Acknowledgments

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Added in Proof

Since preparation of this paper, Dietrich and Montes de Oca (1970), using somewhat different methods, have reported the biosynthesis of heparan sulfate by HeLa, L. mouse embryo, and rat embryo cells in culture.

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Identification of an Ommochrome in the Eyes and Nervous Systems of Saturniid Moths*

Alfred M. Ajami and Lynn M. Riddiford†

ABSTRACT: A substituted phenothiazine pigment has been isolated and characterized from the optic lobes, larval ocelli, and nerve cords of saturniid moths. By comparative spectrophotometric, colorimetric, chromatographic, and chemical degradative techniques we have demonstrated it to be related to ommin—an ancillary pigment found in the compound eyes of insects. The optic lobe, ocellar, and ganglionic ommochrome display absorption maxima in the blue region of the spectrum when oxidized by molecular oxygen or hydrogen peroxide. A shift toward green wavelengths accompanies reduction by ascorbic acid, sodium dithionite, and similar reductants in a variety of solvents. Estimation of the oxidation potential with indicator dyes has placed the E_0' of the pigment at pH 7 between 0.123 and 0.217 mV.

The ommochromes are a group of natural organic pigments widely distributed in the animal kingdom (Fox and Vevers, 1960). They are biochemically defined as products of tryptophan metabolism and arise from the oxidative coupling of 3-hydroxykynurenine molecules. There are three classes of ommochrome pigments, all of which show a characteristic change of color upon oxidation or reduction (Butenandt and Schäfer, 1962; Linzen, 1966).

In arthropods ommochromes have been found mainly in the eyes and ocelli (Becker, 1942; Ziegler, 1961; Butenandt and Schäfer, 1962). In this paper we report the extraction and purification of ommochromes from the compound eyes of two saturniid moths. These pigments are then used as reference standards for the identification of the embryonic ocellar, pupal optic lobe, and ganglionic pigments of the same species.

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Materials and Methods

1. Experimental Animals and Preparation of Tissue Samples. *Hyalophora cecropia* and *Antheraea pernyi* were purchased from dealers or reared indoors on an artificial diet as described by Riddiford (1968) and chilled at 5°. Optic lobes and ganglia excised from diapausing pupae and heads from adults of both species were minced in distilled water, lyophilized, and stored at −20°. Embryos during the last third of embryonic development were removed from the eggs and decapitated. The heads were lyophilized from distilled water and stored at −20° until several grams of material had accumulated.

2. Extraction and Purification of Pigments. Each of the pigment sources was homogenized in 80% acetone (v/v), the homogenate was centrifuged at 10,000*g*, and the supernatant was discarded. The pellet was resuspended and washed twice again using the same procedure, and was then preextracted with 50% methanol (v/v) for 12 hr at 40°. The mixture was centrifuged as above, the supernatant was discarded, and the pellet was washed twice with methanol. Ommochromes were extracted from this pellet into 0.5% concentrated HCl–meth-

anol (w/v) during 10 min and harvested by repeated precipitations from 0.1 M Na_2HPO_4, as described by Butenandt *et al.* (1954, 1958).

We further purified these pigments by ion-exchange chromatography according to Osanai and Koga (1966). Aliquots of eye pigment extract, dissolved in 0.5% HCl–methanol, were placed on an SE-Sephadex C-50 (medium) column (45 × 2.5 cm). For the optic lobe and ocellar pigment extracts, 0.2-ml aliquots were placed on a smaller column (10 × 1 cm) of the same resin. After rinsing with 0.1 N HCl, ommatins were eluted with 0.5 N HCl and ommins with 0.5 N NaOH. Pigment extracts from ganglia were found to be too dilute for convenient purification by this method. Throughout all procedures the temperature was maintained at 0–4° since the extracted pigments are known to be heat labile in acidic or basic solvents.

3. Spectrophotometry. Absorption spectra of the ommochromes purified in this manner were determined in various solvents using the Cary 14 recording spectrophotometer. The solvents in which ommochromes dissolved most readily were 1 N NaOH, 0.033 M sodium phosphate buffer (pH 7.0), glacial acetic acid, absolute formic acid, 0.5% HCl–methanol, and concentrated H_2SO_4. Infrared spectra were measured in a potassium bromide tablet using the Perkin-Elmer 302 recording spectrophotometer. Nuclear magnetic resonance spectra of the pigments and their hydrolysis products were performed on the Varian A-60 in dimethyl sulfoxide or trifluoracetic acid.

4. Oxidation and Reduction Reactions. Solutions of purified ommochromes in 0.033 M sodium phosphate buffer (pH 7.0) and in 0.5% HCl–methanol were reacted with oxidizing and reducing agents. Oxidation was accomplished by adding 3% hydrogen peroxide to a final concentration of 0.75% or by adding 1 volume of saturated sodium nitrite solution to 5 volumes of pigment solution. Reduction was accomplished by adding saturated solutions of either sodium thiosulfate, sodium dithionite, sodium borohydride, ammonium sulfide, or ascorbic acid in the same proportions as used for the oxidation of the pigment with nitrite. Solutions treated in this manner were routinely filtered through a Quickfit microfiltration apparatus in order to remove the fine precipitate that often formed upon standing.

We estimated oxidation–reduction potentials spectrophotometrically using phenol indophenol, 2,6-dichlorophenol indophenol, 1-napthol-2-sulfonate indophenol, toluylene blue, thionine, and methylene blue as redox indicators (Long, 1968). Samples of either reduced or oxidized ommochrome pigment in 0.05 M sodium phosphate buffer (pH 7.0) were added to 1.0×10^{-7} M solutions of dye. Absorbance changes were recorded by difference spectroscopy in the Cary 14 spectrophotometer with untreated indicator solution as the reference.

5. Thin-Layer and Paper Chromatography. We performed thin-layer chromatography of the ommochromes by spotting 10- to 20-μl aliquots of fresh 0.5% HCl–methanol solutions onto cellulose powder plates (MN-Polygram CEL 300, Brinkmann Instruments) and onto silica gel G plates (spread 250 μ thick, activated at 110° for 30 min). These were developed in saturated chambers with the following solvent systems: formic acid–methanol–HCl (15:3:1, v/v) (Butenandt *et al.*, 1958); methanol–butanol–benzene–water–HCl (10:5:5:5:1, v/v); and collidine–lutidine–water (1:1:2, v/v). Paper chromatography was by the descending method on Whatman No. 40 filter paper using the same solvents. We determined visually the position of the pigments on the developed chromatogram.

6. Chemical Assays. Both alkaline and acid hydrolysis products of ommochromes from compound eyes, ocelli, optic lobes, and ganglia were obtained according to Butenandt *et al.* (1958); 50-μl aliquots of the alkaline hydrolysate were spotted onto Whatman No. 1 filter paper, followed by ascending chromatography in butanol–acetic acid–water (4:1:1) with xanthurenic acid and 3-hydroxykynurenine as standards. After 6 hr, the chromatograms were dried and observed under an ultraviolet lamp; the separated compounds then were revealed with either ninhydrin reagent (Moore and Stein, 1948) or nitroprusside reagent (Anson, 1941).

Acid hydrolysis of ommochromes was accomplished by refluxing samples with 4000 parts of formic acid–HCl (1:1) under a nitrogen atmosphere. The reaction products were separated by ascending paper chromatography in butanol–acetic acid–water (4:1:1) as before, and the chromatograms were developed as described above.

The Carr–Price reaction for carotenoids (Carr and Price, 1926) was carried out as follows: freshly excised optic lobes and ganglia were precipitated with acetone, dehydrated with acetic anhydride, and then treated with 0.5 ml of chloroform saturated with antimony trichloride.

7. Materials. Organic solvents were purchased from Fisher Scientific. They were refluxed over powdered zinc metal–KOH to remove sulfurous oxidants and trace contaminants, then distilled under reduced pressure. All other reagents were A grade and used without further purification. Xanthurenic acid and 3-hydroxykynurenine were obtained from Aldrich and the redox indicators from Eastman. Sephadex SE-50 was from Pharmacia.

Experimental Results

1. Solubility Properties. The eye pigments extracted from Cecropia and Pernyi were insoluble in acetone, diethyl ether, methanol, ethanol, propanol, chloroform, petroleum ether, and distilled water. The optic lobe and larval ocellar pigments of both species and the ganglionic pigment of Cecropia showed similar properties. Since they are insoluble in organic solvents, these pigments cannot be carotenoids. Further evidence for this conclusion was afforded by a negative reaction with antimony trichloride. Both purified ommochromes and pigments in acetone-dried tissues failed to develop the intense blue color typical for carotenoids.

After 15-min incubation in the cold 1 N NaOH, 20% NH_4OH, and nitrogen salt buffers of pH greater than 8.0, the pigments were eluted from intact tissues. The pigments also dissolved readily in acidified methanol, acetic acid, formic acid, concentrated HCl, 50% H_2SO_4–methanol, and concentrated H_2SO_4. Because of their solubility in cold bases, HCl, and H_2SO_4, one may conclude they are not melanins (Thomson, 1962).

Ommins from the adult eyes remained stable in 1 N NaOH for more than 24 hr at room temperature and in 10 N NaOH for more than 2 hr as did the ocellar, ganglionic, and optic lobe pigments. This evidence suggests that the latter are not C_7-substituted pteridines—like erythropterin (Pfleiderer, 1963)—nor the alkali-unstable ommatins, a conclusion further substantiated by the adsorption properties of the optic lobe and ocellar pigments on SE-Sephadex. These pigments cochromatographed with the eye ommins when eluted with 0.5 N NaOH.

2. Absorption Spectroscopy. The visible absorption spectra and the oxidation–reduction properties of the pigments extracted from the various tissues were recorded in a variety of solvents and found to be typical of ommochromes. Figure

TABLE I: Comparison of the Visible Absorption Maxima of Optic Lobe and Ganglionic Pigments from *H. cecropia* and *A. pernyi*.

Solvent	Optic Lobe	Ganglia[a]	Ommin	Xanthommatin
1 N NaOH	505	500–505	500	380
0.33 M sodium phosphate, pH 7.0				
oxidized[b]	470		475	435
reduced[c]	520		520	490
Glacial acetic acid	512		510	465
0.5% HCl–methanol				
oxidized[d]	420	420	425	
	470	465	465	450
reduced[e]	390		390	370
	514	515	515	480
Formic acid	480		480	
	516	510	510	
	538	540	540	
H_2SO_4 (concd)	380		380	370
	412		410	410
	445	440	440	440
	545	550	550	525

[a] All ganglionic pigment preparations were from *H. cecropia*. [b] Oxidized with H_2O_2 in 0.75% final concentration. [c] Reduced with sodium dithionite added as solid. [d] Oxidized with $NaNO_2$ added as solid. [e] Reduced with ascorbic acid added as solid.

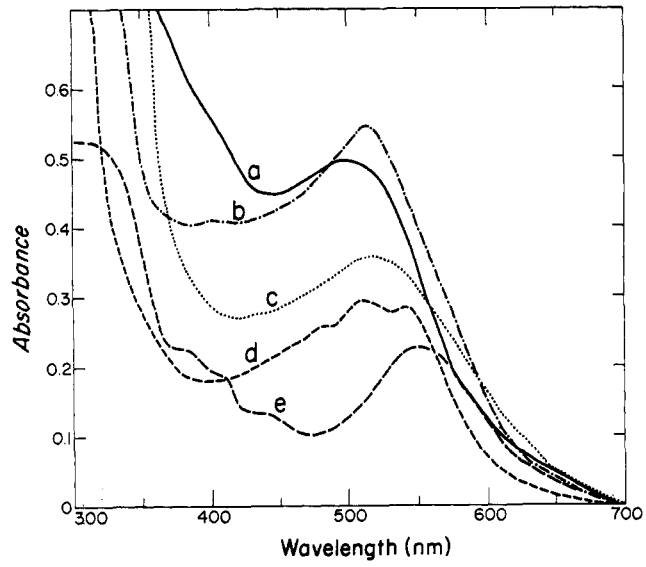

FIGURE 1: Visible absorption spectra of ommin extracted from Cecropia eyes The tracings were zeroed arbitrarily at 700 nm, since the absorbance at that wavelength in each of the solvents did not exceed 0.02. Solvents: a, 1 N NaOH; b, 0.5% HCl–methanol; c, 0.03 M sodium phosphate buffer (pH 7.0); d, formic acid; and e, concentrated sulfuric acid.

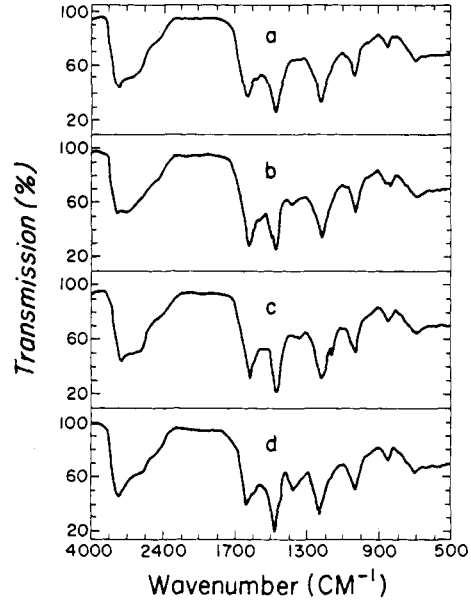

FIGURE 2: Infrared spectra of Cecropia ommochromes taken in KBr tablets. Pigment sources are: a, optic lobes, b, ocelli; c, gangliar and d, eye ommin.

1 presents five of the visible absorption spectra of ommin from the moth eyes, whose absorbances have been arbitrarily zeroed at 700 nm. In each case the maxima of the unknown coincided with those of adult eye ommin (Table I), and preliminary evidence indicates that the molar absorbance coefficients of these pigments in 0.5% HCl–methanol also coincide with a value of 11,000. It should be noted that ganglionic pigment could not be obtained in pure form nor in sufficient concentration so as to display sharp absorption bands, but rather only absorption plateaus over the expected wavelength range.

For the purpose of identification of the chemical nature of the chromophores, sulfuric acid was the best solvent. Adult eye ommins and the three unknown pigments showed a deep purple color, with an absorption maximum at 550 nm (±5 nm), which is characteristic of the ring-substituted phenazothionium ion (Shine and Mach, 1965). In addition, the 545/445 nm absorbance ratios of the unknowns are in good agreement with the value of 1.8 found for eye ommin; this evidence further suggests that the unknown pigments are ommochromes, most likely of the ommin class.

The infrared spectra of optic lobe, ocellar, and ganglionic pigments measured in KBr are shown in Figure 2 (a,b,c). They differ clearly from those of ommatin and ommidin pigments (Butenandt *et al.*, 1954; Linzen, 1966), but resemble those of ommin from adult eyes (Butenandt *et al.*, 1958) (Figure 2d). All samples displayed absorption maxima as follows: a broad band at 3050–3450 cm^{-1}, indicating a carboxylic acid function as well as CH and NH stretching vibrations; at 1650 and 1425 cm^{-1}, characteristic of carboxylic acids and of NH and CH bending vibrations; and at 1240 and 1050 cm^{-1}, indicating CO and CN stretching vibrations (Dyer, 1965). None of the samples showed an absorption peak at 1740 cm^{-1} which is peculiar to ommochromes of the xanthommatin type.

3. Estimation of Oxidation–Reduction Potentials. The oxidized pigment from both optic lobes and ganglia as well as ommin from Cecropia eyes were reduced by the leuco forms of 1-naphthol-2-sulfonate indophenol (E_0' = 0.123 mV at pH 7.0), toluylene blue (E_0' = 0.115), thionine (E_0' = 0.062), and methylene blue (E_0' = 0.011). The reduced ommochromes in

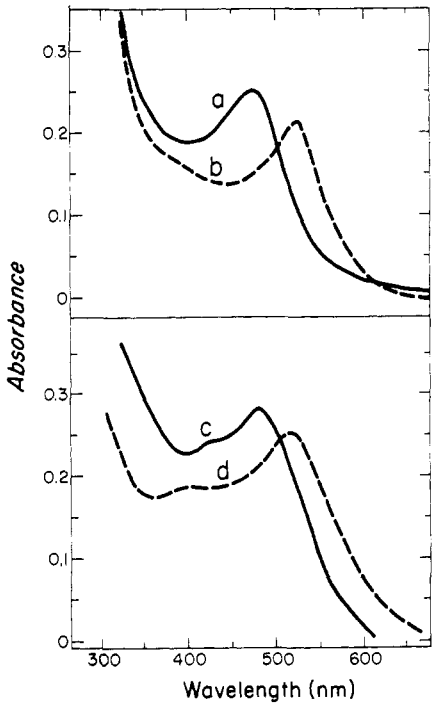

FIGURE 3: Visible absorption spectra of ommin: a, oxidized in 0.033 M sodium phosphate buffer (pH 7.0); b, reduced in sodium phosphate buffer (pH 7.0); c, oxidized in 0.5% HCl–methanol; and d, reduced in 0.5% HCl–methanol.

turn are oxidized by 2-6 dichlorophenol indophenol (E_0' = 0.217) and phenol indophenol (E_0' = 0.227). This evidence suggests that the redox potential of ommochrome from ganglia and optic lobes lies at an intermediate value between 0.123 and 0.217 mV at pH 7.0. Representative absorption spectra of the oxidized and reduced ommochrome in two solvent systems are presented in Figure 3.

4. Thin-Layer and Paper Chromatography. Separation of ommochrome extracts from the four pigment sources gave similar results in three solvents. Ommin from eyes and the unknowns cochromatographed homogeneously as dark red spots in the solvent system formic acid–methanol–HCl (15:3:1) on Whatman No. 4 paper (R_F 0.69), on silica gel G plates (R_F 0.71), and on cellulose plates (R_F 0.72). With methanol–butanol–benzene–water–HCl (10:5:5:5:1) on silica gel G plates the eye pigment was resolved into three components—two reddish brown spots and a purple smear with R_F values of 0.78, 0.74, and 0.50–0.65, respectively. In the same solvent system, optic lobe, ocellar, and ganglionic pigments showed two distinct spots of R_F 0.78 and 0.7. A similar separation was obtained when we used collidine–lutidine–water (1:1:2) namely, three spots for ommin from eyes (R_F 0.76, 0.46, 0.35) and two spots for each of the unknown samples (R_F 0.75 and 0.47) (Table II).

The R_F values of the ommin standards extracted from adult eyes coincide in the three different solvent systems with those of pigments from optic lobes, larval ocelli, and ganglia.

5. Chemical Assays. Xanthurenic acid is known to be one of the products of alkaline digestion of ommin. We chromatographed the alkaline hydrolysates of ganglionic, ocellar, and optic lobe pigment together with commercially available xanthurenic acid. In each case a blue ultraviolet fluorescent spot corresponding to xanthurenic acid was identified (R_F 0.59 ± 0.01).

TABLE II: Comparison of R_F Values of Ommin and of Xanthommatin.

Solvents	Stationary Phase	Ommin	Xanthommatin
Formic acid–methanol–HCl	Paper	0.69	0.80
Formic acid–methanol–HCl	Silica gel	0.71	0.75
Methanol–butanol–benzene–water–HCl	Silica gel	0.78 0.74	0.82
Collidine–lutidine–water	Paper	0.40	0.25
Collidine–lutidine–water	Silica gel	0.76 0.46	0.32

Acid hydrolysis of each pigment yielded two major ninhydrin reactive spots, one of which cochromatographed with 3-hydroxykynurenine (R_F 0.44 ± 0.01). The other spot, a red pigment, remained near the origin. It showed positive nitroprusside and ninhydrin reactions. We further characterized this hydrolysis product of the eye and optic lobe pigment by nuclear magnetic resonance spectroscopy. The spectra of both samples in dimethyl sulfoxide indicated aromatic protons at δ 7.39 (singlet) and 7.15 (singlet) and an amino acid functional group adjacent to a methylene carbon at δ 3.80 (triplet) and δ 3.25 (multiplet). The region between δ 7.4 and 4.08 was obscured due to the presence of water.

Discussion

The most abundant pigments in the lepidopteran eye are ommochromes (Linzen, 1967) which, as seen above, can be readily differentiated from pteridines, melanins, and carotenoids. Therefore, we used these eye ommochromes as reference standards in a variety of chemical procedures as a means of identification of the reddish pigments in the embryonic ocelli and pupal optic lobes of Pernyi and Cecropia and in the ganglia of Cecropia. These latter pigments proved to be ommochromes of the ommin type as their solubility, oxidation–reduction, spectral, and chromatographic properties coincided with the known eye ommins. Also, hydrolyses products of these pigments were identical with those of the eye ommins.

Acknowledgments

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Purification and Characterization of an Ommochrome–Protein from the Eyes of Saturniid Moths*

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ABSTRACT: An ommochrome–protein has been extracted from the eyes of the silkmoth *Hyalophora cecropia* with the cationic detergent cetyltrimethylammonium bromide. The protein was purified by Sephadex G-200 and agarose gel filtration. It is homogeneous as judged by rechromatography and disc electrophoresis and has a molecular weight of 24,000 ± 1500. When oxidized by hydrogen peroxide, the pigment–protein shows absorption maxima at 440 and 470 nm and a shoulder at 510 nm. Upon reduction with ascorbic acid, a single broad peak at 510 nm results. Determination of the oxidation–reduction potential of the ommochrome–protein was accomplished by single point equivalency determinations; a value of 196 ± 7 at pH 7.0 was obtained. The chromophore, a substituted phenothiazine, appears to be covalently linked to its protein carrier and may be hydrolyzed by a variety of acidic or basic solvents. When free in solution at neutral pH, the chromophore chelates divalent cations, including Cu^{2+}, Fe^{2+}, and Ca^{2+}.

These results are discussed in relation to the biological significance of the ommochrome pigments which are found in the optic lobes, ocelli, and nerve cords of certain moths.

Although direct supporting evidence is scanty, the existence of polymeric or macromolecule-bound ommochromes has been suggested with regularity in reviews about the visual system of insects (Ziegler and Harmsen, 1969). Goldsmith (1964) reported that ommochromes in retinula cells "are usually bound to protein in discrete granules," and subsequently three electron microscopic studies have confirmed this opinion (Fuge, 1966; Schoup, 1966; Langer, 1967).

Attempts to identify the pigment–protein complexes by biochemical means have met with partial success. Bowness and Wolken (1959) claimed to have isolated a light-sensitive yellow pigment from housefly eyes, whose spectroscopic properties resembled those of xanthommatin and whose adsorptive properties on calcium phosphate columns indicated a protein. Osanai (1966) obtained an aqueous extract with redox properties from the integument of *Hestina japonica*, and he concluded that it was an ommochrome–protein of the xanthommatin type. In a more detailed study, Yoshida and Ohtsuki (1966) succeeded in partially characterizing, by extraction and gel filtration with cetyltrimethylammonium bromide,[1] a "photoactive" xanthommatin of large molecular size—presumably a protein-bound pigment—from the ocelli of the anthomedusan *Spirocodon saltatrix*.

In the preceding paper we identified an oxidation–reduction pigment from the eyes and nervous system of saturniid moths (Ajami and Riddiford, 1971). Our preliminary results showed that it was bound to a protein, and this paper discusses the isolation and characterization of such a protein from the eyes of *Hyalophora cecropia*.

Materials and Methods

1. Preparation of Tissues for Extraction. H. cecropia were obtained and treated as described previously (Ajami and Riddiford, 1971). Freshly emerged adult moths were decapitated and the compound eyes carefully dissected. These were washed twice with distilled water by homogenization, centrifugation (10,000*g* for 10 min), and decantation of the supernatant. The sediment was lyophilized and stored at −20° until used, but for no more than 48 hr.

2. Buffered Extraction. The eye powder was precipitated with acetone; then after centrifugation and decantation of the supernatant, we extracted for 1 hr at 25° the equivalent of two eyes (either consecutively or separately) with 1 ml of one of the following buffers: 0.05 M potassium chloride–hydrogen chloride (pH 2.0); 0.05 M sodium acetate–acetic acid (pH 5.0); 0.05 M sodium phosphate (pH 7.0); 0.05 M Tris-HCl (pH 7.0); 0.05 M Tris-HCl (pH 8.3); 0.05 M ammonium bicarbonate (pH 8.8); 0.05 M sodium carbonate (pH 10.0). The solutions

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: CTAB, cetyltrimethylammonium bromide.