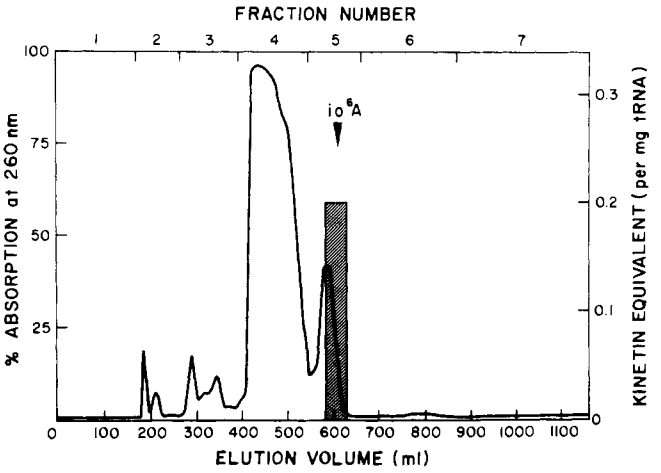

FIGURE 2: Rechromatography of fraction 3 from Figure 1 with water. Fraction 3 taken to dryness was dissolved in 5.5 mL of distilled water and applied to a Sephadex LH-20 column (62.5 × 2.5 cm, 307 mL) and eluted with water at a flow rate of 46.1 mL/h. The eluate was collected, pooled into seven fractions, and bioassayed as above.

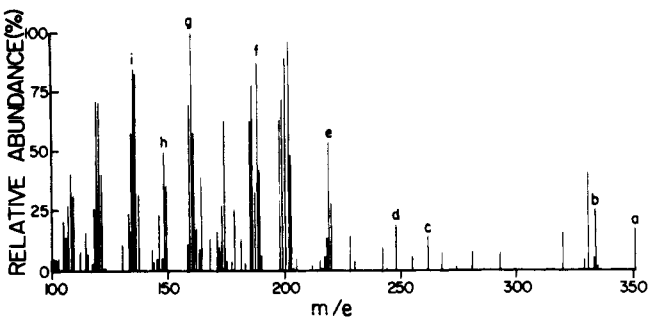

FIGURE 3: Mass spectra of io⁶A isolated from *Euglena* tRNA.

by direct injection of the sample into the ion source at an initial inlet temperature of 150 °C.

Results

Isolation of Cytokinin-Active Ribonucleosides. Dialyzed tRNA (18 700 OD_{260} units) was hydrolyzed enzymatically and extracted with ethyl acetate as described in the Experimental Section. The extract was evaporated to dryness and dissolved in 10 mL of 33% ethanol and fractionated on a Sephadex LH-20 column which had been equilibrated with the same solvent. The eluate from the column was continuously monitored at 260 nm and the elution profile obtained is presented in Figure 1. The eluate was divided into eight fractions, as indicated in the figure, and aliquots were bioassayed for cytokinin activity. Cytokinin activity was found in fractions 3, 5, and 8 as is indicated by vertical bars in Figure 1.

Analysis of Fraction 3. Fraction 3 taken to dryness was dissolved in double-distilled water and fractionated on a Sephadex LH-20 column which had been equilibrated with the same solvent. The elution profile of this column is given in Figure 2.

The eluates were pooled into seven fractions and aliquots were taken for bioassay. Only the fraction corresponding to an elution volume of 560–660 mL showed cytokinin activity. This fraction was evaporated to dryness and the cytokinin was purified by repeated ascending chromatography on Whatman I paper, using 9.5% aqueous ethanol as solvent. The ultraviolet spectrum of this material in 95% ethanol was identical with that of a synthetic sample of io⁶A, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine.

The high-resolution mass spectrum gave ions at the following *m/e* values: *a* = 351.154 (M^+), *b* = 334.156 (M − OH), *c* = 262.129 (M − $C_3H_5O_3$)$^+$, *d* = 248.111 (M − $C_4H_7O_3$)$^+$, *e* = 219.111 (M − $C_5H_8O_4$)$^+$ = (B + 1)$^+$, *f* = 188.095 (B + 1 − CH_2OH)$^+$, *g* = 166.063 (B + 1 − C_3H_7O)$^+$, *h* = 148.061 (B + 1 − C_4H_7O)$^+$, *i* = 135.055 (B + 1 − C_5H_8O)$^+$ the inter alia, typical of io⁶A (Hall et al., 1967; Hecht et al., 1970). The mass spectrum of the purified sample showing the relative intensities of fragments is presented in Figure 3.

The retention volume on Sephadex LH-20 chromatography, R_f values on paper and on thin-layer silica gel chromatography, ultraviolet spectra and mass spectra all indicate the cytokinin in fraction 3 to be io⁶A, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine.

On thin-layer silica gel chromatography, the sample showed an UV-absorbing spot at R_f 0.43 corresponding to the cis isomer of io⁶A. No UV absorption at a R_f of 0.34, corresponding to the trans isomer, was detected. The GLC data also confirm the above observation (Figure 4). Comparison of the chromatograms in Figure 4 shows that the cytokinin-active fraction 3 from Figure 1 has the same retention time as the synthetic *c*-io⁶A and that no evidence of *t*-io⁶A was found in this preparation. Thus the bulk of the io⁶A isolated from *Euglena* tRNA is the cis isomer.

Analysis of Fraction 5. Rechromatography of fraction 5 and bioassay of its elution profile presented in Figure 5 shows cytokinin activity in fractions 4 and 6, corresponding to i⁶A and ms²io⁶A, respectively. Fraction 4 was repeatedly chromatographed on Whatman paper and the UV-absorbing band at R_f 0.61 was eluted and evaporated to dryness. The ultraviolet spectrum of this material in 95% ethanol was identical with that of authentic i⁶A. The high resolution mass spectrum of this material obtained as described in Experimental Section and shown in Figure 6 had peaks at the following *m/e*: *a* = 335.160 (M^+), *b* = 246.137 (M − $C_3H_5O_3$)$^+$, *c* = 232.119 (M − $C_4H_7O_3$)$^+$, *d* = 203.118 (M − $C_5H_8O_4$)$^+$ = (B + 1)$^+$, *e* =

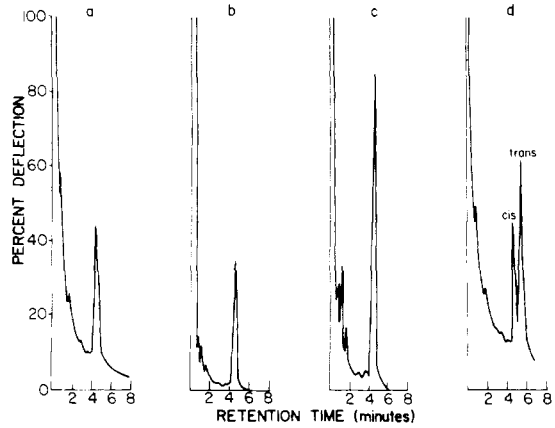

FIGURE 4: Gas–liquid chromatography of Me_3Si derivatives of (a) synthetic *cis*-io^{6}A (0.5 μL); (b) isolated io^{6}A from *Euglena* tRNA (0.4 μL); (c) a mixture of synthetic *cis*-io^{6}A (0.5 μL) and isolated io^{6}A (0.5 μL); (d) a mixture of synthetic *cis*-io^{6}A (0.6 μL) and *trans*-io^{6}A (1.0 μL).

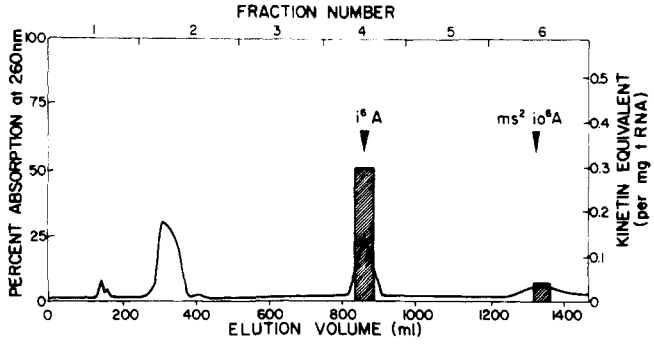

FIGURE 5: Rechromatography of fraction 5 from Figure 1 with water. Fraction 5 taken to dryness was dissolved in 5.5 mL of distilled water and fractionated on a Sephadex LH-20 column (52 × 2.5 cm, 255 mL) which had been equilibrated with water and eluted at a flow rate of 45.5 mL/h. The eluate was pooled into fractions as indicated and bioassayed as in Figure 1.

188.097 $(B + 1 - CH_3)^+$, f = 160.064 $(B + 1 - C_3H_7)^+$, g = 148.063 $(B + 1 - C_4H_7)^+$, h = 135.055 $(B + 1 - C_5H_8)$. These fragments are characteristic of i^{6}A as reported by Hecht et al. (1970) and Biemann et al. (1966). The above data therefore show fraction 4 to be i^{6}A, 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine.

Analysis of Fraction 6 of Figure 5. The eluate corresonding to fraction 6 on paper chromatography showed one UV-absorbing band at R_f 0.66 in addition to a fluorescent band very close to it. After repeated chromatography on paper, a final yield of about 0.50 OD_{260} unit of material was obtained. The ultraviolet absorption spectrum of this resembled that of synthetic ms^{2}io^{6}A. Because of small quantity and low purity, high-resolution mass spectrum could not be obtained. A low-resolution mass spectrum which was obtained showed some characteristic peaks at *m/e*, in the range of 200–300, but the other ion peaks were obscured because of high intensity peaks of contaminants. GLC was used to further purify and characterize the preparation. Comparisons of the GLC's of the Me_3Si derivatives of fraction 6 and authentic *t*-ms^{2}io^{6}A and *c*-ms^{2}io^{6}A alone and coinjected, as presented in Figure 7, show that fraction 6 contained *c*-ms^{2}io^{6}A together with a trace of *t*-ms^{2}io^{6}A. Since GLC data suggested the feasibility of the separation of the contaminants, mass spectrometry was attempted in a Du Pont mass spectrometer coupled to a Varian gas chromatograph. The gas chromatography of the Me_3Si derivative of the isolate showed a peak in the region corresponding to ms^{2}io^{6}A which gave a mass spectra with peaks characteristic of ms^{2}io^{6}A as shown in Figure 8.

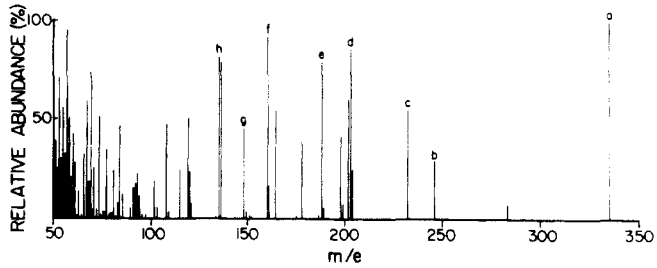

FIGURE 6: Mass spectra of i^{6}A isolated from *Euglena* tRNA.

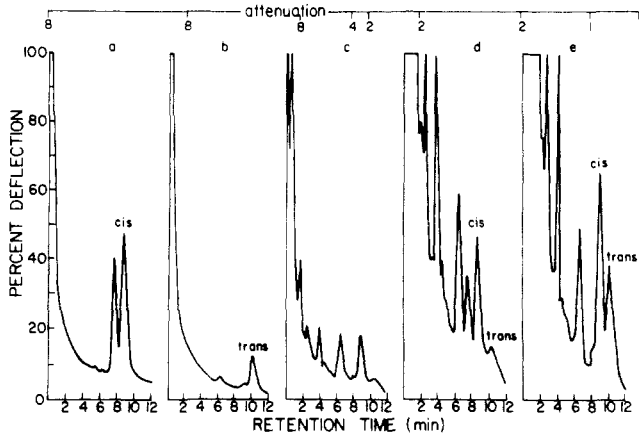

FIGURE 7: GLC of Me_3Si derivatives of (a) synthetic *cis*-ms^{2}io^{6}A (1.0 μL); (b) synthetic *trans*-ms^{2}io^{6}A (2.0 μL); (c) isolated ms^{2}io^{6}A (1.0 μL); (d) mixture of isolated ms^{2}io^{6}A (1.0 μL) and *cis*-ms^{2}io^{6}A (0.5 μL); (e) mixture of isolated ms^{2}io^{6}A (0.7 μL) and *trans*-ms^{2}io^{6}A (0.25 μL).

Further evidence for the presence of ms^{2}io^{6}A in *Euglena* tRNA was obtained by Sephadex LH-20 column chromatography of the tRNA hydrolysate of cells grown in the presence of radioactive sulfur. These chromatographs showed a radioactive peak in the region of ms^{2}io^{6}A (Swaminathan et al., 1977).

Analysis of Fraction 8. The late eluting peak corresponds to the region where ms^{2}i^{6}A is normally eluted from Sephadex LH-20 columns. A radioactive peak corresponding to the elution position of ms^{2}i^{6}A was obtained also in ^{35}S-labeling experiments (Swaminathan et al., 1977), suggesting ms^{2}i^{6}A is indeed responsible for the cytokinin activity in this fraction.

Discussion

Three cytokinin-active ribonucleosides from *Euglena gracilis* var Bacillaris have been identified as 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. The structures of the first two compounds were assigned on the basis of their chromatographic properties, ultraviolet and high resolution mass spectra, which were identical with those of the authentic compounds. The assignment of structure for the third compound is based on chromatographic properties, ultraviolet spectra, ^{35}S-label incorporation and low resolution mass spectra of its Me_3Si derivative. A small amount of cytokinin activity was detected in Sephadex LH-20 column eluates in the region where 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine was normally found, and, furthermore, ^{35}S uptake ex-

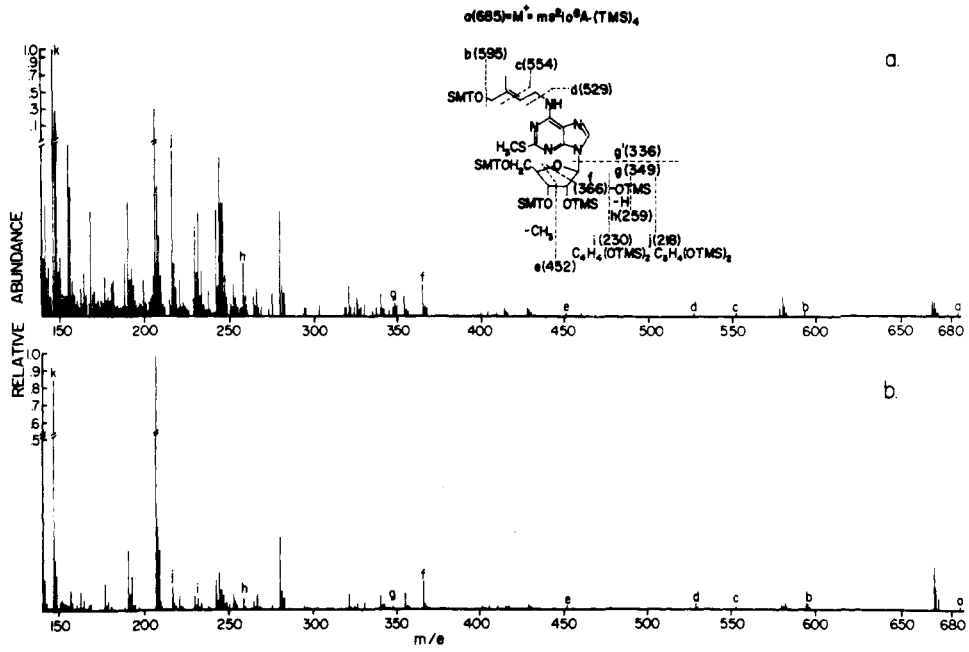

FIGURE 8: Mass spectra of Me_3Si derivatives of (a) synthetic ms^2io^6A; (b) isolated ms^2io^6A.

periments showed label in this region. However, structurally similar compounds might have similar ultraviolet and chromatographic characteristics so that in the absence of mass spectral data, the identification of this latter compound is not definite.

The distribution pattern of cytokinins in different organisms is of interest from the phylogenetic standpoint. To date, four major cytokinins, i^6A, io^6A, ms^2i^6A, and ms^2io^6A, have been identified in tRNA preparations from different sources of wide evolutionary divergence. However, there has been no earlier report of their occurrence in algal tRNAs. These studies on *Euglena gracilis* establish for the first time the occurrence of cytokinins in an algal tRNA.

Of the four cytokinins cited above, only two, i^6A and ms^2i^6A, are generally found in bacterial tRNAs. Plant tRNAs contain in addition io^6A and ms^2io^6A, which have not been found ordinarily in bacterial or animal tRNAs. However, tRNA preparations from certain plant pathogenic strains of *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* have been reported to contain io^6A and ms^2io^6A, respectively (Chapman et al., 1976; Thimmappaya and Cherayil, 1974). In this context, the occurrence of io^6A and ms^2io^6A in *Euglena* is of special interest because of its dual classification as plant or animal. Evidently the cytokinin composition of *Euglena* tRNA is similar to that generally found in plants.

In the plant tRNA preparations that have been found to contain io^6A, the cis isomer is the most abundant. Only a small proportion of trans isomer has been found in tRNAs of pea shoots and roots (0.5–0.8%), wheat germ (0.6%), and alfalfa (5%) (Vreman, 1973). In *Euglena* tRNA no trans isomer of io^6A was detected by TLC and GLC. In the case of ms^2io^6A, the trans isomer content was about 30% as compared with 53, 35, and 4% reported for pea shoots, alfalfa, and wheat germ, respectively (Vreman, 1973). Thus, also the isomeric distribution of cytokinins in *Euglena* conforms to the distribution reported for plants.

Based on the observed KE values and the specific activities for *c*-io^6A and ms^2io^6A (15- and 40-fold lower, respectively, than that of i^6A), one can get rough estimates of the relative abundance and mole percent of the different cytokinins in *Euglena* tRNA. Such a computation gives a mole percent ratio of 83:7:10 for *c*-io^6A:i^6A:ms^2io^6A. Thus, *c*-io^6A is the most abundant cytokinin in *Euglena* tRNA. Since cytoplasmic tRNA constitutes the major portion of the preparation, such a high abundance implies that it is of cytoplasmic origin. The evidence for the compartmentalization of io^6A to cytoplasmic tRNA and of ms^2io^6A to chloroplast tRNA is presented elsewhere (Swaminathan et al., 1977).

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Selective Chemical Modification of *Escherichia coli* Elongation Factor G: Butanedione Modification of an Arginine Essential for Nucleotide Binding†

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ABSTRACT: Treatment of *Escherichia coli* elongation factor G with the arginine reagent, 2,3-butanedione, leads to the inactivation of the enzyme when performed in sodium borate buffers. The inhibition follows pseudo-first-order kinetics until 95% of the activity has been lost and further incubation results in complete inhibition. Removal of the borate by exhaustive dialysis results in the restoration of approximately 85% of the original activity. The pH dependence of the reaction suggests that the ionization of a group in the protein with a pK_a of approximately 8.8 facilitates the reaction with butanedione. A reaction order of 1.01 ± 0.13 was calculated for the inhibition reaction, indicating that the incorporation of one butanedione per elongation factor G results in the inactivation of the enzyme. The kinetics of inhibition in the presence of GTP indicate that the elongation factor G·GTP complex is refractory to butanedione inhibition. Elongation factor G which has been partially inactivated by butanedione has the same apparent K_m for GTP as does the native enzyme. These results indicate that elongation factor G contains only one essential arginine residue which is reactive with butanedione and that this residue is located at its nucleotide binding site.

In spite of its central role in protein synthesis, the nature of the structural features of elongation factor G (EF-G)[1] which are important for its function remains largely unknown. Although EF-G has long been known to catalyze the hydrolysis of GTP in the presence of the ribosome (Nishizuka and Lipmann, 1966), it was not known until recently whether the nucleotide binding site was on EF-G, the ribosome, or was generated by the interaction of these two molecules. Several lines of evidence now indicate that the nucleotide binding site exists on EF-G. These include equilibrium binding measurements (Arai et al., 1975; Baca et al., 1976), steady-state kinetic analysis (Rohrbach and Bodley, 1976), and the protection of EF-G by guanine nucleotides against sulfhydryl reagents (Marsh et al., 1975; Baca et al., 1976).

The existence of the binding site on EF-G for the negatively charged nucleotide suggested that basic amino acid residues in EF-G play an important part in the binding of nucleotide. Indeed with the development by Riordan (1973) of 2,3-butanedione as a highly selective arginine modifying reagent, a number of enzymes which act upon phosphate-containing substrates have been shown to contain arginyl residues at their active sites. Some notable examples of these enzymes are alcohol dehydrogenase (Lange et al., 1974), mitochrondrial and supernatant malate dehydrogenase (Foster and Harrison, 1974; Bleile et al., 1975), alkaline phosphatase (Daemen and Riordan, 1974), fructose-1,6-bisphosphate aldolase (Lobb et al., 1975), and mitochondrial ATPase (Marcus et al., 1976).

With this background, it seemed likely that arginyl residues could be important in the binding of guanine nucleotides to EF-G. In this report we describe the results of our study on the interaction of butanedione with EF-G.

Experimental Section

Materials. Elongation factor G was purified to homogeneity from *Escherichia coli* B (obtained from Grain Processing Corp.) by the method of Rohrbach et al. (1974). 2,3-Butanedione was purchased from Sigma Chemical Co. [^{3}H]GTP and [α-^{32}P]GTP were obtained from New England Nuclear Inc. Fusidic acid was a generous gift of Dr. W. O. Godtfredsen of Leo Pharmaceutical Products. All other chemicals were of the highest purity commercially available.

pH Dependence of Butanedione Inhibition. Reaction solutions (103 μL) containing 1.8 μM EF-G, 4.06 mM butanedione in either 50 mM sodium citrate–50 mM sodium borate–5 mM β-mercaptoethanol (pH 5.74 to 8.98) or 50 mM sodium bo-

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[1] Abbreviations used: EF-G, elongation factor G; Tris, tris(hydroxymethyl)aminomethane.