however, the presence of one flavonoid in anthers (a glucose rhamnoside of isorhamnetin) and one in corresponding stigmas of the above plant (possessing an aglycone similar with isorhamnetin and the unusual combination, glucose and fructose). Kaempferol-3-O-rutinoside and rutin have now been isolated from stigmas of *C. pepo* but the flavonoid containing fructose has not been found in this material; these results may be related to phytogeographical factors[8]. From the physiological view point, it is of interest that differences have been found between the flavonoid patterns of pollens and corresponding stigmas of the 4 species (table).

Flavonoids of pollens and stigmas of 4 species of the genus *Cucurbita**

Species	Pollen	Stigma
Cucurbita pepo	**1**	**2,4**
C. maxima	**1,2,3,5,6**	**1,2,3**
C. moschata	**1,2,3**	**2**
C. ficifolia	**1,4,7**	**1,2**

* Flavonoids 5-7 were not present in sufficient amount for analysis; preliminary investigations suggest that they may be flavonol-3-O-monoglycosides.

1 Acknowledgments. The author thanks Dr Chr. Karl (Weleda, Schwäbisch Gmünd) for a sample of isorhamnetin and Mr A. D'Urso (Botanic Institute, University of Catania) for help in acquiring the plant material.
2 J.N. Hartshorne, Nature, Lond. *182*, 1382 (1958).
3 G.A. Barber, Arch. Biochem. Biophys. *64*, 401 (1956).
4 T.J. Mabry, K.R. Markham and M.B. Thomas, The Systematic Identification of Flavonoids. Springer, Berlin 1970.
5 C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, J. Am. chem. Soc. *85*, 2497 (1963).
6 F. Petek, Bull. Soc. Chim. Fr., p. 263 (1965).
7 The 3-rutinoside and 3-robinobioside of kaempferol which occur together in *Cucurbita maxima* and *C. moschata* (table) were not separated; the mixture of the pair of glycosides was identified by total acid hydrolysis (which gave kaempferol, rhamnose, glucose and galactose) and methylation followed by acid hydrolysis (which gave 5,7,4′-tri-O-methylkaempferol, 2,3,4-tri-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-galactose and 2,3,4-tri-O-methyl-L-rhamnose).
8 J.B. Harborne, T.J. Mabry and H. Mabry, in: The Flavonoids, p. 1086. Chapman & Hall. London 1975.

A possible pathway for the biosynthesis of adenochromines

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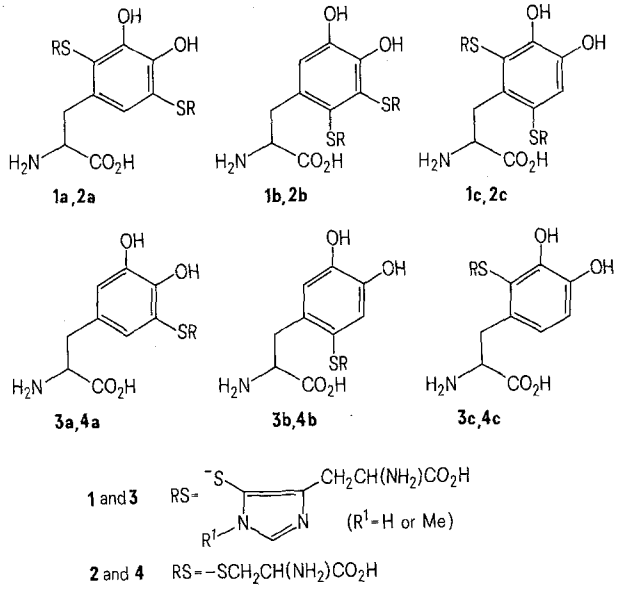
Summary. 5-Thiolhistidine reacted with dopaquinone produced in vitro by tyrosinase oxidation of dopa to give high yields of secoadenochromines (**3**) along with a small amount of adenochromines (**1**) which are the amino-acids responsible for the iron-(III)-binding properties of adenochromes.

Adenochromines (**1a**, **1b**, **1c**) are component amino-acids of a unique group of iron-(III)-binding peptides, adenochromes, which occur in the branchial heart of *Octopus vulgaris*[3].

Suggestions for the biosynthesis of the adenochromines followed from their close structural similarity with the cysteinyldopas[4], especially 2,5-S,S-dicysteinyldopa[5] (**2**), which are formed by addition of cysteine to dopaquinone produced by tyrosinase oxidation of dopa[6]. When examined in vitro this reaction leads mainly to the formation of the monoadducts i.e. 5-S-(**4a**), 2-S-(**4c**) and 6-S-(**4b**) cysteinyldopas (74%, 14% and 1%) along with a small amount (5%) of the diadduct 2,5-S,S-dicysteinyldopa (**2**). Therefore a similar reaction between 5-thiolhistidine and dopaquinone could account for the biosynthesis of adenochromines by way of the monoadducts **3a**, **3b**, **3c**, known as secoadenochromines.



To test this hypothesis we have studied the enzymic oxidation of dopa and secoadenochromines A, B and C in the presence of 5-thiolhistidine.

A) *Tyrosinase oxidation of dopa and 5-thiolhistidine.* A solution of L-dopa (12.5 mg; 0.063 mmoles) and 5-thiol-L-histidine dihydrochloride[7] (35.2 mg; 0.125 mmoles) in 0.05 M sodium phosphate buffer, pH 6.8, was vigorously stirred at 24 °C (oxygen not bubbled into the solution) in the presence of mushroom tyrosinase (4.4 mg; 2750 units/mg; from Sigma Chem. Co.) and the course of the reaction was followed by monitoring the UV spectrum (in 0.1 N HCl) of aliquots taken at suitable intervals. Since after 45 min the formation of secoadenochromines (**3**) became evident (increase of absorbance at 293 nm), the oxidation was stopped by acidification to pH 1 with 6 N HCl and the reaction mixture was passed through a column (0.9×26 cm) of Dowex 50 W-X2 (200-400 mesh, H^+ form). After washing with 1 N HCl (10 ml) the column was eluted with 3 N HCl at a flow rate of 9 ml/h and fractions of 5 ml were collected and monitored spectrophotometrically. Fractions 12-15 gave 13.3 mg of 5-thiolhistidine disulphide; 16-20, 13.3 mg of a mixture of **3b** and **3c** contaminated with some (~ 10%) disulphide; 22-29, 19.8 mg (about 55%) of **3a** containing a trace of **1b**, and 32-38, 1.6 mg (3%) of **1a** and **1c** which were not separable from each other.

Fraction 16–20 was eventually purified from the accompanying disulphide by preparative TLC on cellulose with n-propanol-1 N HCl (3:2) to give a 2:1 mixture of **3b** and **3c** as determined by PMR spectroscopy (in 2 N DCl).

B) *Tyrosinase oxidation of secoadenochromines and 5-thiolhistidine.* A mixture of secoadenochromine A (**3a**, 6.63 mg; 0.0125 mmoles) and 5-thiol-L-histidine dihydrochloride (7 mg; 0.025 mmoles) in phosphate buffer, pH 6.8, was oxidized in the presence of mushroom tyrosinase (2.2 mg) at room temperature. After 2.5 h the oxidation was stopped by acidification. Fractionation of the reaction mixture on Dowex 50 WX2 (200–400 mesh, H^+ form) gave: the disulphide of 5-thiolhistidine (~ 2 mg); adenochromine A (**1a**, 1.1 mg, 12%) and **1b** (0.2 mg, 2.16%) which were separated by preparative TLC on cellulose with n-propanol-1 N HCl (3:2). Similar results were obtained in the enzymic oxidation of a mixture of secoadenochromines B (**3b**) and C (**3c**), in a molar ratio 4:1, leading to: adenochromine B (**1b**, 1.7%); adenochromines A and C (**1a** and **1c**, 7%) along with 82% of unreacted material.

Results and discussion. Under biomimetic conditions, the reaction of 5-thiolhistidine with dopaquinone, generated by tyrosinase oxidation of dopa, proceeds smoothly and clearly to give, along with a small amount (3%) of the 3 adenochromines, the parent monoadducts secoadenochromines A, B and C in 55, 20, 10% yields, respectively.

Attempts to increase the yields in adenochromines under different conditions, or with a crude preparation of *Octopus* tyrosinase[8], were unsuccessful because of the inability of the enzyme to oxidize efficiently the intermediary secoadenochromines into the corresponding o-quinones. It is relevant, however, that oxidation of secoadenochromines, in the presence of excess of 5-thiolhistidine, gave a 10% yield of adenochromines. In spite of the fact that under in vitro conditions the formation of monoadducts **3** prevails, these experiments are consistent with the view that the formation of adenochromines in *Octopus* may be regarded as a result of deviation of the normal eumelanin pathway[6], involving a nonenzymic reaction between dopaquinone (enzymically produced) and 5-thiolhistidine. The analogy of such a process with phaeomelanin biosynthesis is remarkable, especially when compared with the early stages leading to the formation of 5-S-cysteinyldopa (**4a**) and 2-S-cysteinyldopa (**4c**), as well as 2,5-dicysteinyldopa (**2**). A major difference between the addition of 5-thiolhistidine and cysteine to dopaquinone is that in the former case the addition takes place to a significant extent (about ¼) at C-6 position (by 1,4-addition), which accounts for the in vivo formation of all the 3 possible isomers of **1**.

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3 S. Ito and G. Prota, J. chem. Soc. chem. Commun. *24*, 1042 (1976) and S. Ito, G. Nardi, A. Palumbo and G. Prota, Tetrahedron, submitted.

4 S. Ito and G. Prota, Experientia, *33*, 1118 (1977).

5 S. Ito and J.A.C. Nicol, Tetrahedron Lett. *1975*, 3287; Biochem. J. *161*, 499 (1977).

6 For recent reviews on melanogenesis, see R.H. Thomson, Angew. Chem. Int. Edit. *13*, 305 (1974) and G. Prota and R.H. Thomson, Endeavour *35*, 32 (1976).

7 The product used in this study was in fact a 3:1 mixture of 5-thiolhistidine and its 1-Methyl homologue obtained as described by Ito and Prota[3].

8 An active preparation of *Octopus* tyrosinase could be easily obtained after finding that the enzyme is present in the supernatant liquid of the ink sac after spinning off the melanin. Details of the preparation will be described elsewhere.

On the relationship of hemoglobin oxidation with the conformation of hemoglobin

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Summary. The rate of hemoglobin oxidation by various oxidants was studied under aerobic and anaerobic conditions, and the mechanism of hemoglobin oxidation was discussed in relation to the conformation of hemoglobin.

Recent advances on stereochemistry of hemoglobin revealed the allosteric behavior of this protein, which is characterized by the 2 conformational models including the oxy or R state and the deoxy or T state[1-3]. The shift of the R to the T state has been shown to be induced by the binding of organic phosphates such as 2,3-diphosphoglycerate (2,3-DPG) and inositol hexaphosphate (IHP)[4-7].

On the other hand, although the oxidation of hemoglobin by various oxidants has been studied under various conditions, few studies have so far been carried out from the standpoint of the conformation of hemoglobin. With regard to oxidation of hemoglobin by ferricyanide and nitrite and autoxidation of this protein, the effect of 2,3-DPG is mentioned in relation to the oxygen affinity of hemoglobin[8-10]. Recently we investigated the effect of IHP, as a stronger effector, on the redox reaction of this hemoprotein by some oxidoreductants and showed that the redox reaction of this protein may be affected by the conformational changes due to the binding of IHP[11,12]. In this paper, reaction mechanism of hemoglobin oxidation by several oxidants is surveyed in relation to hemoglobin conformation including the R and T state.

Methods. Hemoglobin A, obtained from fresh human red cells by hemolysis was passed through Sephadex G 25 (fine) which was previously equilibrated with 0.05 M bis-tris buffer (pH 7.0) with 0.1 M NaCl. By this procedure, hemoglobin free from organic phosphates was obtained. Catalase-free hemoglobin was prepared according to the method of Huisman and Dozy[13]. Experiments were performed as follows. A 0.2-ml sample of hemoglobin solution (25 μM as hemoglobin tetramer) and 2 ml of 0.05 M bis-tris buffer (pH 7.0) containing 0.1 M NaCl were mixed with or without IHP (90 μM). After mixing, and 5 min standing, the reaction was started by the addition of 20 μl solution of oxidants such as ferricyanide, hydroxylamine, chlorate, H_2O_2, β-naphthoquinone-4-sulfonate or nitrite and the rate of hemoglobin oxidation was spectrophotometrically measured at 25 °C by following the increase in absorbance at 630 nm. The catalase-free hemoglobin was used for the experiment with H_2O_2. The measurement of the rate of