centration of Mg^{++} ion[10], which might influence the enzyme systems localized in outer mitochondrial membrane. Therefore we have examined the effect of Mg^{++} ion on the external NADH oxidation in the presence of cytochrome c by human placental, human and rat skeletal muscle mitochondria.

Figure 3 presents the effect of increasing concentrations of $MgSO_4$ on the oxidation of NADH in the presence of added cytochrome c in human and rat skeletal muscle and human placental mitochondria. The results are expressed as a percent of the control oxidation rate in the absence of $MgSO_4$. It may be seen that $MgSO_4$ inhibited NADH oxidation in the presence of cytochrome c in the case of both human skeletal muscle and placental mitochondria. The inhibition depended on $MgSO_4$ concentration reaching about 50% and 75% at 9 m*M* and 16 m*M* $MgSO_4$ respectively in the case of human skeletal muscle mitochondria; the same degree of inhibibition in the mitochondria from human placenta was observed at 5 m*M* and 13 m*M* $MgSO_4$, respectively. In the case of rat skeletal muscle mitochondria, $MgSO_4$ exerted a stimulatory effect on cytochrome c induced NADH oxidation. Maximal stimulation was obtained at the concentration range 2–5 m*M* $MgSO_4$. $MgSO_4$ concentration higher than 8 m*M* stimulated only slightly NADH oxidation in the presence of cytochrome c. At 16 m*M* no stimulatory effect was observed. $MgCl_2$ exerted similar effect as $MgSO_4$ (not shown), indicating that Mg^{++} ion is responsible for the inhibition in the case of human skeletal muscle mitochondria and placental mitochondria, and for the stimulation in the case of rat skeletal muscle mitochondria. In human placental and skeletal muscle mitochondria, $MgSO_4$ inhibited NADH oxidation to the same extent both in the presence and absence of rotenone. In the case of rat skeletal muscle, mitochondria NADH plus cytochrome c oxidation was studied in the absence of rotenone only because this inhibitor exerted inhibitory effect on NADH plus cytochromec oxidation[3]. Data presented in Figure 3 indicate that with regard to the action of Mg^{++} ion on NADH oxidation in the presence of cytochrome c, rat skeletal muscle mitochondria resemble rat liver mitochondria[9], whereas in human mitochondria Mg^{++} ion exerted an inhibitory effect on this process.

The question arises whether the inhibitory effect of $MgSO_4$ is caused by other contaminating ions. We checked the $MgSO_4$ solution in atomic absorption spectrophotometer and showed that Zn^{++} ion is present, although at a concentration which was without effect on NADH oxidation. At present it is difficult to say what is the mechanism of Mg^{++} action on NADH oxidation in the presence of cytochrome c. It seems that two possibilites may be taken into consideration: a) that Mg^{++} is either inhibiting or stimulating NADH-cytochrome b_5 reductase localized in the outer mitochondrial membrane, b) that Mg^{++} is limitting the permeability of outer membrane to cytochrome c, inhibiting in this manner electron flow from cytochrom b_5 to cytochrome oxidase of the inner mitochondrial membrane. It is not excluded that both mechanisms are responsible for the observed Mg^{++} ion effect on NADH plus cytochrome c oxidation.

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The Extracellular Protease from *Pseudomonas fluorescens*

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Summary. An extracellular protease has been purified from cultures of *Pseudomonas fluorescens*. It is a metalloenzyme with a molecular weight of 37,000 ± 3,700, able to digest casein, hemoglobin and gelatine.

The extracellular proteolytic enzymes produced by some *Pseudomonas* strains are important in the process of meat spoilage at low temperatures[2]. Extracellular proteases have been isolated from the psychrophile *Ps. fragi*[3], and from *Ps. maltophilia*[4] and *Ps. aeruginosa*[5]. We report in this communication the purification and some properties of the extracellular protease produced by *Ps. fluorescens*, a fluorescent facultatively psychrophilic *Pseudomonas*, which differs from *Ps. aeruginosa* by its ability to grow at 4 °C and from *Ps. putida* by its ability to hydrolize gelatine[6].

Methods. Ps. fluorescens, strain R-12, was isolated from water of the Paraná River at Rosario[7] and grown at 25 °C in Nutrient Broth No 1 (Oxoid), 13 g/l, with the addition of $CaCl_2$ (0.3 g/l), in a New Brunswick water bath girotory shaker. When the cultures reached the stationary phase ($A_{680\,nm}$ of 1.4), the cells were separated by centrifugation at 15,000 *g* for 20 min at 4 °C. To the clear coloured supernatant was added solid ammonium sulfate to 50% saturation at 0 °C. The precipitate was separated by centrifugation at 37,000 *g* for 20 min at 4 °C and discarded. The supernatant was brought to 60% saturation with solid ammonium sulfate. The precipitate was dissolved in 1 ml of 20 m*M* *Tris*-HCl buffer (pH 7.6) containing 2 m*M* $CaCl_2$ and filtered through a Sephadex G-200 column (52 × 1.2 cm) equilibrated with the same buffer. The active fractions were pooled and dialyzed against 20 m*M* glycine-NaOH buffer (pH 9.7) for 21 h

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Purification of the extracellular protease from *Ps. fluorescens*

Step	Volume (ml)	Total protein (mg)	Enzyme activity Total (units)	Enzyme activity Specific (units/mg)	Purification	Yield (%)
Crude supernatant	519	761	24.3	0.03	1	100
Ammonium sulphate fractionation	0.8	14.8	8.0	0.54	18	33
Sephadex G-200 column	16.4	5.1	7.3	1.43	48	30
DEAE-cellulose column	14.5	1.7	3.0	1.76	59	12

The enzyme was purified and assayed as described under Methods.

at 4 °C. The dialysate was chromatographed on a column of DEAE-cellulose (16×1.2 cm) equilibrated with the same buffer; the elution was performed with a gradient of NaCl from 0 to 250 m*M* in the same buffer. The protease was eluted between 65 and 135 m*M* NaCl. The concentration of NaCl was determined by flame photometry.

The standard reaction mixture contained 1% casein, 40 m*M* *Tris*-HCl buffer (pH 7.5) and enzyme, in a final volume of 1 ml. After 10 min incubation at 30 °C with shaking, the reaction was stopped and the casein remaining undigested was precipitated by the addition of 2.5 ml of a 3% trichloroacetic acid solution. After centrifugation at 15,000 *g* for 10 min at room temperature, the $A_{280\,nm}$ of the supernatant was determined in a Unicam SP 1800 B spectrophotometer. Blanks, to which trichloroacetic acid was added before the enzyme, were substracted in all cases. One unit of proteolytic activity is defined as the amount of enzyme able to liberate in 1 min at 30 °C peptides soluble in 2.14% trichloroacetic acid giving an $A_{280\,nm}$ of 1 absorbance unit. The reaction was linear with time for at least 10 min, with amounts of purified enzyme up to 0.1 mg.

The protein concentrations of the different fractions obtained in the purification method was determined by the spectrophotometric method of WARBURG and CHRISTIAN[8].

Results and discussion. The production of the protease in Nutrient Broth No. 1 required the addition of $CaCl_2$ to the culture medium, as has been shown for the *Ps. aeruginosa* enzyme[5]. As in the case of the extracellular proteases of *Ps. maltophilia*[4] and the marine bacterium strain SA 1[9], the *Ps. fluorescens* enzyme was produced in the late logarithmic and early stationary phase of growth.

The method outlined in the Table produced 59-fold purified enzyme, with a yield of 12%. The purified preparation presented one rather diffuse, slowly migrating, protein band when subjected to polyacrylamide gel electrophoresis[10]; similar behaviour has been described for the *Ps. maltophilia* enzyme[4], and might be due to autoproteolysis. The protein band was identified as the protease by its ability to digest gelatine when the polyacrylamide gels were applied to agar-gelatine plates[11].

The purified enzyme was able to digest casein, hemoglobin and gelatine. With casein as the substrate, the pH curve was broad with nearly maximal activity between pH values of 6.5 and 10.

The molecular weight of the enzyme was estimated to be 37,000 ± 3,700 by gel filtration[12] on a Sephadex G-100 column (36×1.2 cm). The elution volumes of cytochrome c (mol.wt. 12,400), protease, bovine serum albumin (mol.wt. 68,000), citrate synthase (mol.wt. 100,000) and Blue Dextran 2000 were 32; 23.6; 20.1; 19.2 and 17.1 ml, respectively.

The enzyme was not inhibited by the sulfhydril reagent *p*-chloromercuribenzoate at concentrations up to 1 m*M*. EDTA did not inhibit the enzyme activity when added to the reaction mixtures at concentrations up to 2 m*M*, nor when the protease was dialyzed against 20 m*M* *Tris*-HCl buffer (pH 7.6) containing 1 m*M* EDTA for 3 h at 7 °C. However, when the enzyme was dialyzed against the same buffer, containing 5 m*M* EDTA, for rather long periods of time (3–7 days), eventually all enzyme activity was lost, and was not recovered after dialysis against distilled water. This inactivation could be reversed by the addition of different divalent cations to the reaction mixture, showing that a stable apoenzyme had been obtained. At a concentration of 1 m*M* Cd^{2+} and Mg^{2+} were ineffective as reactivators; Ca^{2+} and Mn^{2+} were little effective, and Zn^{2+} and Co^{2+} were able to restore a considerable fraction of the original activity. Figure 1 shows that Co^{2+} and Zn^{2+} were similarly effective at con-

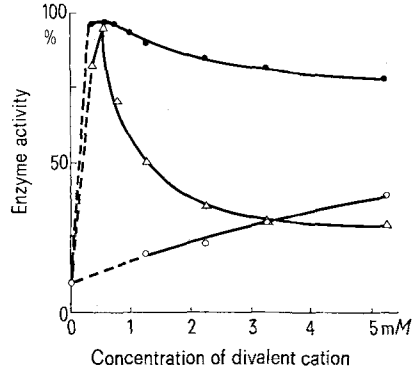

Fig. 1. Reconstitut. of protease activity by addition of divalent cation to the apoenzyme. 4 ml of purified enzyme preparation were dialyzed against 500 ml of 20 m*M* *Tris*-HCl buffer (pH 7.6) containing 5 m*M* EDTA for 70 h at 7 °C. The enzyme activity was assayed as described under Methods, except for the buffer, which was 30 m*M* *Tris*-acetate (pH 6.6), and $CaCl_2$ (○), $ZnCl_2$ (△) or $CoCl_2$ (●), which were added to the assay mixtures at the concentrations stated on the abcissa. Since 0.25 m*M* EDTA was carried over with the enzyme solution (0.05 ml), the lowest cation concentration tested was 0.35 m*M*. The enzyme activity is expressed as percent of the original activity before dialysis (0.24 units/ml).

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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

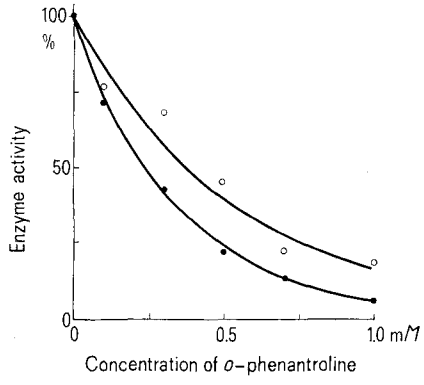

Fig. 2. Inhibition of the extracellular protease from *Ps. fluorescens* by *o*-phenantroline. 0.1 ml of purified enzyme preparation, previously exhaustively dialyzed against 20 m*M* *Tris*-HCl buffer (pH 7.6), was assayed as described under Methods except for the buffer, which was 30 m*M* *Tris*-acetate (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