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INFLUENCE OF SOME FLAVONOIDS ON RETICULATION OF COLLAGEN FIBRILS *IN VITRO*

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Abstract—Reconstituted collagen fibrils obtained from neutral-salt soluble type I collagen of lathyritic rat skin are incubated at 37° with flavanoids, dihydroquercetin, rutin, naringin and mainly (+)-catechin, alone or in presence of cupric ions. The influence of these compounds on the solubility, thermal stability and cross-links of the collagen fibrils is studied. With (+)-catechin, collagen fibrils become insoluble, but their thermal stability is only slightly modified. In presence of (+)-catechin–Cu(II) complex, collagen fibrils become totally insoluble and their thermal stability increases with formation of many cross links precursors (allysine and hydroxyallysine) and reducible cross-links (hydroxylysinonorleucine and lysinonorleucine). These last compounds are also found with the other flavonoids. The formation of these compounds indicates the oxidative desamination of lysine and hydroxylysine residues of collagen molecules by flavonoid and Cu(II) mixture. This reaction has also been observed with type II collagen and with albumin and is not specific to connective tissue proteins.

Most mechanical properties of tension resistance in connective tissues result from the collagen fibres. Synthetised and excreted from the cell as procollagen, the collagen molecule (a rigid rod 300 nm × 1.4 nm) first gives rise to fibrils, and then forms a reticulated three dimensional fibrous network. The steps in this fibrillogenesis, its regulation and the three-dimensional structure of the resulting fibres are still little known. Fibrillogenesis has been studied by the *in vitro* reconstitution of fibrils having the native structure as observed in the tissue, starting from purified collagen molecules [1]. Better knowledge of these mechanisms is essential for the exploration of pathological modifications of connective tissues seen in certain syndromes (Ehlers-Danlos, Marfan for example) or in hepatic or pulmonary fibrosis. It would also be very useful to have active molecules acting specifically at different stages of collagen maturation.

Earlier studies showed that flavonoids had a high affinity for connective tissue in general and for collagen in particular. Schlebusch and Kern [2] noted that (+)-catechin increases collagen stability *in vivo*. Cetta *et al.* [3] and Orloff *et al.* [4, 5] demonstrated the protective action of some flavonoids against lathyrism. Collagen solubility in fibroblast cultures from skin biopsies of patients with the Ehlers–Danlos type V or the Marfan syndromes or Cutis Laxa is exceptionally high, but it is decreased by addition of (+)-catechin to the culture medium [6, 7]. More recently, in the case of osteogenesis imperfecta for which alteration of collagen and glycosaminoglycans has been suggested, a three months treatment with (+)-catechin gave marked improvement [8]. Consequently these results are in favour of an action of (+)-catechin on the factors regulating collagen solubility (mainly on cross-links formation). The mode of (+)-catechin action on *in vitro* cross-links formation in elastin has been studied by Cetta *et al.* [9]. A (+)-catechin–copper complex induces the formation of aldehydic groups in elastin, even without lysyl-oxidase, the enzyme normally responsible for this transformation.

(+)-Catechin also affects collagen biosynthesis. It inhibits, prolyl and lysyl hydroxylases [10, 11]. It also inhibits the enzyme of collagen degradation, collagenase [12].

In this work the effect of (+)-catechin, alone or in the presence of cupric ions, on the solubility of collagen fibrils reconstituted *in vitro* and on the formation of intra- and inter-molecular cross-links is studied.

MATERIALS AND METHODS

Molecules studied. Flavonoids, (+)-catechin, dihydroquercetin, rutin and naringin, are prepared by Zyma S.A. (Nyon, Switzerland). The copper (II) chloride is purchased from Prolabo (Paris, France).

The flavonoid–Cu(II) complex was prepared by a method similar to that used by Cetta *et al.* [9]: at 4° mix, in a ratio of 2:1, 0.1 M (+)-catechin and 0.1 M $CuCl_2$ in 0.5 M ammonium hydroxide: the pH is lowered to 5.8 with 3 N HCl; the resulting brown precipitate is washed in the cold and then dried by lyophilisation. It contains about 13% copper—that is, two copper (II) molecules for every three molecules of (+)-catechin.

Collagen fibril formation and incubation with various compounds. Type I collagen is extracted from the dorsal skin of rats made lathyritic by β-aminopropionitrile intoxication (600 mg/kg/day during 3 weeks). The skins are shaved, stripped of adipose tissue and then cut into pieces and ground in liquid nitrogen with a laboratory grinder IKA A10. Neutral-salt soluble collagen is extracted by 0.05 M Tris, 1 M NaCl buffer pH 7.5 and purified by the method described by Chandrakasan *et al.* [13].

Type II collagen is prepared from bovine articular cartilage. The cartilage is ground up as described for skin and the collagen extracted by pepsin after pretreatment with 4 M guanidinium chloride in 0.05 M sodium acetate pH 5.8 as described by Herbage *et al.* [14].

The purified collagen solution, in 0.05 M CH_3COOH at about 2 mg/ml is dialysed against 0.05 M KH_2PO_4, 0.14 M NaCl buffer pH 7.4 during 24 hr at 4° with at least two bath changes. The dialysed collagen is centrifuged at 38,000 r.p.m. for 1 hr at 4°. Fibrils are formed by gradually warming to the solution from 4° to 32° over 2 hr as described by Robins and Bailey [15]. The formation of a network of native fibrils at 32° is verified by electron microscopy.

At this stage of fibrillogenesis at 32° the compounds under examination are added: (+)-catechin, (+)-catechin–Cu(II) complex, freshly mixed (+)-catechin and $CuCl_2$, $CuCl_2$ and other flavonoid–Cu(II) complex, dihydroquercetin, rutin and naringin. The (+)-catechin–Cu(II) complex is nearly insoluble at neutral pH values, so all the test compounds are dissolved in 0.5 M ammonium hydroxide. They are added to the incubation medium at concentration of 20 to 80 μg/mg of collagen, in volume of solution giving variations in pH of less than 0.5. The incubation medium containing fibrils plus the test compound is warmed at 37° for 5 hr.

Crystallized bovine albumin (Sigma) dissolved in 0.05 M KH_2PO_4, 0.14 M NaCl buffer pH 7.4 is also incubated with (+)-catechin–Cu(II) complex as described for the collagen.

Analysis of incubated fibrils

Ultrastructure. Ultrastructural studies are performed using the Philipps EM 300 electron microscope at the Centre de Microscopie Electronique Appliquée à la Biologie of the University "Claude Bernard", Lyon, France, after staining with phosphotungstic acid (0.4% pH 3.5) and unbuffered uranyl acetate solution (0.1%) as described by Stark and Kuhn [16].

Solubility. After incubation at 37° the fibrils are cooled and stored at 4° for 20 hr then centrifuged (25,000 r.p.m., 30 min). The percentage of redissolved collagen fibrils in the pellet and supernatant is determined by measuring the hydroxyproline content according to the method of Stegemann [17].

Thermal stability. The lyophilised collagen fibrils (5 mg) are swollen by deionised water (0.5 ml) and sealed in the cell of a Setaram programmed differential calorimeter. The thermogram is obtained with a heating rate of 3° min starting at 4°. The temperature of denaturation is measured by extrapolation of the tangent to upward slope of the peak to the baseline. According to Mc. Clain and Wiley [18] the value given at the baseline–tangent intersection gives a good estimate of the denaturation temperature of collagen.

Reduction of collagen and identification of the cross-links. The fibrils, buffered at pH 7.4 as for the incubation are reduced by KB^3H_4 with a specific activity of 10 mCi/mmole in the ratio of 1 mg of KBH_4 for 30 mg of collagen. The reduced fibrils are hydrolysed by 6 N HCl at 120° for 24 hr and the radioactive compounds then analysed by ion exchange chromatography as described by Bailey *et al.* [19]. The nature of the precursors of the collagen cross-links, (dihydroxynorleucine (diOHNL), hydroxynorleucine (OHNL) produced by reduction of hydroxylysine and lysine aldehyde and chlorohydroxynorleucine (ClNL) formed by acid hydrolysis of OHNL) is confirmed by gas chromatography, using known standards, according to Perier *et al.* [20]. Confirmation of the identity of the reduced cross-links (dihydroxylysinonorleucine (diOHLNL), hydroxylysinonorleucine (OHLNL) and lysinonorleucine (LNL), Schiff base type bonds produced by reaction of allysine or hydroxyallysine with lysine or hydroxylysine) is achieved by comparison with authentic standards using the extended basic column of the Jeol 5 A H aminoacids analyser, eluted with pH 5.28 citrate buffer.

Amino-acid analysis. The fibrils are hydrolysed by 6 N HCl at 120° for 24 hr and the amino-acids, particularly lysine and hydroxylysine, are identified using a Jeol JLC 5 AH amino-acid analyser eluted with citrate buffers pH 5.10 and 3.10.

RESULTS

Ultrastructure

The fibrils formed after heating the collagen solution from 4° to 32° for 2 hr show the periodic striation (640 Å) characteristic of native collagen fibrils. This structure remains unchanged even after 5 hr incubation at 37° alone or with flavonoid and copper (not shown).

Solubility

Most of the type I collagen fibrils (95%) redissolve at 4° (Table 1). Fibrils incubated with copper (II) chloride are slightly less soluble. With (+)-catechin the percentage solubility decreases (15–17%) whatever the concentration used; when copper (II) is added to the (+)-catechin the fibrils become insoluble (<1%). A similar result is obtained with dihydroquercetin and rutin in presence of Cu(II), only naringin–Cu(II) is less efficient (16% resolubilised).

Thermal stability

Figure 1 shows typical thermograms for collagen fibrils alone, with (+)-catechin and with the (+)-catechin–CU(II) complex. Table 1 gives the temperature measured at the onset of denaturation. For fibrils reconstituted from type I neutral-salt soluble collagen, incubated alone or with (+)-catechin this temperature is 40°. However in the presence of (+)-catechin a shoulder appears (arrow Fig. 1(b)) at a higher temperature, higher concentration of (+)-catechin giving more clearly defined shoulder.

The stability of fibrils incubated with (+)-catechin–Cu(II) complex or mixture is greatly modified. The main peak appears at a higher temperature (57° to 60°) whilst at lower temperature, around 40°, a slight peak persists (Fig. 1(c)). The position of the main peak does not depend upon the concentration of the (+)-catechin–Cu(II).

Table 1. Solubility at 4° and thermal stability by differential calorimetry of collagen fibrils formed at 37° in the presence of various molecules at different concentrations

	Collagen fibrils solubility (% at 4°)	Thermal stability denaturation temperature (T_D°,)
Type I collagen (neutral-salt soluble)	95	40
+ $CuCl_2$ 40 μg	79	40
+ (+)-catechin 20 μg	16	40
40 μg	17	40 shoulder 56
80 μg	15	40 shoulder 56
+ (+)-catechin–Cu(II) 20 μg	1.5	53 2nd peak 40
40 μg	1.5	59 2nd peak 45
60 μg	0	60 2nd peak 47
80 μg	0	60 2nd peak 47
+ (+)-catechin + $CuCl_2$ 80 μg (mixture)	4	

Reducible compounds

Type I collagen. The neutral-salt soluble collagen fibrils incubated alone at 37° gives two reduced compounds OHLNL and histidinohydroxymerodesmosine (HHMD, produced by condensation of hydroxylysine, the aldol condensation product and histidine residue, followed by reduction). These compounds are typical of soft tissues (skin, tendon) (Fig. 2(a)). There are only very few of these bonds. This is normal since the collagen used, extracted from lathyritic animals, has only very few aldehydic groups. The same radioactive compounds are found in presence of (+)-catechin (Fig. 2(b)). Their concentrations are independent of the quantity of (+)-catechin. However when very high concentrations of (+)-catechin (≥ 80 μg) are used, some precursors can be detected (Table 2). Incubation with $CuCl_2$, induces formation of a few other compounds: bonds precursors (diOHNL and OHNL) and the reduced bonds LNL, with no modification of the OHLNL concentration (Fig. 2(c)). Fibrils incubated with the (+)-catechin–Cu(II) complex or mixture give high amounts of bonds precursors (diOHNL) and compounds produced by cross-linking (Fig. 2(d) and (e)). The variation of the concentration of these compounds with that of the (+)-catechin–Cu(II) shows that with increasing (+)-catechin–Cu(II) there is a great increase in reduced aldehydes diOHNL and OHNL, the OHLNL ratio remains the same and the LNL ratio increases regularly (Table 2). The changes in precursors and reducible bonds during incubation with a given quantity of (+)-catechin–Cu(II) show that the precursors diOHNL and OHNL increase rapidly at a high concentration, and that the OHLNL bond reaches a maximum after 2 hr, whilst the LNL bond stabilizes much later (Fig. 3). The similar formation of reducible compounds is found with dihydroquercetin, rutin and naringin in presence of Cu(II), but at a lower ratio (Table 2).

It must be pointed out that the (+)-catechin–Cu(II) complex whatever the concentration and action time does not give rise to aldol bonds, as demonstrated by the absence after reduction of reduced aldol and HHMD after acid (Fig. 2) or alkalin hydrolysis (not shown).

Type II collagen. For type II fibrils incubated alone at 37° for 5 hr the diOHLNL bond, characteristic of hard tissues (bone, cartilage), is found. These fibrils, when incubated with (+)-catechin–Cu(II) complex

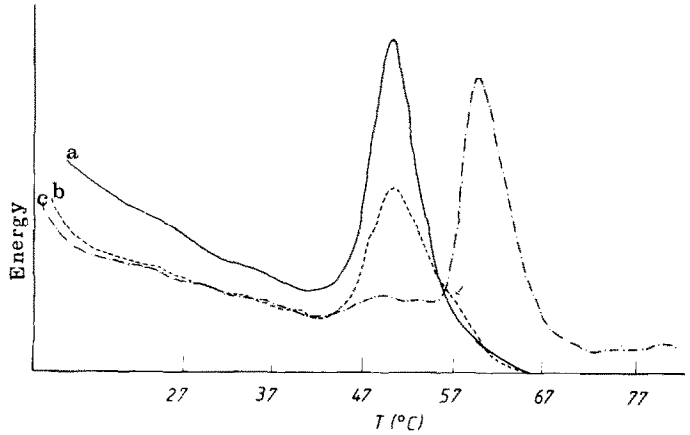

Fig. 1. Collagen fibrils stability by programmed differential calorimetry: (——) neutral-salt soluble collagen alone, and with (- - -) (+)-catechin 40 μg/mg, (·····) (+)-catechin–Cu(II) 20 μg/mg collagen.

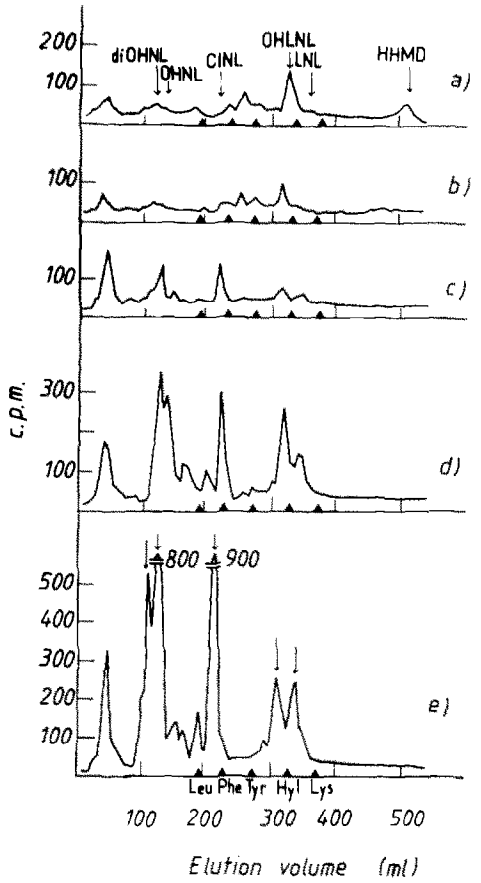

Fig. 2. Chromatography in pyridine formate buffer of derivatives linking collagen fibrils formed at 37°: (a) standard fibrils; fibrils incubated with (b) (+)-catechin 40 μg/mg collagen, (c) Cu^{2+} 40 μg (d) and (e) (+)-catechin–Cu(II) respectively 20 and 40 μg.

or mixture show as well as a great increase in diOHLNL, a sudden appearance of a great deal of reduced aldehydes diOHNL and OHNL, and fewer OHLNL and LNL derivatives (Fig. 4).

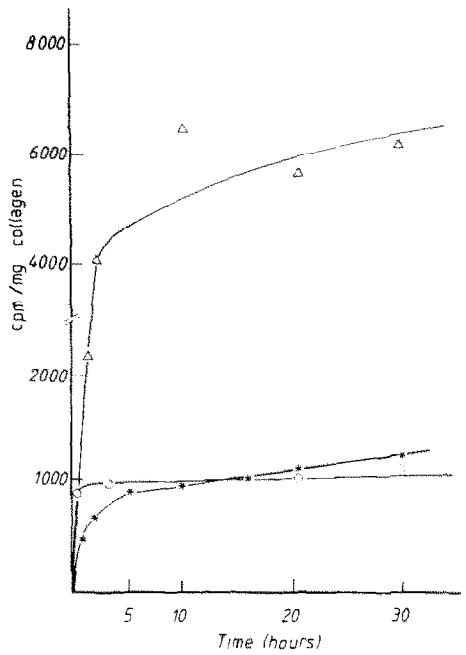

Fig. 3. Correlation between substituted group constants (π-values) and K_m-values. Ordinate: -log K_m-values, abscissa: π-values ($r = -0.9510$; $P < 0.05$). For number of experiments see Table 1.

Non-collagen proteins. The albumin incubated alone, after reduction, acid hydrolysis and chromatography, shows no sign of any reducible compounds resembling those obtained from collagen. On the other hand, when incubated in presence of (+)-catechin–Cu(II) complex or mixture, the OHNL and LNL derivatives are found (Fig. 5).

Amino-acids analysis

For type I collagen alone, lysine and hydroxylysine residues are 29.5 and 5.7 residues ‰. After incubation with 80 μg of (+)-catechin–Cu(II), these residues decrease to 25.8 and 4.0 respectively.

Table 2. Reducible compounds from type I collagen fibrils incubated at 37° for 5 hr with or without different concentrations of $CuCl_2$, (+)-catechin and (+)-catechin-Cu(II) and different flavonoids

Incubations	Reducible compounds		
	diOHNL-OHNL-CINL (cpm/mg collagen)	OHLNL (cpm/mg collagen)	LNL (cpm/mg collagen)
Type I collagen	traces	390	traces
+ $CuCl_2$ 40 μg	990	290	300
+ (+)-catechin 20 μg	traces	480	traces
40 μg	traces	380	traces
80 μg	440	340	traces
+ (+)-catechin–Cu(II) 20 μg	3115	1030	655
(complex) 40 μg	3900	1175	880
60 μg	6385	1080	1315
80 μg	6760	1460	1670
+ (+)-catechin 60 μg + $CuCl_2$ 20 μg (mixture)	7600	675	1365
+ dihydroquercetin–Cu(II) 80 μg	2180	270	395
+ rutin–Cu(II) 80 μg	1110	345	325
+ naringin–Cu(II) 80 μg	1015	570	415

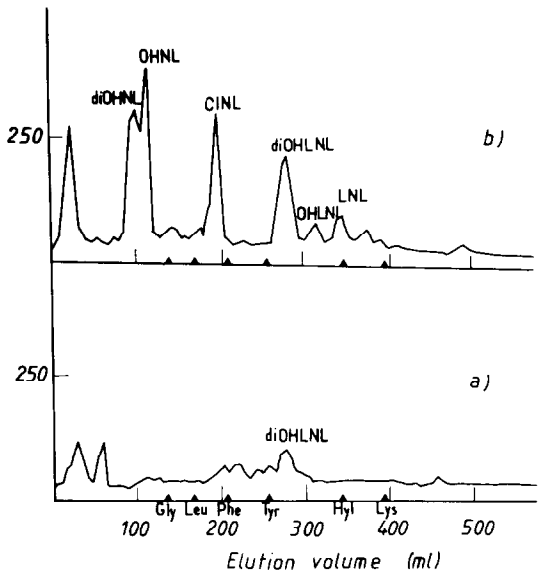

Fig. 4. Reducible compounds from type II collagen fibrils (a) alone (b) incubated with (+)-catechin–Cu(II) 80 μg/mg collagen.

DISCUSSION

The fibrils formed after a short incubation at 32° have the typical periodicity (640Å) of native collagen fibrils. These reconstituted collagen fibrils are used in this study, to test the effects of (+)-catechin alone or with Cu(II) upon their solubility and cross-linking.

In the presence of (+)-catechin, collagen fibrils become insoluble. As demonstrated in this work, this insolubilisation is not due to formation of reducible intermolecular cross-links and should result from interaction between the (+)-catechin and collagen, as described by Schlebusch and Kern [2]. This would explain observations made during the study of fibroblast cultures from patients suffering from the Ehlers–Danlos type V [6] or Marfan syndromes or Cutis Laxa [7], showing a decrease in the abnormally high collagen solubility in presence of (+)-catechin. These results seem difficult to extrapolate to *in vivo* conditions. There have been a few positive results with lathyritic animals treated with (+)-catechin [3–5] but the exact nature of the active metabolite is unknown. Also the metabolism of (+)-catechin varies between species [21] so that results obtained for one species cannot be applied to another. In experiments on rats *in vivo*, it was not possible to show any action of (+)-catechin on either the rate of collagen synthesis or its maturation [22].

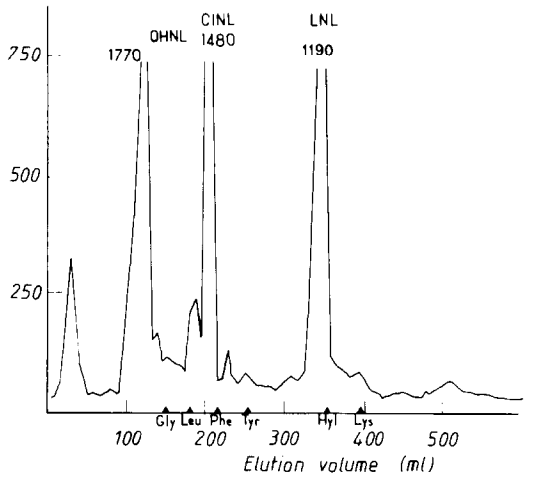

Fig. 5. Reducible compounds from albumin incubated with (+)-catechin–Cu(II) 80 μg/mg collagen.

The thermal stability of the fibres depends simultaneously on their solubility and reticulation ratio. The fibrils alone or in presence of (+)-catechin denature at the same temperature than the collagen molecule in solution. However in presence of (+)-catechin the insolubilisation of fibrils can explain the appearance of the 'shoulder' for higher temperature. On the other hand, the thermal stability of collagen fibrils incubated with (+)-catechin–Cu(II) is identical with that obtained with fibrils present in tissues such as skin or tendon. This can be explained by the appearance of fibrillar reticulation. The type I collagen used is aldehyde deficient and reconstituted fibrils can form only a few cross-links, as in the initial tissue. With (+)-catechin–Cu(II), many OHLNL bonds appear, as well as many LNL bonds which are only rarely found in normal tissue. Similarly the type II collagen, extracted from cartilage, gives many diOHLNL bonds characteristic of the tissue, as well as two other types of cross-links OHLNL and LNL, which are less numerous and not tissue specific. The presence of these bonds implies an oxidative deamination of the lysine and hydroxylysine of the collagen molecules with formation of aldehydes. These have been detected in their reduced state as diOHNL and OHNL. This oxidation was confirmed by the simultaneous decrease in number of lysine and hydroxylysine in the fibrils incubated with (+)-catechin–Cu(II) complex.

In presence of $CuCl_2$ only, there is a slight oxidation but in this case the Cu(II) is 4 to 16 times more concentrated than in the (+)-catechin–Cu(II) complex. So it would not seem possible that the addition of copper (present in complex) to the incubation medium would activate the lysyl-oxidase which could have been extracted with the collagen. This enzyme needs copper as its coenzyme. These results agree with those of Cetta *et al.* [9] on elastin: the (+)-catechin–Cu(II) complex induces oxidative deamination of elastin in absence of lysyl-oxidase. However, this oxidizing action of (+)-catechin–Cu(II) complex is not specific to connective tissue proteins (elastin and collagen) as Cetta *et al.* [9] thought; the present work shows the same effect on albumin molecules. Until now, formation of aldehydes in collagen from the lysine and hydroxylysine residues in absence of lysyl-oxidase, has only been mentioned by Fowler *et al.* [23]. They used pyridoxal phosphate and copper, but in this case there was no subsequent cross-linking in the reconstituted collagen fibrils.

It is well known that flavonoids are good chelating agents for copper ions [24]. But they are even better known for their anti-oxidizing action when in the presence of metal ions, especially on ascorbic acid.

This anti-oxidizing activity can be explained, at neutral and alkaline pH, by the chelation of metal ions; in acidic solutions there is only a weak chelating action but the activity can be explained by their ability to accept free radicals [25]. In this study, in neutral solutions, the (+)-catechin–Cu(II) is an oxidizing agent. Similar results were obtained with another flavonoids notably flavanones: dihydroquercetin, rutin, naringin but their action is less marked. The reaction mechanism has not been defined and a structural study of the (+)-catechin–Cu(II) has not been undertaken. Little work has been done on the (+)-catechin–Cu(II) complex; only Takamura and Sakamoto [26] made a spectrophotometric study, showing the site of Cu fixation on the flavonoid and defining the molarity ratio, one to two molecules of copper per flavonoid in acid solution and one copper for two flavonoids in alkaline solution. The copper: flavonoid molarity ratio measured in this study is about 2:3. This suggests the presence of a chelate encompassing several (+)-catechin molecules; moreover the strong alkalinity of the solutions favorises polymerization of the (+)-catechin molecules.

Further studies are thus necessary to elucidate the exact mechanism of that reaction and to evaluate its potential *in vivo* application.

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