centrations up to 0.5 m*M*, giving nearly 100% reactivation, but higher Zn^{2+} concentrations caused a considerable inhibition. Ca^{2+} was considerably less effective, and much higher concentrations were required. The small amount of purified enzyme obtained did not allow the determination of the metal contents of the metalloprotein.

Since $CaCl_2$ was required for the production of the protease, it might be supposed that the divalent cation present in the metalloenzyme was Ca^{2+}. However, as shown in Figure 2, *o*-phenantroline at concentrations up to 1 m*M* inhibited the enzyme even in the presence of 10 m*M* $CaCl_2$, thus indicating that the divalent cation

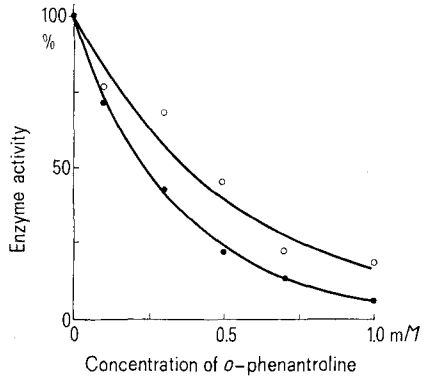

Fig. 2. Inhibition of the extracellular protease from *Ps. fluorescens* by *o*-phenantroline. 0.1 ml of purified enzyme preparation, previously exhaustively dialyzed against 20 m*M* *Tris*-HCl buffer (pH 7.6), was assayed as described under Methods except for the buffer, which was 30 m*M* *Tris*-acetate (pH 6.6), and *o*-phenantroline, which was directly added to the assay mixtures at the concentrations stated on the abcissa, in the absence (●) or in the presence (○) of 10 m*M* $CaCl_2$. In order to avoid interference by *o*-phenantroline, after centrifugation a 1 ml-aliquot of the supernatant of the assay mixture was added 2 ml of 1 *M* NaOH and 0.5 ml of Folin-Ciocalteau's phenol reagent, diluted $^1/_3$[15]. After 30 min at room temperature, the absorbance of the solution at 750 nm was read and taken as an expression of the enzyme activity. Blanks, to which trichloroacetic acid was added before the enzyme, were substracted. The enzyme activity is expressed as percent of the enzyme activity in the absence of the inhibitor ($\Delta A_{750\,nm}$/min = 0.11).

acting as prosthetic group is not Ca^{2+}, being probably one of the cations able to form strong complexes with *o*-phenantroline, such as Co^{2+} or Zn^{2+}. A similar finding has been reported for the alkaline protease of *Ps. aeruginosa*[13].

$CaCl_2$, which is known to stabilize some extracellular protease[3], did not protect the *Ps. fluorescens* enzyme, which was almost completely inactivated after incubation at 50 °C for 10 min, either in the absence or in the presence of 5 m*M* $CaCl_2$.

The results presented in this communication show that *Ps. fluorescens* produces an extracellular protease able to digest casein within a pH range wider than that reported for the enzymes purified from other *Pseudomonas*[3,5]. The molecular weight of the enzyme is similar to that reported for the proteases from *Ps. maltophilia*[4] and *Ps. fragi*[3], but significantly lower than that of the alkaline protease of *Ps. aeruginosa*[14]. The *Ps. fluorescens* protease is a metalloenzyme, as the enzymes produced by other *Pseudomonas*[3,4,5], but in our case a stable apoenzyme was obtained by dialysis against EDTA for rather long periods of time. The lack of enzyme reactivation by dialysis against distilled water suggests that in the case of the *Ps. fluorescens* protease the metal prosthetic group was actually removed, and not simply masked, by the chelating agent. The second possibility is thought to be true for the *Ps. aeruginosa*[14] enzyme. The divalent cation bound to the apoenzyme might be Co^{2+} or Zn^{2+}, as suggested by the greater effectiveness of these cations for the reconstitution of the holoenzyme (Figure 1) and by the inhibition by *o*-phenantroline in the presence of $CaCl_2$ (Figure 2).

Since only one proteolytic enzyme was detected in the culture supernatants of *Ps. fluorescens*, and it was able to digest casein at neutral pH, the protease described here can be considered responsible for the gelatine hydrolysis which is one of the phenotypic characters of *Ps. fluorescens*[6].

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Occurrence of Trichochromes in the Urine of a Melanoma Patient[1]

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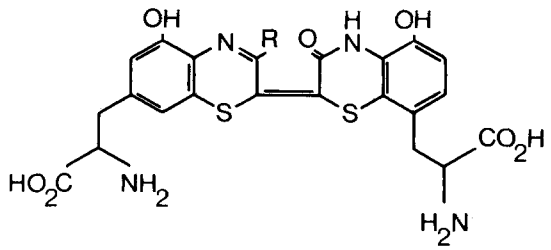
Summary. The presence of two phaeomelanic pigments, trichochrome B and C, was demonstrated in the urine of a patient with malignant melanoma metastases.

Trichochromes[3–5], formerly named trichosiderins, are a unique group of amino acidic pigments possessing a $\Delta^{2,2'}$-bi-(2H-1,4-benzothiazine)chromophore (Figure), which accounts for their characteristic pH-dependent visible spectra. They are the simplest group of phaeomelanic pigments, and are formed in melanocytes by a deviation of the eumelanin pathway involving as key step the 1,6-addition of cysteine to dopaquinone to give 5-S- and 2-S-cysteinyldopa.

Unlike eumelanin, which occurs in several types of pigmented tissues, trichochromes have so far been found only in certain red hair and feathers, and it has been suggested that their formation is somehow restricted to these keratinized structures. The recent finding of large amounts of 5-S-cysteinyldopa in the urine of melanoma patients[6,7] led us to look for trichochromes in the urine of a patient with melanoma metastases and markedly



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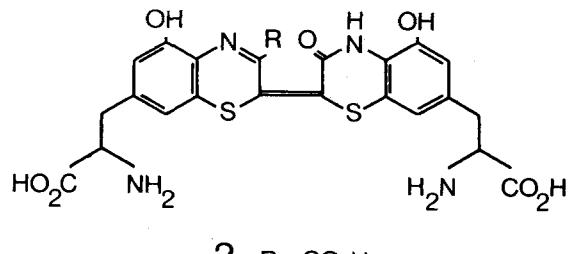
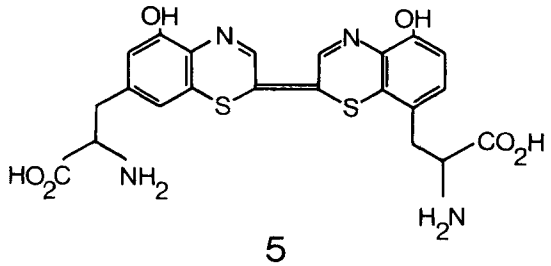
3 : R = H



2 : R = CO_2H
4 : R = H



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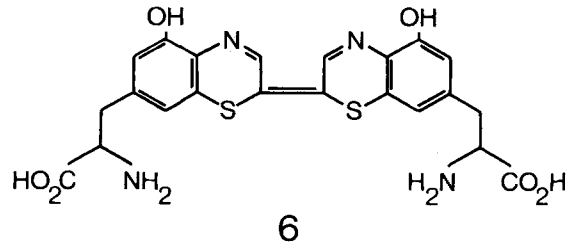

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1. Trichochrome B. 2. Trichochrome C. 3. Decarboxytrichochrome B. 4. Decarboxytrichochrome C. 5. Trichochrome E. 6. Trichochrome F.

elevated urinary excretion of 5-S-cysteinyldopa and related metabolites.

Material and methods. Urine was obtained from a male patient aged 46 years with red-blond hair. He had been operated on for a cutaneous melanoma, and had widespread metastases. 24-hour specimens were collected in plastic bottles containing 50 ml of acetic acid and 1 g of sodium metabisulphite. Determination of 5-S-cysteinyldopa and dopa + dopamine was carried out by methods previously described[8,9].

Authentic samples of decarboxytrichochrome B and C were obtained as follows, 22 mg of a mixture of 2-S- and 5-S-cysteinyldopa (molar ratio c. 1:9) was dissolved in a small volume of 0.1 *N* HCl. The solution was passed on a column (2×10 cm) of Dowex 50W-X4, 200–400 mesh, H^+ form. After washing with water (200 ml), the column was treated with 0.5 *N* NaOH, and yielded a brownish-yellow eluate which was acidified to pH 1 with 6 *N* HCl and boiled for 20 min. The reddish-brown solution so obtained was re-chromatographed on a Dowex 50W column equilibrated with 0.1 *N* HCl. After washing as before, elution was first performed with 0.2 *M* sodium acetate, which removed most of the coloured material, and finally with 0.1 *N* NaOH. The alkaline eluate was adjusted to pH 3 and the precipitated pigments were collected by centrifugation, dissolved in 0.1 *N* HCl, and fractionated by paper chromatography on Whatman 3MM with isopropanol – formic acid – concentrated HCl (30:70:1, v/v). The red bands corresponding to decarboxy trichochrome B (lower Rf value) and decarboxy trichochrome C were cut out and eluted with 0.1 *N* NaOH. Quantitation of the pigments so obtained was done by spectrophotometry.

Isolation of trichochromes from the melanoma urine was carried out by the following procedures. 1. A urine sample (10 ml) was acidified to pH 1 with 6 *N* HCl and boiled for 10 min to convert trichochromes B and C into the corresponding decarboxy derivatives. Isopropanol (10 ml) was then added and the mixture was saturated with NaCl. After shaking for 5 min, the isopropanol layer was withdrawn and evaporated to dryness. The residue was dissolved in a small volume of 0.1 *N* HCl and analyzed directly by TLC on cellulose using isopropanol-formic acid-concentrated HCl (30:70:1) as eluent.

2. A 24-hour urine sample was acidified to pH 1 with 6 *N* HCl and passed on a column (2×15 cm) of Dowex 50W-X4, H^+ form. After washing with *N* HCl (450 ml), 2 *N* HCl (1200 ml), and water (200 ml) to remove cysteinyldopas and related metabolites, the column was eluted with 0.5 *N* NaOH, and a brownish-yellow fraction containing trichochromes was collected, acidified to pH 1, and boiled for 15 min. The resulting reddish-brown so-

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lution was passed on a smaller column (2 × 5 cm) of Dowex 50W which was then washed with *N* HCl (100 ml), water and 0.2 *M* sodium acetate until the pH of the effluent rose to 5. Subsequent elution with 0.1 *N* NaOH gave a brownish-yellow fraction which, after acidification and evaporation, was further purified by chromatography on a column (1.5 × 20 cm) of Sephadex LH-20 using methanol-2 *N* HCl (95:5) as eluent. Under these conditions, a well-defined red band appeared which was collected and evaporated to dryness. The residue was dissolved in a minimum volume of 0.1 *N* HCl. The resulting solution was first analyzed spectrophotometrically to determine the total yield of the pigments, and was subsequently fractionated by paper chromatography on Whatman 3MM with isopropanol-formic acid-concentrated HCl (30:70:1).

3. A sample of urine (100 ml) was adjusted to pH 13 with 5 *N* NaOH and left at room temperature for 72 h under an oxygen current to convert cysteinyldopas into stable, colourless benzothiazine derivatives[10]. The oxidized urine was then acidified to pH 1 with 6 *N* HCl and passed on a column (2 × 10 cm) of Dowex 50 W, H^+ form. After washing with *N* HCl (150 ml) and water, the column was eluted with 0.5 *N* NaOH to give a trichochrome-containing fraction which was worked up as described in method 2.

As a control to method 3, a solution of 5-S-cysteinyldopa in 0.1 *N* NaOH was treated exactly as the melanoma urine.

Results. Because of the presence of large amounts of 5-S-cysteinyldopa and related metabolites, examination of the melanoma urine was carried out by the 3 procedures described above, all of which gave similar results.

1. *Isopropanol extraction of trichochromes.* Chromatographic analysis of the isopropanol extract obtained from melanoma urine revealed the presence of 3 red pigments, 2 of which showed pH-dependent colours and Rf values identical to those of decarboxytrichochrome B and C. The 3rd spot showed no colour change when exposed to ammonia vapour, and showed an Rf value which did not correspond to any hitherto known trichochrome.

2. *Isolation of trichochromes after removal of pigment precursors by ionic exchange resin.* Fractionation of the melanoma urine on Dowex 50W columns followed by chromatography of the crude pigment mixture on a Sephadex LH-20 column with methanol-2 *N* HCl (95:5) gave a single red band with absorption maxima at 533 nm in 2 *N* HCl and 460 nm in 0.1 *N* NaOH, corresponding to decarboxytrichochrome C+B. The total amount of these pigments, determined spectrophotometrically, was found to be 5 mg/24 h. Subsequent chromatography of the mixture on Whatman 3 MM paper led to the isolation of pure decarboxytrichochrome C and B, along with trace amounts of trichochrome E and F identified by their chromatographic and spectral properties.

3. *Isolation of trichochromes after removal of pigment precursors by oxidation in alkaline medium.* Work-up of the melanoma urine by this procedure, involving initial oxidation of the pigment precursors present with oxygen at pH 13, led again to the isolation of decarboxytrichochrome C and B, whereas no trichochrome E or F was obtained. The total amount of decarboxytrichochrome C+B excreted was estimated spectrophotometrically after purification on Sephadex LH-20, and found to be 9 mg/24 h.

Discussion. During the initial phase of our work, search for trichochromes in the melanoma urine was carried out by a modification of the procedure previously described for the isolation of pigments from red hair and feathers[11]. The method involves preliminary purification of the urine on strongly cationic exchange resin, and subsequent heating of the pigment mixture in acid medium to convert the yellow unstable trichochrome B and C into the corresponding decarboxyderivatives, which possess more favourable analytical properties.

Using this procedure large amounts (up to 30 mg/24 h) of decarboxytrichochrome C and B along with smaller quantities of trichochrome F and E were obtained from the urine of several patients with melanoma metastases and high urinary excretion of 5-S-cysteinyldopa. Subsequent experiments, however, revealed that part of the trichochromes isolated from urine could be artifacts arizing from aerial oxidation of 5-S-cysteinyldopa and related metabolites during the work-up procedure. Indeed, when a solution of pure 5-S-cysteinyldopa was used in the abovementioned procedure instead of melanoma urine a good yield of decarboxy trichochrome C was obtained[12]. The same experiment using a mixture of 5-S- and 2-S-cysteinyldopa also led to the formation of decarboxytrichochrome B. In fact, the procedure proved to be ideal as a biogenetic-type synthesis for preparing decarboxytrichochrome B and C from cysteinyldopas.

In the light of the above findings, we then went on to develop the 3 analytical procedures reported, which proved to be of value for ascertaining the actual excretion of trichochromes in melanoma urine without artifact formation due to the co-presence of pigment precursors.

Analysis of the urine with methods 1 or 3 showed only the presence of trichochrome B and C, whereas using method 2 small amounts of trichochrome E and F were also obtained. It should be noted, however, that large amounts of trichochrome E and F are produced by aerial oxidation of cysteinyldopas present in the urine during acid elution of the Dowex column[13]. Although the bulk of these pigments is eluted by prolonged washing with 2 *N* HCl, some material evidently remains on the resin and is then found in the alkaline eluate. The small amounts of trichochrome E and F detected in the urine with method 2 must therefore be regarded as artifacts.

The finding of trichochrome B and C in the urine of a patient with melanoma has important implications for the biochemistry of mammalian pigmentation, and has also potential clinical significance. Indeed, until now in mammals trichochromes had been found only in red hair, and it seemed that their formation was in some way dependent on the particular biochemical environment of the hair follicle melanocyte. The present findings strongly suggest that trichochromes are formed also in other types of melanocytes, and that they are excreted as a result of the pathological activity of malignant melanocytes.

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[12] Decarboxytrichochrome C was also obtained simply by oxidation of 5-S-cysteinyldopa in 0.1 *N* NaOH with atmospheric oxygen for a few minutes, followed by acidification and boiling of the reaction mixture.

[13] Work at our laboratory has demonstrated the presence of large amounts of 2-S-cysteinyldopa in the urines of melanoma patients.