Reports

Chlamydomonas: Colored Excretion Products

Abstract. Particle-free media in which pure cultures of the green alga *Chlamydomonas moewusii* have been growing are yellow. In its solubility, chromatographic behavior, fluorescence, response to changes of *p*H, and optical absorption, the coloring matter closely resembles mixtures of substances previously held responsible for the yellow color of some pond waters. Our findings suggest that the yellow materials of natural waters may originate from algal metabolism.

To understand the ecology of aquatic organisms many chemical studies of the dissolved organic materials in natural waters have been carried out (*1*, *2*). Yet the origin, nature, and role of these substances remains largely obscure. Shapiro (*3*) characterized the organic materials responsible for the yellow color of many lakes as a mixture of carboxylic acids, some of which fluoresce in ultraviolet light. He suggested that these substances arise from decomposition in soil.

While studying the metabolism of the flagellated alga *Chlamydomonas*, we observed that pure cultures produced a yellowish supernatant liquid which, after concentration, fluoresced. We are reporting this observation for its possible relation to the origin and role of organic materials in natural waters.

Chlamydomonas moewusii Gerloff ("+" strain, Indiana University Culture Collection No. 97) were grown in a mineral medium in 1-liter culture flasks (*4*), exposed to alternating light (white fluorescent, approximately 8500 lux) and dark periods of 12 hours at 19° to 21°C. The cultures were mechanically agitated during the light periods. To prepare material for chemical analysis, rapidly growing cultures containing 10^6 cells per milliliter were centrifuged in the cold at 500*g* for 10 minutes. The cell-free supernatant fluid was removed and centrifuged at 25,000*g* for 2 hours. The resulting clear, yellow liquid was evaporated to dryness under reduced pressure and at a temperature not exceeding 50°C; the resulting dry powder was suspended in 80 percent aqueous ethanol and placed in the freezer overnight. Filtration of the cold alcoholic solution removed most of the inorganic salts; evaporation to a dry powder, as described above, completed the preparation.

In descending paper chromatography with Whatman No. 1 filter paper, two solvent systems were used: *n*-butanol, water, and acetic acid (4:4:1 by volume); and pyridine, water, tertiary amyl alcohol, and diethylamine (25:25:25:1 by volume). Fluorescence was observed with a Sylvania "Blacklite" long-wavelength ultraviolet lamp, titration curves were made automatically with a Radiometer Titrigraph, and the ultraviolet absorption spectra were determined with a Beckman model DU spectrophotometer.

Titration curves of the first supernatant liquid and the fresh medium gave pK_a^* values of the excreted material from 5.8 to 6.0. When concentrated 150-fold, the supernatant gave a deeply colored yellow solution with a *p*H of 9.1 and a titration curve similar to that of the unconcentrated material. Actively growing organisms, washed, suspended in a small volume of 0.006*M* phosphate buffer at *p*H 6.8, and incubated for 5 hours at 25°C in the dark, produced a soluble, yellow material which showed the same type of curve.

The dried, yellow material was water soluble and dialyzable; it changed reversibly to a lighter color when it was acidified. It was soluble in ordinary ethyl ether and 80 percent aqueous ethanol or ethyl acetate, and less soluble in ligroin or anhydrous ethyl ether. Ether and ethyl acetate extracts were bright yellow and exhibited brilliant blue fluorescence. The ethanol extracts, also yellow, exhibited a greenish fluorescence. Ultraviolet and visible absorption spectra of these extracts lacked definite maxima or minima; the optical density gradually increased (end-absorption), independently of *p*H, at shorter wavelengths.

Chromatograms of ethanol extracts in the butanol and acetic acid solvent showed a yellow, fluorescent spot which moved with the solvent front and which fluoresced bright blue after elution and suspension in ethanol. Part of a larger, more slowly moving spot gave a yellow color with ninhydrin while the fringes and tails fluoresced greenish before and after ninhydrin treatment. Both spots, eluted with 80 percent ethanol and examined spectrophotometrically between wavelengths of 220 mμ and 600 mμ showed only end-absorption. All of the fluorescent portion of the sample, some of it colorless, moved with the solvent front when the pyridine mixture was the solvent.

Although we attempted neither complete separation of the components nor their purification, it seems worth while to compare our findings with Shapiro's more thorough analysis of pond waters (*3*). Points of similarity between his extracts and ours include: resolvability of the mixture by chromatography into several components, some of them yellow; fluorescence of the colored components; buffering action; dialyzability; decolorization by the addition of acid; absence of maxima or minima in visible or ultraviolet absorption; and solubility characteristics. Observed differences are: Shapiro's extract, by our estimate from his Fig. 5, had maximal buffering action at *p*H 4 and *p*H 10, while our maximum was *p*H 5.8 to 6.0; Shapiro's material (see also *2*) showed a gradual color change when the *p*H was lowered, while ours showed sudden change (*5*). With Gorham's observation of no color change in samples from yet another locality (*6*), the differences suggest to us that the sharpness of the color change may be related to the relative purity of the mixtures.

The similarities just mentioned are insufficient to establish that the extracts are chemically similar since many mixtures of naturally occurring substances could give the same results. However, the natural habitat of *Chlamydomonas* in soil and fresh water, together with our finding that this organism produces these substances in the processes of

metabolism, suggests once more the interesting possibility that the substances studied by Shapiro may have their origin in algal metabolism (*3*). Allen has reported that for pure cultures of six species of *Chlamydomonas*, 10 to 45 percent of the oxidizable organic matter formed by the cells is excreted into the medium (*7*).

An extension of this analysis to other groups of algae and a rigorous study and comparison of their excretion products with the organic material found in natural waters may clarify the nature, origin, and role of these substances (*8*).

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References and Notes

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Fate of a Synthetic Polynucleotide Directing Cell-Free Protein Synthesis
I. Characteristics of Degradation

Abstract. Tritiated-polyuridylic acid was degraded rapidly in extracts of *Escherichia coli* and degradation was not dependent on concomitant polyphenylalanine synthesis. Since a large portion of the polymer was degraded before appreciable polyphenylalanine was synthesized, the catalytic activity of the undegraded polyuridylic acid in directing protein synthesis was suggested. The predominant breakdown products were 5′-mononucleotide phosphates. Possible enzymatic routes of polyuridylic acid breakdown are discussed.

Polyuridylic acid (*1*) has been found to direct the incorporation of phenylalanine into polyphenylalanine in a cell-free *Escherichia coli* protein synthesizing system (*2*). The ease of preparation of this synthetic polynucleotide from isotopically labeled UDP and its uniform base composition have facilitated the study of the fate of this compound which is believed to represent a simple model of "messenger RNA." This and the accompanying report (*3*) describe several aspects of the fate of poly U in extracts of *E. coli* which are actively synthesizing polyphenylalanine.

Experimental procedure. An extract of *E. coli*, "pre-incubated S-30" (*2*), was separated into ribosomal and supernatant fractions by centrifugation at 105,000*g* for 3 hours. The upper two-thirds of the supernatant solution was saved. The lower one-third was gently swirled for 5 seconds over the ribosomal pellet and decanted, and the ribosomes were resuspended in one-third the original volume of 0.1*M* tris-HCl, *p*H 7.8, 0.01*M* magnesium acetate, 0.05*M* KCl, and 0.006*M* β-mercaptoethanol. By systematically varying the proportions of ribosomal and supernatant solutions in the presence of a constant amount of poly U [80 mμmole (pU) per milliliter of reaction mixture] the optimal proportion of these solutions for maximal polyphenylalanine synthesis was determined and used in all the experiments. Protein was determined by a modification of the method of Lowry *et al.* (*4*).

To prepare H^3-poly U, UDP (*5*) was tritiated by the Wilzbach procedure (*5*) and subjected to paper electrophoresis for 2 hours at 1000 v in 0.05*M* ammonium formate buffer *p*H 3.5 followed by radioautography. There was only one radioactive spot, and this migrated with authentic UDP. The H^3-UDP, without purification, was converted by polynucleotide phosphorylase which had been obtained from *Micrococcus lysodeikticus* and purified through the $(NH_4)_2SO_4$ step (*6*), to H^3-poly U which was then deproteinized (*7*). The resulting polymer had a specific radioactivity of approximately 3×10^5 count/min μmole (pU) when counted under optimal conditions in a liquid scintillation counter. The average sedimentation constant of this material ($s_{20,w}$) was 8.2 as determined in 0.01*M* sodium cacodylate, *p*H 6.9, and 0.1*M* NaCl in the Beckman model E analytical ultracentrifuge with ultraviolet optics. The approximate weight-average molecular weight of the H^3-poly U, estimated from the relationship between $s_{20,w}$ and molecular weight of poly U described by Fresco (*7a*) was found to be 5×10^5. The degree of heterogeneity of the polymer is shown in Fig. 1. The theoretical distribution of $s_{20,w}$ for a homogeneous poly U with an average $s_{20,w}$ of 8.2 was calculated (*8*) and fell within a very narrow range. The diffusion coefficient for poly U was given to us by J. Fresco. The width of the observed distribution indicated that the polymer was very heterogeneous.

Bray's solution (*9*) was used for counting H^3-poly U and its degradation products, in a Packard Tri-Carb liquid scintillation counter. Appropriate corrections were made for quenching produced by extraneous materials.

After paper chromatography of H^3-poly U and its degradation products, the paper was cut into 1- by 1½-inch pieces which were placed vertically into counting vials. The contents of the paper were eluted by

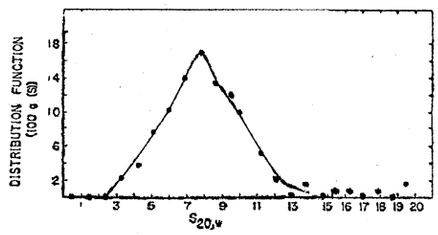

Fig. 1. Heterogeneity of H^3-poly U. A solution containing 30 μg/ml H^3-poly U in 0.1*M* NaCl, 0.01*M* sodium cacodylate, *p*H 6.9, was centrifuged in the Beckman model E analytical ultracentrifuge equipped with ultraviolet optics. Photographs were taken at 8-minute intervals. Calculation of the sedimentation coefficients were made, as described by Schumaker and Schachtman (*22*), using a photograph obtained after 44.8 minutes of centrifugation at 56,100 rev/min.

wetting its upper edge with 1 ml of H_2O, whereupon 16 ml of Bray's solution was added. The vial was stored at 0°C in the dark for 24 hours, and then counted. Although addition of H_2O to wet and elute the paper caused quenching, fewer counts were obtained if this step was omitted, possibly due to trapping of the H^3-material in the interstices of the paper. In control experiments with the H_2O technique, about 80 percent of counts that were placed on paper strips could be counted. Therefore, this correction was made when determining the radioactivity of chromatographic strips.

C^{14}-phenylalanine (uniformly labeled, specific activity 45 μc/μmole) (*5*) was diluted with C^{12}-phenylalanine as needed. The composition of the reaction mixture used for determination of C^{14}-phenylalanine incorporation into protein (*2*) is given in the legend for Fig. 2. The protein precipitates were counted in a proportional gas flow counter with a Micromil window. The presence of tritium in the poly

Table 1. Identification of H^3-products formed from poly U. The concentration of H^3-poly U was 50 mμmole (pU) per millimeter of reaction mixture.

Probable degradative enzyme	Mononucleotide	Percentage of total H^3-poly U added after incubation time	
		3 min	30 min
Polynucleotide phosphorylase (*13*)	UDP	20	13
	UTP	24	11
	5′-UMP	14	48
"Ribosomal ribonuclease" (*19*)	2′-, 3′-cyclic UMP	6	5
	2′- and 3′-UMP	0	1
Total*		65	80

*Refers to the total percentage of H^3-poly U recovered as mononucleotide at the indicated times. The values include 1 percent and 2 percent uridine found, respectively, after 3 and 30 minutes of incubation.