Results. Evidence has been obtained that the two melanoma tissues examined differed both with regard to the concentration of the pigment key intermediates and the type of pigments produced (Tables I and II). In particular the melanoma metastasis from the woman with red-brown hair contained only a dark insoluble pigment with rather high sulfur content. By contrast, the melanoma tissue from the patient with red-blonde hair contained a mixture of alkali-soluble pigments.

Purification of the alkali-soluble fraction on Sephadex G 75 column led to the isolation of 2 reddish-brown polymeric pigments containing nitrogen and sulfur in a ratio characteristic of phaeomelanins isolated from red hair and feathers.

Discussion. Most of our knowledge on mammalian melanins derives from studies on hair shown to contain 2 separate but biogenetically interrelated classes of pigments, the dark, insoluble eumelanins derived from enzymic oxidation of tyrosine, and the alkali-soluble phaeomelanins, ranging from yellow to reddish-brown, which arise from a deviation of the eumelanin pathway by intervention of cysteine.

The chemical nature of melanins has been subject to little attention in current studies on melanomas. The results reported in this study provide evidence that the melanoma from the red-blonde patient was pigmented by phaeomelanins. The melanin present in the melanoma of the patient with red-brown hair illustrates the difficulties involved in classifying melanins. The pigment resembles the eumelanins with respect to colour and insolubility in alkalies. It cannot, however, be regarded as a typical eumelanin because of its high sulfur content (5,8%), which is incompatible with a polymer formed only by tyrosine and related metabolites. Unfortunately, the small amount of material available precluded further experiments to gain information on the structure of this melanin pigment. The fact that human malignant melanocytes may produce different types of pigments has important implications for pigment cell biology, and may provide a chemical basis for the classification of melanomas.

It has previously been demonstrated that 5-S-cysteinyldopa, a key intermediate in phaeomelanin formation, is present in substantial amounts in many different melanomas irrespective of the type of pigmentation[10,11]. The relationship between 5-S-cysteinyldopa content and pigment formation in melanoma has not been defined, however. As shown in Table I, both melanomas examined contained 5-S-cysteinyldopa although in different amounts. In the patient with red-blonde hair, the high content of 5-S-cysteinyldopa is quite consistent with the presence of phaeomelanin-forming melanocytes. The presence of smaller amounts of this metabolite in the other melanoma can also be explained in terms of pigment formation by assuming that the sulfur-containing 'eumelanin' produced in the melanocytes arises by a copolymerization process involving both dopa and cysteinyldopa intermediates. So far the content of sulfur in eumelanin has been attributed to SH bindings of the pigment to protein[12]. However, the high sulfur content found in the insoluble melanoma pigment from the patient with red-brown hair certainly cannot be explained as deriving from sulfur of proteins.

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Resistance of Purified Cholera Toxin to Enzymatic Treatment with Pancreatic Elastase and Papain

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Summary. Treatment of Cholera toxin with pancreatic elastase and papain in vitro showed a high resistance of the toxin molecule to these enzymes, under non-denaturing conditions or in the presence of 2 *M* urea. These experiments support the hypothesis of a particularly stable molecular structure of the toxin, as an explanation of its activity in the intestinal lumen where the pancreatic proteases are active.

The exotoxin produced by *Vibrio cholerae* (Choleragen) is a protein of molecular weight 84,000, constituted of two different types of subunits[1], and it exerts its toxic activities in the intestinal lumen where the pancreatic proteases are active. The activity of the toxin in the presence of proteolytic enzymes could be explained by a particular molecular structure of the toxin itself, resistant to the enzymes, as well as to an increased Choleragen production by the *Vibrio*, prevailing over the inactivation by proteases[2].

In order to ascertain the first hypothesis, we tried to digest the toxin with trypsin and chymotryps in in vitro experiments, and we demonstrated the resistance of the molecule to these enzymes[3]. To explore further this aspect of the problem, we studied the treatment of Choleragen with pancreatic elastase, another enzyme present in the intestinal lumen with differing specificity in comparison with the enzymes previously mentioned.

Non-physiological conditions, such as digestion with papain or the presence of 2 *M* urea, were also studied, in order to evidence a particularly stable molecular structure of the Cholera toxin.

Materials and methods. Highly purified Cholera toxin was prepared according to Saletti et al.[4]. The toxin was characterized[4] by chemico-physical, immunological and biological methods: polyacrylamide gel electrophoresis and immunodiffusion on agar against a specific antiserum

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Table I. Digestion experiments with pancreatic elastase

Conditions adopted	Enzyme activity (U/mg)
0.01 *M* Tris + 0.1 *M* KCl; pH 8.0	
a) 25°C	5.3
b) 37°C	7.6
c) 37°C; 2 *M* urea	5.0

Protein concentration: 0.8 mg/ml. Ratio toxin to elastase: 10/1. Incubation time: 180 min.

Table II. Digestion experiments with papain

Conditions adopted	Enzyme activity (U/mg)
0.4 *M* NaCl; 37°C	
a) pH 8.0	2.8
b) pH 8.0; 2 *M* urea	2.0

Protein concentration: 0.8 mg/ml. Ratio toxin to papain: 100/1. Incubation time: 180 min.

showed a main band, accounting for most of the protein content; the toxic activity, as determined by the 'blueing doses' assay (skin test) in the rabbit, was 700,000 blueing doses per mg of protein.

The digestions were performed at constant pH, under nitrogen in a Combi-Titreur apparatus (Metrohm AG, Switzerland); the alkali uptake (index of enzymatic digestion) was recorded automatically. Pancreatic elestase (3.4.4.7, Serva, Germany) and Papain (3.4.4.10, Type II, Sigma, USA) were titrated with the synthetic substrates N-benzoyl-L-alanine methylester (BAME[5]) and N-benzoyl-L-arginine ethylester (BAEE[6]), respectively, before their use in the digestion experiments. The conditions adopted for the enzymatic treatments are shown in Tables I and II.

Results and discussion. None of the digestion schemes adopted showed evidence of alkali uptake during the treatment of the toxin with either pancreatic elastase or papain. On the other hand, it was possible to evidence complete activity of the enzymes at the end of each single digestion experiment by titrating an aliquot of the digestion mixtures with the specific synthetic substrates.

Samples of the toxin incubated with pancreatic elastase at 25° and 37°C were also tested in order to assay the effect of the enzymatic incubation on the electrophoretic pattern, agar immunodiffusion and biological toxic activity. The toxin did not show any changes in these parameters, which were identical to those found in the toxin samples maintained under the same experimental conditions but in the absence of the enzyme (blank experiments). On the other hand, the enzymes showed no effects in any of the tests.

The blank experiments for the samples incubated in 2 *M* urea showed a partial modification of the properties of the toxin; this denaturation, however, was not sufficient to allow the Choleragen to be attacked by the two enzymes.

The results of these experiments show that Choleragen, in the conditions adopted, is resistant to the action of pancreatic elastase and papain, and they support the hypothesis of a particular molecular structure as an explanation of the activity of the toxin in the intestinal lumen.

More detailed studies regarding enzymatic digestion of Choleragen, also in the presence of denaturants, could clarify the relationship between molecular structure and mechanism of action of the toxin.

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Localization of Peroxidase Activity in *Trypanosoma cruzi* Microbodies[1]

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Summary. Electron microscopic observation of *Trypanosoma cruzi* epimastigotes reveals the presence of microbody-like structures (microperoxisomes) in which 3,3′-diaminobenzidine (DAB) is peroxidized to electron-opaque material. The role of peroxidase in DAB peroxidation is supported by the enzyme demonstration in disrupted epimastigotes and the microbody-containing cell fractions.

Microbodies ('peroxisomes', 'microperoxisomes') are defined as cytoplasmic structures characterized by the association of one or more hydrogen peroxide-producing oxidases with catalase, which destroys the hydrogen peroxide[4–7]. The organelles are recognized as ubiquitous structures in living cells[8], including protozoa[9]. In the bloodstream, forms of *Trypanosoma cruzi* (the agent of Chagas disease), oval bodies have been postulated to be peroxisomes[10]. Incubation of aldehyde-fixed cells in alkaline 3,3′-diaminobenzidine (DAB) media is a suitable procedure for demonstrating peroxisomes since DAB peroxidation determines the formation of electron-opaque material, easily visualized by electron microscopy[11,12]. In this paper we demonstrate the existence of DAB positive, microbodylike structures (microperoxisomes) in the epimastigote (culture) form of *T. cruzi*.

Materials and methods. The Tulahuen strain of *T. cruzi* was grown in a diphasic medium at 28°C, as described before[13]. 4 days after inoculation, the cells were collected and reinoculated in a liquid medium made of NaCl (9 g); Na_2HPO_4 (7.5 g); KCl (0.4 g); glucose (4.0 g); tryptose (Difco; 15 g); yeast extract (Difco; 5 g); liver extract (5 g); inactivated calf serum (5 ml); hemin (Sigma Chemical Company; 20 mg, dissolved in 8 ml of 0.1 *N*