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The Intracellular Blue Pigment of *Pseudomonas lemonnieri**

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Summary. The large-scale production, isolation, and purification are described of the blue insoluble intracellular pigment of the bacterium *Pseudomonas lemonnieri.* The pigment, $C_{26}H_{37}N_5O_6$, occurs in the cells as a salt (cation unknown) of 6-octanoylamino-3-hydroxy-2-aza-benzoquinone-(1,4)-4-[5-octanoylamino-2,6-dihydroxy-pyridyl-(3)-imide] (I). Nitric acid oxidation of pigment I yields IV, 6-octanoylamino-3-hydroxy-2-aza-benzoquinone-(1,4). Further hydrolysis of IV splits off *n*-octanoic acid, which is free of homologues. The structures given for the pigment and its degradation products have been proven by identification with authentic preparations.

Although they have different chromophores, the pigment (I) of *Pseudomonas lemonnieri* and N,N'-dioctanoyl-indigoidine (VI) nevertheless resemble one another in IR-absorbances, NMR-spectra, and chromatographic behavior, because of homogeneous functional groups and ring structures. I and VI are indeed chemically related, as can be seen from the facts that aminocitrazinic acid is a common starting material for the in vitro syntheses of both compounds, and that the diaza-indophenol (I) can be converted to the diaza-diphenoquinone (VI) by hydrogenation and subsequent autoxidation.

Pseudomonas lemonnieri (Lasseur) BREED *et al.* forms an intracellular insoluble pigment which colors its colonies a brilliant blue. During collaborative studies with Professor RICHARD KUHN on indigoidine (STARR, 1958; KUHN, STARR *et al.*, 1965; KUHN *et al.*, 1965), we turned our attention briefly to the blue pigment of *P. lemonnieri* and suggested rather cautiously (STARR *et al.*, 1960) that it might be a phenazine. Recently, we have undertaken a more thorough study of this pigment which leads us to reject our earlier suggestion.

The taxonomic position of *Pseudomonas lemonnieri* has been discussed (HUGO and TURNER, 1957; STARR, BLAU, and COSENS, 1960). The monumental study of STANIER *et al.* (1966) refers this organism to biotype *F* of *Pseudomonas fluorescens.* The significance of this reclassification for our present purposes is that *Pseudomonas lemonnieri* is considered not to be closely related to *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* biotypes *D* and *E*, or *Pseudomonas multivorans*, which together produce

* Dedicated with devotion and admiration to Professor C. B. VAN NIEL on his seventieth birthday.

most of the familiar bacterial phenazine pigments—notably, pyocyanine, chlororaphine, oxychlororaphine, and phenazine α-carboxylic acid—as well as some phenazines of undetermined structure. It was, thus, taxonomically significant to learn from the present study that the intracellular blue pigment of *P. lemonnieri* is in fact not a phenazine. This finding supports the systematic placement (STANIER *et al.*, 1966) of this organism outside of the phenazine-producing groups of the genus *Pseudomonas*. To avoid the circumlocution which would result from using the revised nomenclature, we retain here the designation *Pseudomonas lemonnieri* with the intended meaning: *Pseudomonas fluorescens*, biotype *F*, strain which produces the "*Pseudomonas lemonnieri* pigment".

This report corrects information published previously (STARR *et al.*, 1960), and explains the basis for our belief that the chemical structure of the *P. lemonnieri* pigment is a salt (cation unknown) of 6-octanoylamino-3-hydroxy-2-aza-benzoquinone-(1,4)-4-[5-octanoylamino-2,6-dihydroxy-pyridyl-(3)-imide] (I). A detailed study of the structure and synthesis of the pigment is presented elsewhere (KNACKMUSS, 1967).

Production of Pigment

In the interest of better yield and higher purity of the product, we have modified the conditions previously reported (STARR *et al.*, 1960) for the large-scale production of the *Pseudomonas lemonnieri* pigment.

The culture medium used ("YDC") contained Difco yeast extract, 1.0%; glucose, 2.0%; calcium carbonate (USP light powder, no. 4052 of Mallinckrodt), 2.0%; Difco agar, 1.5%. Autoclaving was at 15 lbs for 30 min, which caramelizes the glucose somewhat. (It was observed that the medium which was sterilized at 10 lbs for 20 min yielded only about one-fifth the pigment produced on the caramelized medium.) The agar medium in flasks was cooled to 47° C, the flasks were swirled gently to suspend the calcium carbonate and the medium was poured into 100 mm petri plates. This departure from the large-scale production method recommended previously (STARR *et al.*, 1960) results from our finding that the large trays retained too much moisture, a factor which appeared to interfere with a high yield of pigment.

Several darkly pigmented colonies of the Stanier strain of *Pseudomonas lemonnieri* (*ICPB-2148* of the International Collection of Phytopathogenic Bacteria) were suspended in 2 ml nutrient broth and used for inoculum. The YDC medium was streaked heavily in two directions with this inoculum, using a standard transfer loop. The plates were incubated at room temperature, about 25° C, for three weeks.

The blue growth was scraped from the plates with a bent glass rod and the cell mass was suspended in a small amount of water, and then centrifuged at 5,000 rev/min (3,440 × g) in a Servall SP-X centrifuge for 30 min to pack the cells. The water was poured off and the pellet frozen in the centrifuge tubes until it could be extracted. Lyophilization, which was previously recommended, is not necessary.

Isolation of Crude Pigment

The crude pigment was isolated by the following procedure, which is scaled to the yield of *P. lemonnieri* cells from 120 petri plates:

In order to liberate the free acid of the pigment, the wet packed cell material (30—40 ml) was treated with 200 ml of methanol plus 4 ml of concentrated HCl in

a Waring Blendor for about a minute. After addition of 200 ml of water, the pigment was extracted as fast as possible, in several batches, with a total of 800 ml of chloroform using the Waring Blendor. The blue-red chloroform-methanol layers were separated and the several batches were combined. Evaporation of the solvents in vacuum (bath temperature, 40° C) left behind a sticky blue-brown mass. The pigment was freed from the lipid material by washing with petroleum ether (B. R., 30—60° C) so that a flaky, blue, red-lustered material was obtained. The average yield was 1.4 mg of crude pigment per petri plate, based on experience with over 3000 plates.

Purification of the Pigment

The crude pigment of *P. lemonnieri* may be recrystallized from small amounts of dimethylsulfoxide, dimethylformamide, or acetic acid. For the analyses, the pigment was recrystallized twice from hot dimethylformamide.

50 mg of the crude pigment were dissolved in 5 ml of dimethylformamide at about 100° C. After filtration through a G3 fritted glass filter and cooling in an ice bath, the pigment separated in crystals with a copper-like luster. The crystals were washed with a few drops of cold dimethylformamide and then thoroughly with methanol. After drying at 110° C in vacuum, the yield was 30—31 mg. The purified pigment (I) melts at 288° C.

Properties and Structure of the Pigment

Elemental analyses of the extracted and purified bacterial pigment (I) agree with the empirical formula $C_{26}H_{37}N_5O_6$ (found: C, 60.51; H, 7.24; N, 13.47; $C_{26}H_{37}N_5O_6$ requires: C, 60.56; H, 7.23; N, 13.58). By the Kuhn-Roth determination, two CH_3-groups per molecule can be detected (found: C—CH_3, 6.15; calc.: 5.86). If powdered, pigment I shows a deep blue color; if pressed on a porous plate, it shows a copper-like metallic sheen. The purified pigment is sparingly soluble in the common solvents, and slightly more soluble in the more polar solvents acetic acid, dimethylformamide, and dimethylsulfoxide. Only in trifluoroacetic acid, have we found very good solubility. The pigment (I) and its alkali salts are insoluble in water, which explains why they cannot be reduced by sodium dithionite. Methanol must be added to effect hydrolysis of the pigment, because of its insolubility in hydrochloric acid.

The mass spectrum shows no molecular peak. Two peaks of equal intensity are observed for the masses 265 and 252.

The visible absorbance spectra show maxima at 627 mμ, log ε 4.96, in dimethylsulfoxide (with a weak shoulder at 595 mμ); at 625 mμ in dimethylformamide; at 635 mμ in pyridine; at 634 mμ in acetic acid; at 636 mμ in trifluoroacetic acid; and at 638 mμ in chloroform (with a shoulder at 600 mμ).

The IR-spectrum (Fig. 1) of the pigment (I) resembles that of diacetylindigoidine (KUHN, STARR *et al.*, 1965; KUHN *et al.*, 1965) because of

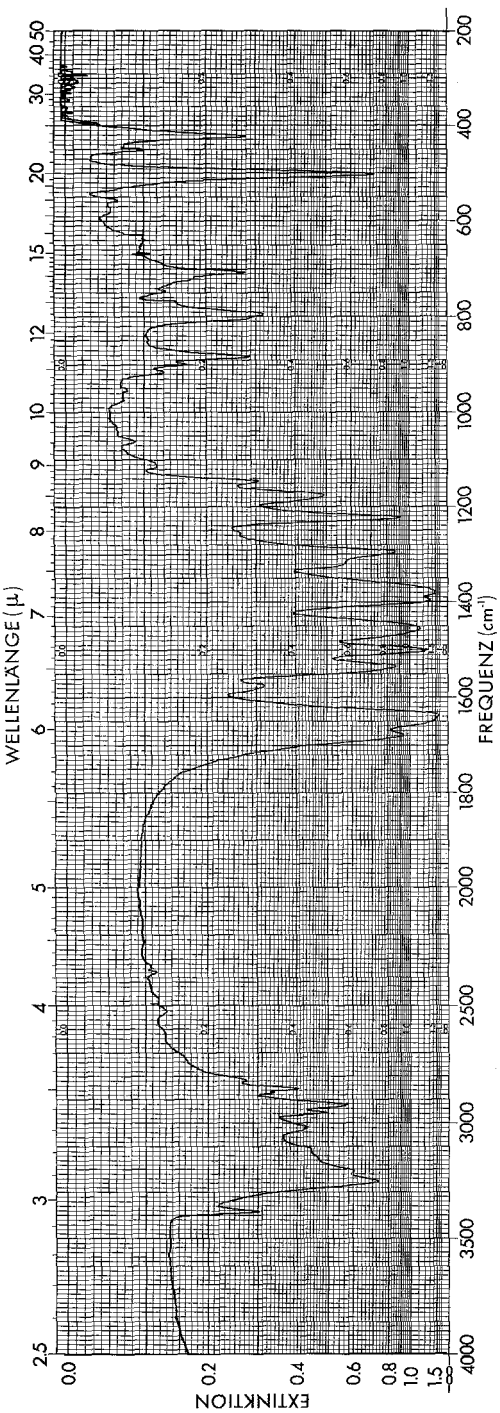


Fig. 1. Infrared-spectrum of the pigment (1) from *Pseudomonas lemonnieri* in KBr

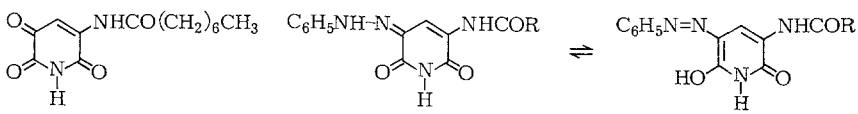
similar NH- and OH-vibrations and amide bands. A characteristic group of bands between 2830—2980 cm^{-1} is attributable to the octanoic acid residues.

The pigment (I) is degraded by cold nitric acid. A yellowish crystalline product (IV) is obtained in high yield:

Oxidation of I by Nitric Acid. 150 mg of crude pigment were dissolved in cold concentrated nitric acid (~ 2 ml; D: 1.4). The reaction mixture was stirred and kept in an ice bath until a clear yellow solution was obtained. When water was added (~ 20 ml), yellowish crystals separated; yield 106 mg (71 per cent). In another preparation, 50 mg of pure pigment yielded 40 mg (77 per cent) of the azaquinone IV. For chemical analysis, the crude product (106 mg) was recrystallized twice from hot water, yielding faintly yellow flat needles (57 mg), mp. 157°C (uncorr.). The analysis of IV agrees with the empirical formula $C_{13}H_{18}N_2O_4$ (found: C, 58.53; H, 6.98; N, 10.34; calc.: C, 58.63; H, 6.81; N, 10.52). The molecular weight determined osmometrically is 277, by mass spectrometry it is 266 (calc.: 266.3).

When IV is hydrolyzed by hot 6 N hydrochloric acid, an almost colorless oil, identified as *n*-octanoic acid, separates from the reaction mixture; it solidifies when cooled below 10°C.

Hydrolysis of IV. 5 mg of pure IV were hydrolyzed by 0.5 ml 6 N HCl in a sealed tube for 6 hours at 80°C. The oil was extracted with chloroform. The chloroform solution was washed with water, dried with $MgSO_4$, and the solvent evaporated. The oily residue was distilled in vacuum (75—80°C, 1 Torr). The product is identical with *n*-caprylic (= *n*-octanoic) acid in IR-spectrum and in melting point (mp 13.9—14°C uncorr.; authentic sample from E. Merck: 14.3°C uncorr., mixed mp. 14.1—14.2°C uncorr.).



IV Va Vb

R = $(CH_2)_6CH_3$

The azaquinone structure IV is likely for the nitric acid degradation product—based on its UV absorbance (λ_{max} 345 mμ, log ε 3.57), its IR-spectrum (γ_{CO} 1740 cm^{-1}) [which also has been found for 2,2′-dihydroxy-5,6,5′,6′-tetraoxo-5,6,5′,6′-tetrahydro-bipyridyl-(3,3′) (KUHN *et al.*, 1965)], and its NMR-spectrum [which showed one vinylproton (τ 2.43) and two OH- or NH-protons (τ 0.05 and —2.32) in addition to fifteen aliphatic protons]. The structure was confirmed for its phenylhydrazone Va (λ_{max} 438 mμ, log ε 4.54), for which the tautomeric azo-dye Vb can be written. The phenylhydrazone was identified by electron-, IR-, and Debye-Scherrer-spectra, as compared with an authentic sample synthesized from aminocitrazinic acid. (For analysis, the phenylhydrazone was recrystallized from dimethylsulfoxide with 10 per cent water added.

Found: C, 64.02; H, 6.77; N, 15.90; $C_{19}H_{24}N_4O_3$ requires: C, 64.02; H, 6.79; N, 15.72.)

As of now, no derivative of the blue pigment of *P. lemonnieri* (I) has been obtained directly. Acetylation destroys the chromophore or gives complex mixtures of unstable reaction products. Reductive acetylation produces a colorless oil, in which a number of products can be detected by thin-layer chromatography. The colorless spots, when exposed to moist air for several hours, regenerate the deep blue color of the pigment. No defined material could be isolated from the complex mixtures resulting from methylations.

Catalytic hydrogenation in trifluoroacetic acid and reoxidation by air converts the blue pigment (I) to a red one (VI), which is identical [visible absorbance spectrum (λ_{max} 515 mμ, log ε 4.43), IR-spectrum (γ_{CO} 1665 and 1520 cm^{-1}), and Debye-Scherrer-diagram] with 5,5′-dioctanoylamino-4,4′-dihydroxy-3,3′-diaza-diphenoquinone-(2,2′) (N,N′-dioctanoyl-indigoidine) (VI) prepared by octanoylation of indigoidine.

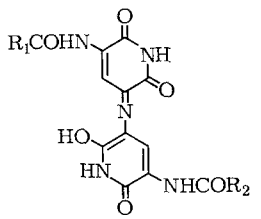
Hydrogenation and Subsequent Autoxidation of Pigment I. 149 mg (0.29 mmole) of pure pigment I were hydrogenated in 20 ml of trifluoroacetic acid with 100 mg of a Pd-$BaSO_4$ catalyst. Two equivalents of H_2 were quickly absorbed, while the color of the solution changed from blue to faint yellow. The catalyst was filtered off and the trifluoroacetic acid evaporated in vacuum. Methanol was added and evaporated several times to remove traces of the acid; the hydrogenation product turned more and more violet in the air. Finally red-violet crystals of VI separated. The reaction product was washed with a small volume of methanol and dried. Yield 87 mg, 60 per cent. For identification with the authentic sample (see next paragraph), the crude product was recrystallized from acetic acid.

Octanoylation of Indigoidine. 620 mg (5/2 mmole) of purified lyophilized indigoidine were suspended in 6.25 g octanoic acid anhydride and 8 drops of $BF_3 \cdot ET_2O$. The mixture was stirred and kept at 110° C for 3.5—4 hours. N,N′-dioctanoyl-indigoidine VI was separated by diluting the octanoic anhydride with a small amount of benzene, followed by centrifugation. The crude product was washed several times with benzene. It can be recrystallized from hot dimethylformamide or acetic acid. Found: C, 62.40; H, 7.26; N, 11.47; $C_{26}H_{36}N_4O_6$ requires C, 62.38; H, 7.25; N, 11.19.

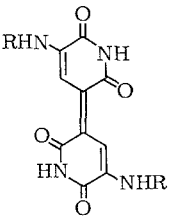
Discussion and Conclusions

An improved method is given for the large scale production, isolation, and purification of the blue insoluble intracellular pigment of the bacterium *Pseudomonas lemonnieri*. The pigment occurs in the cells as a salt (cation unknown) of 6-octanoylamino-3-hydroxy-2-aza-benzoquinone-(1,4)-4-[5-octanoylamino-2,6-dihydroxy-pyridyl-(3)-imide] (I). Nitric acid oxidation of pigment I yields IV, 6-octanoylamino-3-hydroxy-2-aza-benzoquinone-(1,4). Further hydrolysis of IV splits off *n*-octanoic acid, which is free of homologues.

The structures given for the pigment and its degradation products are certified by identification with authentic preparations (KNACKMUSS, 1967). Pigment I can be resynthesized in 80 per cent yield from IV by



I $R_1 = R_2 = (CH_2)_6CH_3$
II $R_1 = R_2 = CH_3$
III $R_1 = (CH_2)_6CH_3$; $R_2 = CH_3$



VI $R = CO(CH_2)_6CH_3$

condensation with amino-octanoylaminocitrazinic acid. Amino-acetaminocitrazinic acid can easily be converted to the diacetamino homologue dye (II), for which the molecular weight 347 (calc.: 347.3) was found by mass spectrometry. Finally, the acetamino-octanoylamino homologue dye (III) can be synthesized by condensation of IV with amino-acetaminocitrazinic acid, which demonstrates the presence of the imide bridge in pigment I. The phenylhydrazone of IV (Va $\leftrightharpoons$ Vb) is identical with an authentic product prepared from aminocitrazinic acid. Details about the chemistry of these compounds are reported elsewhere (KNACKMUSS, 1967).

The properties of the pigment (I) from *P. lemonnieri* resemble to a certain extent those of 5,5-dioctanoylamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′), also designated as N,N′-dioctanoyl-indigoidine (VI). Nearly the same solubility and chromatographic behavior were observed. The substances resemble one another in IR- and NMR-spectra (a signal for the acidic OH-proton of I, II, or III was not detected). However, they have completely different chromophores.

The two compounds (I and VI) are indeed chemically related. The pigment (I) of *P. lemonnieri* can easily be converted to N,N′-dioctanoyl-indigoidine (VI) by hydrogenation and subsequent autoxidation. Because of the similar ring structures, aminocitrazinic acid is a common starting material for the chemical syntheses of both compounds. Therefore, it is likely that indigoidine and the pigment of *Pseudomonas lemonnieri* might be closely related in their biosyntheses.

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