Production and Identification of *N*-Glucosylrubropunctamine and *N*-Glucosylmonascorubramine from *Monascus ruber* and Occurrence of Electron Donor-Acceptor Complexes in These Red Pigments

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The filamentous fungus *Monascus ruber* produces water-soluble red pigments in a submerged culture when grown in a chemically defined medium containing glucose as a carbon source and monosodium glutamate as a nitrogen source. Two new molecules with polyketide structures, *N*-glucosylrubropunctamine and *N*-glucosylmonascorubramine, constituting under some conditions 10% of the total extracellular coloring matter when glucose as a carbon source was in excess (20 g/liter), were isolated and structurally characterized by high-pressure liquid chromatography, Dionex methods, ¹H and ¹³C nuclear magnetic resonance spectroscopy, and mass spectrometry. The occurrence of the electron donor-acceptor complex effect was demonstrated by UV spectroscopy, polarography, and thin-layer voltammetry. The use of *n*-butanol as an extraction solvent stabilized the pigments against the effects of daylight for several months, promoting the stability of this type of complex.

Currently most natural colorants are extracted from plants or animals, but microorganisms could be a promising source of food colorants. *Monascus* species are an important source of natural food pigments. Currently more than 50 patents concerning the use of *Monascus* pigments for food have been issued in Japan, the United States, France, and Germany (9). The annual consumption of *Monascus* pigments in Japan increased from 100 tons in 1981 to 600 tons in 1992 (8, 18) and was valued at \$12 million according to a survey published in 1992 (8). New food applications, such as the coloration of processed meats (sausage and hams), marine products like kamaboko (fish paste), and tomato ketchup, have been described (18).

The six well-known *Monascus* pigments include yellow (monascin and ankaflavin), orange (monascorubrin [MBN] and rubropunctatin [RTN]), and red-purple (monascorubramine and rubropunctamine). These pigments are produced mainly in the cell-bound state. They have low solubility in water, are sensitive to heat, are unstable in the pH range of 2 to 10, and fade with exposure to light (19). Thus, patents have focused mainly on the solubilization, stability, and extraction in solution of pigments. The pigments react readily with amino group-containing compounds in the medium, such as proteins, amino acids, and nucleic acids (9, 19), to form water-soluble pigments.

For a better control of the production of the pigment, a

chemically well-defined medium with submerged fermentation, with glutamate as a nitrogen source, was studied. This promotes better production and excretion of extracellular pigments (10, 13) and also leads to a majority of the pigment being in the form of a complex between the pigment and glutamic acid (1).

In addition to the molecules already identified (1), several others having the same physicochemical properties as the pigments linked to glutamate have been detected in the culture broth; thus, it was interesting to characterize them. These molecules can represent 10% of the total coloring matter.

A loss in the extracellular pigment concentration was observed at the end of the culture, especially when the culture duration was long (more than 120 h). This loss can be explained by the photochemical (daylight) degradation of the pigments. They are stable during several months when preserved in organic solvents, while in aqueous solutions, they are degraded in a few days. The stability is favored by an electron donor-acceptor (EDA) complex property that was shown for these extracellular molecules (quinone-amine).

Many molecular complexes can be formed between an electron donor (D) and an electron acceptor (A) (11). There are three main types of EDA complexes: (i) those between neutral molecules (D, A), (ii) those between an anion and a neutral molecule (D⁻, A; D⁺, A), and (iii) those between oppositely charged ions (D⁻, A⁺) (6). These complexes can be characterized by several properties and are responsible for the intense color of these pigments. This phenomenon is associated with the transfer of an electron from the donor to the acceptor (11). It is known that many acceptor cations are directly involved in important biological processes or exhibit a pronounced biological activity and photochemical properties (6, 17).

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This paper describes the extraction, purification, and characterization of the structures of molecules involved in the extracellular water-soluble pigments, as well as the occurrence of EDA complexes in these extracellular pigments (*N*-glutaryl-rubropunctamine [GTR], *N*-glutarylmonascorubramine [GTM], *N*-glucosylrubropunctamine [GCR], and *N*-glucosylmonascorubramine [GCM]) and the determined physicochemical properties of extracellular red pigment molecules, i.e., EDA complexes, which are required for light stability.

MATERIALS AND METHODS

Microorganism and growth conditions. The strain used is a high-pigment-producing strain obtained from anka, *Monascus ruber* ATCC 96218.

The chemically defined fermentation medium developed in previous studies (1, 14) was used; it contained (grams per liter of distilled water) glucose, 20; monosodium glutamate, 5; K_2HPO_4 , 5; KH_2PO_4 , 5; $CaCl_2$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; and $MnSO_4 \cdot H_2O$, 0.03. The initial pH of the medium was adjusted to 6.5 with phosphoric acid (H_3PO_4).

The stock culture was kept on potato dextrose agar (Difco). Spores were prepared by growth on potato dextrose agar slants for 10 days at 28°C. The spores were washed with sterile water. A suspension of 10^8 spores was used to inoculate a 1-liter baffled Erlenmeyer flask containing 200 ml of inoculum medium, which was incubated at 28°C for 2 days. This inoculum was then transferred to a 2-liter fermentor containing 1.6 liters of synthetic medium. The culture was incubated at 30°C at an aeration rate of 0.4 vol/vol/min, and the agitation speed was increased from 250 to 600 rpm.

Isolation and purification of new red pigments from fermentation broth. The mycelium was separated from the culture broth by filtration through a M14 membrane (0.8- μ m pore size; Tech-sep, Bollene France), and then the filtrate was lyophilized. The crude extract was recovered with deionized water, and this solution was then extracted several times with water-saturated *n*-butanol. The organic phase was dried with anhydrous Na_2SO_4 and vacuum concentrated. The extract was applied to chromatographic columns packed with silica gel (70-230 mesh) suspended in chloroform. The adsorption column was first eluted with chloroform-methanol (90:10, vol/vol) to elute less-polar products containing free pigments, and then the water-soluble pigments were eluted with chloroform-methanol (50:50, vol/vol). The pigments were then isolated from fractions obtained by preparative thin-layer chromatography on silica gel with chloroform-methanol-water (65:25:4, vol/vol) as the solvent system.

The water-soluble red pigments thus isolated were purified by high-pressure liquid chromatography (HPLC) on a C_{18} column by using a separation gradient of water-methanol (80:20 to 0:100 [vol/vol] over 30 min). The flow rate was 0.8 ml/min. The detector used was a Waters Lambda Max spectrophotometer at 480 nm.

NMR. For nuclear magnetic resonance (NMR) spectroscopy, a Bruker ARX 400- or 300-MHz instrument was used. The pigments were dissolved in CD_3OD (99.8%).

Mass spectrometry. The molecular weights of pigments were determined by fast atom bombardment on a NERMA R10-10 instrument. The matrix was glycerol acidified with trifluoroacetic acid. The solvent was a 1:1 (wt/vol) acetonitrile-water mixture containing 2% formic acid. Desorption chemical ionization with NH_3 was also used.

UV spectra. The spectra were recorded with a Hewlett-Packard UV-visible diode array spectrophotometer with a sample concentration of 100 μ g/ml.

Dionex methods. Monosaccharides were separated on a CarboPac PAI anion-exchange column (4 by 250 mm; Dionex, Sunnyvale, Calif.) equipped with a CarboPac PA guard column (3 by 25 mm; Dionex). Elution was performed with 15 mM NaOH. Monosaccharides were detected with a PAD II pulsed amperometric detector equipped with a gold electrode (4). Standard monosaccharide solutions were used to determine retention times and the ratios between peak areas and concentrations.

Identification and purification of *N*-glucosamine. The hydrolysis of GCM or GCR was attained by the addition of 10 ml of 1 N H_2SO_4 to 35 mg of the fraction (recovered on 1.5 ml of methanol), and the mixture obtained was refluxed for 2 h. Methanol was evaporated, and the mixture was extracted three times with 10 ml of chloroform. The aqueous phase was neutralized with Na_2CO_3 and centrifuged. The supernatant was dried, solubilized in methanol, and analyzed by thin-layer chromatography (ethyl acetate-acetic acid-methanol [3:1:1]). The spots were revealed with sulfuric anisaldehyde (anisaldehyde-sulfuric acid-ethanol [0.5:0.5:9]). The aqueous phase was analyzed by the Dionex method.

Absorption spectra of red pigments. Samples (0.25 mg) of the separated fraction were dissolved in 12 ml of various solvents and used as standards in 1-cm glass cuvettes for measurements of absorbance within the visible light area.

Electrochemical study. The electrochemical behavior of the red pigment molecules was studied by two electrochemical methods in order to assess heterogeneous electronic transfer between an electrode and these compounds. The first method was thin-layer voltammetry. The working electrode was made of a carbon film (CFT 3000 10; COMAIP France) set between two glass slides delimiting a compartment with a volume of 20 μ l. The choice of carbon was

FIG. 1. Culture development in fermentor. X, biomass; S, glucose; MSG, monosodium glutamate.

justified by the high overvoltages for the hydrogen and oxygen discharges (-0.8 and 1.1 V with respect to a saturated calomel electrode) at pH values higher than or equal to 3.

The reference electrode, with respect to which all potentials are expressed, was an Hg-Hg $_2$ Cl $_2$ -Cl $^-$ saturated-type electrode. The auxiliary electrode was made of platinum. The electrochemical apparatus used was an EG&G Princeton Applied Research model 273 potentiostat associated with a Luxytrace type Sefram X-Y recorder.

The second electrochemical method used was fast polarography at a drop mercury electrode. The electrochemical apparatus used was a type 626 (METROHM, Herisau, Switzerland) polarograph equipped with a 663 VA STAND station or an E 505 station. The potentials are expressed relative to an Ag-AgCl-Cl $^-$ saturated-type reference electrode. The auxiliary electrode, which had a large surface area, was made of tungsten.

Resistance to daylight. Pigment mixtures of GTR and GTM or GCR and GCM were dissolved in *n*-butanol or in water, at a concentration giving an A_{500} of approximately 0.5. These pigment solutions were placed in Pyrex tubes and exposed to daylight. The A_{500} of these solutions before and after exposure were compared.

Degassed samples (in the absence of oxygen) were sealed and exposed to light under the same conditions.

RESULTS AND DISCUSSION

The filamentous fungus *Monascus* is a source of various secondary metabolites derived from polyketides (5). The strain of *Monascus* used in these experiments produces mainly extracellular pigments, members of the fungal sclerotiorin metabolites (5, 16).

Growth and production kinetics. The growth kinetics with respect to both glucose and monosodium glutamate consumption in culture broth are presented in Fig. 1. Growth was insignificant up to 40 h of culture and then increased sharply with increasing time of culture up to 6 g (dry weight) per liter at 110 h before stationary phase began, concomitant with the depletion of glucose. Production of extracellular pigments was proportional to the biomass production and reached the highest level, 13 A_{480} units, after 102 h of fermentation, while the residual concentrations of glucose and monosodium glutamate were 3.8 and 1.8 g/liter, respectively. A 15% decrease in pigment concentration, most probably due to photochemical degradation of pigments, was observed after its maximal value was reached. The highest specific growth rate observed was 0.04 h $^{-1}$, which was maintained for the majority of the fermentation. Pigment production and substrate consumption rates were similar to those previously observed (1, 13).

FIG. 2. Major pigments produced by members of the genus *Monascus* during culture production (18).

Isolation and purification of new red pigments from fermentation broth. The whole broth of the medium used previously contained the six well-known *Monascus* pigments (Fig. 2), with most of them being cell bound (9). However, the major part of the pigments produced in our new fermentation broth was extracellular. Various solvent systems were used for extraction of extracellular red pigments. The choice of extraction with water-saturated *n*-butanol has a considerable advantage, since this system is more efficient than other systems described in the literature (1, 9). Effectively, from 1 g of lyophilized extract, we extracted 120 mg of red pigments; however, only 90 mg of red pigments was extracted when other systems were used. Moreover, this system is more rapid, since the lyophilized extract was easy to dissolve in water.

Among the extracellular pigments, four pigment molecules (GTR, GTM, GCR, and GCM) were isolated from the fermentation broth. GTR, GTM, GCR, and GCM were purified twice by HPLC in order to separate GTR from GTM and GCR from GCM; the retention times of these molecules are given in Table 1.

The production of GCR and GCM depended on the initial concentration of glucose in the medium. At a high glucose concentration (20 g/liter), GCR and GCM represented 10% of the red pigment, while GTR and GTM accounted for 80% of the pigment production. The respective quantities of these molecules are given in Table 1. At a low glucose concentration (5 g/liter), the concentrations of GTR and GTM were lower than they were with excess glucose, while GCR and GCM were not produced. Under glucose-excess conditions, 1-D-glucosamine was formed from residual glucose and ammonia issued from desamination of sodium glutamate (one nitrogen source in culture) (Fig. 3). There were fewer secondary reactions

observed when the cells were subjected to glucose-limiting conditions (5 g/liter).

Structure determination. The mass spectrum of GTM showed a molecular ion MH^+ at m/z 512, corresponding to the known GTM with the molecular formula $C_{28}H_{33}O_8N$ (1). The UV spectrum showed absorption at $\lambda_{max} = 500$ nm ($\epsilon = 4.4 \times 10^4$) and 422 nm ($\epsilon = 3.8 \times 10^4$).

The mass spectrum of GCM showed a molecular ion MH^+ at $m/z = 544$, which could correspond to the molecular formula $C_{29}H_{37}O_9N$. The UV spectrum showed absorption at $\lambda_{max} = 500$ nm ($\epsilon = 4.4 \times 10^4$) and 422 nm ($\epsilon = 3.8 \times 10^4$).

The structural elucidation of GCM was carried out by chemical degradation methods, NMR, and mass spectroscopy.

Identification and purification of *N*-glucosamine. Analysis of the aqueous fraction from acid hydrolysis of GCM gave

TABLE 1. Retention times of extracellular pigments in HPLC analysis and amount in the total extracellular coloring matter in relation to the glucose concentration in the medium

Molecule	Retention time (min)	Amt (mg) ^a with the following glucose concn (g/liter) in medium:	
		5	20
GTR	16	20	30
GTM	20	35	50
GCR	14.5	ND ^b	4
GCM	18	ND	7

^a Amount obtained from 1 liter of culture medium.

^b ND, not detected.

FIG. 3. Possible reaction mechanism for the formation of extracellular red pigments (GTR, GTM, GCR, and GCM) from 1-D-glucosamine or monosodium glutamate (nitrogen source) and RTN and MBN molecules in the culture medium.

D-glucose, which demonstrates that the MBN is excreted in the medium as the soluble *N*-glucosamine derivative GCM, with an *N*-glucosidic bond between MBN and 1-glucosamine (Fig. 3). Moreover, the analysis by desorption chemical ionization

with NH_3 showed a peak at m/z 180, which corresponds to the mass of glucosamine.

NMR spectroscopy. Carbon and proton chemical shift assignments were based on the analysis of one- and two-dimen-

FIG. 4. GTM (A) and GCM (B). The chemical shifts (δ) at each carbon atom are presented in Tables 2 and 3, respectively.

sional NMR data by methods such as correlated spectroscopy, heteronuclear multiple quantum coherence, and heteronuclear multiple-bond connectivity. Because of the large number of carbons and protons with close chemical shifts, compound GCM appeared to be similar to GTM (Fig. 4). GCM exists as a mixture of α and β sugar anomers (3:1), which could not be separated by reverse-phase HPLC. However, the excellent signal dispersion in the NMR spectrum enabled each anomer to be distinguished. The signal attributions of GTM and GCM are given in Tables 2 and 3, respectively.

GTR was identified with an MH^+ signal at m/z 484 (1) and had the same physicochemical properties as GTM (Table 2). GCR was isolated and characterized (by NMR and mass spectrometry) with an MH^+ at m/z 516.

The ^1H and ^{13}C NMR spectra of GTR and GCR were determined. Both the ^1H and ^{13}C NMR spectra of GTR are very similar to those of GTM (Table 2), and the spectra of GCR are similar to those of GCM (Table 3).

(i) ^1H NMR. The H-12 and H-13 signals were superimposed, although the H-13 signal has to be more deshielded than the H-12 signal. Modeling of GTM gave the lowest-energy conformation, with the CO of the carboxylate near H-13, deshielding it. This explains the absence of a J_3 coupling constant between H-12 and H-13 (coupling degeneration); a correlation spot can be seen by the correlated-spectroscopy ^1H - ^1H technique, showing correlation between these two protons.

The signals at 4.6 and 5.15 ppm correspond to the chemical shifts of the anomeric protons H-2a α and H-2a β of a sugar. The chemical shifts of H-2f and H-2f' were 3.28 and 3.46 ppm, respectively, which corresponds to the chemical shift of two geminal protons in the α position of an asymmetric carbon. At about 3.75 ppm, four protons belonging to a sugar (C_6 ; aldose) can be observed. H-1 is more deshielded than the other pro-

tons, because it is localized in the β position to a carbonyl function.

(ii) ^{13}C NMR. C-13 and C-1 are not distinguished but we could see a correlation spot by heteronuclear multiple quantum coherence at 141.45 ppm for the two protons H-13 and H-1 (6.57 and 8.3 ppm).

C3 and C4' are also superimposed in CD_3OD ; however, in dimethyl sulfoxide- d_6 (DMSO- d_6), the chemical shifts of these carbons are different.

The chemical shifts at 90 and 93 ppm correspond to the

TABLE 2. ^1H (400 MHz) and ^{13}C (100 MHz) NMR analysis of GTM^a

Carbon	δ_{C} (ppm ^b)	δ_{H} (ppm)	Carbon	δ_{C} (ppm)	δ_{H} (ppm)
1	141.50	8.30	15	30.68	1.67 s
3	153.33		16	198.60	
4	119.46	7.07	17	41.25	2.79 t
4'	153.444		18	26.07	1.61 m
5	98.95	6.72	19	30.30	1.32 m
6	173.47		20	30.59	1.32 m
7	86.98		21	33.00	1.32 m
8	196.74		22	23.26	1.32 m
8'	119.59		23	14.39	0.91 t
10	173.87		2g	35.09	2.17 m
11	101.78		2h	30.80	2.19 m (H), 2.65 m (H')
			2i	73.70	4.92 dd
12	123.82	6.58	2j	178.25	
13	141.45	6.57	2k	174.06	
14	19.23	1.98 dd			

^a J (Hz) for GTM: 2i = 9/4.8; 14 = 5/2.3; 17 = 6.9; 23 = 6.9.

^b In CD_3OD .

TABLE 3. ^1H (400 MHz) and ^{13}C (100 MHz) NMR analysis of GCM α and β anomers^a

Carbon	10 α anomer		10 β anomer	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	141.50	8.30	141.50	8.30
3	153.33		153.33	
4	119.46	7.07	119.46	7.07
4'	153.44		153.444	
5	98.95	6.72	98.95	6.72
6	173.47		173.47	
7	86.98		86.98	
8	196.74		196.74	
8'	119.59		119.59	
10	173.87		173.87	
11	101.78		101.78	
12	123.82	6.58	123.82	6.58
13	141.45	6.57	141.45	6.57
14	19.23	1.98 dd	19.23	1.98 d
15	30.68	1.67 s	30.68	1.67 s
16	198.60		198.60	
17	41.25	2.79 t	41.25	2.79t
18	26.07	1.61 m	26.07	1.61 m
19	30.30	1.32 m	30.30	1.32 m
20	30.59	1.32 m	30.59	1.32 m
21	33.00	1.32 m	33.00	1.32 m
22	23.26	1.32 m	23.26	1.32 m
23	14.39	0.91 t	14.39	0.91 t
2f	62.50	3.28 m (H), 3.46 m (H')	62.50	3.28 m (H), 3.46 m (H')
2d	71.65	3.75 m	71.65	3.75 m
2b	72.98	3.75 m	77.96	3.75 m
2e	72.98	3.75 m	76.16	3.75 m
2c	74.75	3.75 m	76.16	3.75 m
2a	93.00	4.60 d	98.00	5.15 d

^a J (Hz) for GCM α : 2a = 8; 14 = 5/2.3; 23 = 6.9. J (Hz) for GCM β : 2a = 4; 14 = 5/2.3; 23 = 6.9.

anomeric carbons α and β . The region between 62.5 and 93 ppm corresponds to the chemical shift of the hydroxylated carbons of a pyranose and clearly distinguishes the chemical shifts of the two anomeric carbons α and β .

Absorption properties and light stability: EDA complex. The GCR and GCM molecules were prepared by hemisynthesis from RTN and MBN (18). They are very water soluble, in contrast to RTN and MBN. It was shown that these molecules are very stable to light, temperature, and extremes of pH (from 2 to 10) (9). Formation of molecular complexes between amines as donors and π -acceptors (i.e., quinones) has been described (2). This stability is favored by the formation of molecular complexes that are usually of the EDA complex type (6, 11). Moreover, by using the UV spectroscopy and electrochemical methods generally used to determine these kinds of

TABLE 5. Dilution effect in *n*-butanol on molar extinction coefficient (ϵ) and the absorption bands, $\pi \rightarrow \pi^*$ (hyperchromic and bathochromic effect), for an initial pigment concentration of 100 $\mu\text{g/ml}$

Dilution factor	K $\pi \rightarrow \pi^*$		B $\pi \rightarrow \pi^*$	
	λ_{max} (nm)	$\log \epsilon$	λ_{max} (nm)	$\log \epsilon$
2/5	434	4.5	512	4.6
1/2	434	4.5	516	4.6
2/3	436	4.6	516	4.8
1	436	4.6	518	4.8

complexes (6, 7), we have also observed the formation of EDA complexes in the analyzed extracellular red pigments, which should stabilize them against the effects of light exposure (15, 18).

(i) Solvent effect. The solvent effects on these bands (K and B) are quite analogous to those on the absorption band characteristic of binary charge transfer complexes (7, 12). The bathochromic effect (solvatochromism effect) is a function of the hydrophobicity of the solvents; this is true for the two K and B bands for our products, and it can be noted that there is about a 38-nm difference between the results for aqueous medium and DMSO (Table 4).

(ii) Dilution effect. The molar extinction coefficient (ϵ) varied as a function of the dilution factor (in butanol or water); bathochromic and hyperchromic effects were observed, depending on the concentration (Tables 5 and 6).

These results (solvent effect and dilution effect) show evidence of the occurrence of complexes of the EDA type between the different compounds of the mixture of colorants.

Tast polarography. The current-potential curves obtained by tast polarography are presented in Fig. 5. Curve 1 is the residual current corresponding to a deaerated 0.2 M KCl solution. Curve 2 was obtained with 10^{-3} M pigment in a deaerated 0.2 M KCl solution and shows three cathodic waves, the half-wave potentials of which are located, respectively, at -0.65 , -0.97 , and -1.35 V.

The amplitude (I) of the first wave was directly proportional to the square root of the height (h) of the mercury column: $I_{\text{limiting}} - 0.88 \text{ V } (\mu\text{A}) = -0.44 + 0.109h^{0.5}$ ($40 \text{ cm Hg} < h < 83.5 \text{ cm Hg}$). Under the same conditions, when the solution of pigment is diluted in 0.2 M KCl, the height of the diffusional wave at -0.88 V decreases linearly with the residual concentrations of which are responsible for this wave.

For the second wave the same results were obtained. Its amplitude was directly proportional to the square root of the height of the mercury column and directly proportional to the concentration of the pigment present in the solution: $I_{\text{limiting}} - 1.2 \text{ V } (\mu\text{A}) = 0.98 + 0.22h^{0.5}$ ($40 \text{ cm Hg} < h < 83.5 \text{ cmHg}$);

TABLE 4. Solvent effect on the K and B absorption bands, $\pi \rightarrow \pi^*$ (solvatochromism effect), at a final pigment concentration of 40 $\mu\text{g/ml}$

Solvent	λ_{max} (nm)	
	K $\pi \rightarrow \pi^*$	B $\pi \rightarrow \pi^*$
H ₂ O	422	498
Methanol	422	498
Ethanol	424	516
Butanol	424	518
DMSO	426	536

TABLE 6. Dilution effect in water on molar extinction coefficient (ϵ) and the absorption bands, $\pi \rightarrow \pi^*$ (hyperchromic and bathochromic effect), for an initial concentration of pigment of 100 $\mu\text{g/ml}$

Dilution factor	K $\pi \rightarrow \pi^*$		B $\pi \rightarrow \pi^*$	
	λ_{max} (nm)	$\log \epsilon$	λ_{max} (nm)	$\log \epsilon$
2/5	412	4.3	492	4.5
1/2	416	4.4	492	4.6
2/3	416	4.4	492	4.7
1	416	4.2	496	4.3

FIG. 5. Current-potential curves. (Left) Curves obtained by fast polarography at a dropping mercury electrode with the following parameters: 1 drop/s; $P_{Hg} = 1.1$ atm ($h_{Hg} = 83.5$ cm); counter electrode, Pt; reference electrode, Ag-AgCl-Cl⁻; r (scan rate), $0.3 \text{ V} \cdot \text{min}^{-1}$; N₂; KCl, 0.2 M; gelatin, 0.008%. Curve 1, residual current; curve 2, 10^{-3} M red pigment. (Right) Curves obtained with a thin film of carbon enclosed in a thin-layer cell with 10^{-3} M red pigment in 0.2 M KCl. $r = 15 \text{ mV} \cdot \text{min}^{-1}$; $\theta = 20^\circ\text{C}$. Oxidation: volume of thin layer, 42.5 μl ; order, 3a $\rightarrow$ 3b $\rightarrow$ 3c. Reduction: volume of thin layer, 43.5 μl ; order, 4a $\rightarrow$ 4b. SCE, saturated calomel electrode.

$I_{\text{limiting}} - 1.2 \text{ V } (\mu\text{A}) = 2,700 [\text{Red pigment}] \text{ mol} \cdot \text{liter}^{-1} (10^{-3} > [\text{Red pigment}] \text{ mol} \cdot \text{liter}^{-1} > 0.3 \cdot 10^{-3})$.

The first wave, corresponding to the reduction of an impurity, and the second wave, attributed to the reduction of the main compounds (pigment) present at a concentration of about $10^{-3} \text{ mol} \cdot \text{liter}^{-1}$, were obtained under conditions of diffusion limitation.

The height of the mercury column had no influence on the amplitude of the third wave, showing that it was not correlated to a single faradaic process but probably was caused by an adsorption phenomenon.

By this method it is also possible to show that the pigment is reducible on mercury at a cathodic range potential of -0.97 to -1.22 V , but it is difficult to determine the number of electrons exchanged.

Thin-layer electrochemistry. Thin-layer electrochemistry has several advantages. Due to the low volume of the treated solution, it is possible to transform all of the electroactive molecules present in a solution in a short time and, particularly, while the current-potential curve is being processed, during a period in which when the scanning rate is well suited to the selected concentration range. The potential scan rate was 15 mV/min .

The amount of charge, under these conditions, facilitated the easy determination of the number of electrons exchanged during the electrode reaction. To achieve this, the experimental amount of electricity, determined by means of the surface area under the current-potential curve, which corresponds to the total transformation of the product, was compared with the theoretical amount of charge calculated from the number of moles of electroactive substance contained in the thin layer.

The current-potential curves obtained when 10^{-3} M red pigment in 0.2 M KCl solution was subjected to oxidation on a

carbon electrode are presented in Fig. 5 (curves 3a, 3b, and 3c). Curve 3a shows that the oxidation of pigment begins at 0.4 V and peaks at 0.665 V . The measurement of the corresponding charge leads to an electron number of 1 per molecule of pigment (on the basis of a concentration equal to $10^{-3} \text{ mol} \cdot \text{liter}^{-1}$), showing that the oxidation gives a radical R $\cdot$.

Curve 3b in Fig. 5, obtained on the return scan, shows a signal in the potential range between 0.9 and 0 V . For potentials lower than 0 V , the curve shows one peak at -0.28 V , corresponding to about 1.5 electrons. This cathodic signal is located in the range of oxygen reduction to water, which requires four electrons. The difference between the two electron numbers (4 and 1.5) is probably due to a chemical reaction between the radical R $\cdot$ and the oxygen dissolved in the solution. This chemical reaction can explain the important decrease of the amplitude of the anodic peak at 0.665 V in curve 3c.

The current-potential curve for a solution of pigment subjected directly to reduction (Fig. 5, curves 4a and 4b) indicates a signal at -0.35 V , which was attributed to the dissolved-oxygen reduction. This reduction gives many intermediates (the final step of the reduction is water) that are very reactive, such as H₂O₂ and OH $\cdot$. These intermediates can oxidize the pigment and give unidentified products, which are responsible for the four signals present as shoulders at 0.23 , 0.5 , 0.57 , and 0.75 V .

These results confirm the donor-acceptor behavior of the red pigment molecules. Indeed, the electrochemical study permits the conclusion that the red pigments (mixtures of four compounds) behave towards the carbon or mercury electrodes as pure compounds free of all impurities. For all of the compounds, the function which exchanges the electrons is an amine-quinone group.

FIG. 6. Daylight decoloration of red pigments dissolved in *n*-butanol and water (50 µg/ml). OD, optical density.

The following reaction can be written for the oxidation step:

In the case of the cathodic signal at -0.97 V, the electron acceptor group is again the chromophore by means of the quinone function. The signal at -0.65 V is attributed to an impurity.

Photochemical tests. Photobleaching tests with degassed solutions show that pigment degradation continues in aqueous solution, and the bleaching kinetics were the same in the presence or absence of oxygen. This means that pigment degradation is due to the light energy. We also checked that samples protected from light (in the presence or absence of oxygen) do not degrade after a long period of time. We can thus say that pigment degradation is photochemical ($h\nu$) and not due to oxidation by the dissolved oxygen. In contrast, the pigments dissolved in butanol are stable to light exposure for several weeks (Fig. 6). We think that water dissociates the EDA complexes. It seems that EDA complexes in polar solvents are very unstable (3), thus diminishing the stability of the solution in daylight; this does not happen in *n*-butanol solutions, where the complexes are more stable.

It is clear that cultures of long duration, such as is often the case with fed-batch fermentations (>120 h) (1, 13), should be avoided, since a loss in the extracellular pigment concentration was observed before fungal growth stopped. The pigments were not consumed by the fungus, as neither carbon nor nitrogen limitation occurred. The electrochemical study has shown EDA complex properties within these molecules (GTR, GTM, GCR, and GCM) and also their irreversible reduction or oxidation. In light of this, it might be asked if these pigments play a physiological role for the fungus. These molecules (quinones) can absorb electrons under certain conditions and may also liberate electrons (amine) by oxidation.

In summary, this work has elucidated two new molecules (GCR and GCM) that constitute 10% of the total extracellular coloring matter isolated and identified under culture conditions with glucose as the carbon source and in excess and with 5 g of monosodium glutamate per liter as a nitrogen source.

^{13}C NMR study of GTR, GTM, GCR, and GCM has enabled the structures of these molecules to be determined and should enable their metabolism (polyketide pathway) to be elucidated. UV spectroscopy and electrochemical study of these molecules enabled the description of the formation of EDA complexes, which can stabilize them against degradation by light. *n*-Butanol was the best extraction solvent and enabled red pigments to be stabilized against the effect of light for 6 months, while pigments solubilized in water faded after only 1 week. It seems that *n*-butanol can promote this molecular organization of the EDA complex type, while water can destabilize it.

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