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Oxidation products are responsible for the resistance to the action of collagenase conferred on collagen by (+)-catechin

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Among many other pharmacological properties [1, 2], (+)-catechin [or (+)-cyanidanol-3], a flavonoid, has been attributed [3, 4] a stabilizing effect on collagen which renders collagen more resistant to the action of mammalian collagenase (EC 3.4.24.3). However, in aqueous solutions (pH 6 to 8), (+)-catechin spontaneously becomes oxidized and polymerizes [5–7]. We report here that the resistance to the action of collagenase conferred on collagen by its preincubation with (+)-catechin is likely to be due to the action on collagen of oxidation products derived from (+)-catechin and not to the direct action of intact (+)-catechin.

Materials and methods

[^{14}C]collagen-coated Multiwell plates, prepared from acid-soluble guinea-pig skin (type I) collagen, were used to monitor collagen degradation following our described technique [8], using as a source of collagenase trypsin-activated mouse bone culture fluids [9] containing approximately 10 units of collagenase/ml (1 ml/well) or occasionally, bacterial collagenase (EC 3.4.4.19), either crude (Type I, from Sigma, St Louis, MO; 2 mg/well) or highly purified (Type VII, from Sigma; 20 μg/well). For some experiments, the plates were coated with pepsin-extracted bovine articular cartilage (type II) collagen [10] or with pepsin-treated acid-soluble type I collagen (2 mg/ml in 0.5 M-acetic acid, incubated for 48 hr at 4° with pepsin, 0.2 mg/ml, then dialyzed against pH 7.5 buffer). All collagens were ^{14}C-labelled by acetylation [11]. Whenever required, the collagen films covering the plates were pretreated with (+)-catechin by incubating the collagen-coated plates for up to 2 days at 35° with, into each well, 1 ml of TNCN buffer (50 mM-Tris/HCl, pH 7.5, with 0.14 M-NaCl, 5 mM-$CaCl_2$ and NaN_3, 0.2 mg/ml) containing the drug [(+)-catechin chlorhydrolysinate, kindly provided by Dr P. Niebes, Continental Pharma, Mont-Saint-Guibert, Belgium]; the plates were then extensively rinsed with TNCN buffer to remove unbound catechin. Control plates were treated with TNCN buffer alone. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of collagen degradation products was done as previously reported [12], after incubation of collagen with mammalian collagenase for 18 hr at 25° in the presence or not of 0.5 mM (+)-catechin. The oxidation of catechin was followed either directly by taking the complete absorbance spectra of the preparations in a Perkin-Elmer model 555 spectrophotometer, or indirectly, after a colorimetric reaction with *p*-dimethylaminocinnamic aldehyde [13].

Results and discussion

Preincubation of collagen (type I)-coated plates for 2

days with (+)-catechin rendered the collagen more resistant not only to the action of mammalian collagenase (Fig. 1), as already shown [3, 4], but also to that of bacterial collagenase, either crude (Fig. 1) or highly purified (not shown). Stabilization towards bacterial collagenase had not been noted before [3, 4], apparently because conditions for limited proteolysis of the collagen substrate were not investigated. The same protective effect of (+)-catechin towards mammalian collagenase was manifested with catechin-treated coated-plates prepared with pepsin-treated type I-collagen (indicating that intact telopeptides are not necessary for the interaction of collagen with catechin) or with pepsin-extracted type II-collagen (not shown). Similarly, (+)-catechin prevented the appearance on slab gel electrophoresis of the typical three-quarter and one-quarter fragments obtained from collagen on incubation with mammalian collagenase at 25° (not shown), as already noted by others [4].

In our initial experiments, we observed that the resistance of collagen-coated plates to the action of collagenase at 35° was almost not affected by a 3-hour preincubation with 10^{-4} M (+)-catechin taken from a freshly prepared (+)-catechin solution but that it was considerably increased when the catechin was taken from a concentrated stock solution that had been "aged" in TNCN buffer for a few weeks at room temperature (or for 5 days at 35°). A similar, almost complete stabilization of collagen toward collagenase could be obtained with 10^{-4} M "fresh" (+)-catechin when the preincubation of the plates with the drug was extended to 2 days or more. However, "aged" catechin solutions, but not fresh solutions, are yellowish and upon prolonged preincubation of the plates at 35° with solutions of (+)-catechin in TNCN buffer, pH 7.5, a yellow to brown coloration of the solution and of the collagen-coated plates became manifested, suggesting that a product different from catechin could have been fixed on the substrate beside or instead of catechin. Pure catechin is indeed colourless. The catechin solutions had initially no absorbance in the visible range but upon incubation, 3 absorption maxima appeared. As the maxima increased, indicating an increasing amount of reaction products, their positions moved slightly (spectra in TNCN buffer, pH 7.5: λ_{1max} = 394–410 nm, λ_{2max} = 424–436 nm, λ_{3max} = 468–480 nm) and their relative values changed. The bands became wider and finally the visible spectrum showed a single wide absorption maximum in the range 300–450 nm. Obviously, complex and multiple steps reactions were involved. The reaction was inhibited by $Na_2S_2O_5$ but considerably accelerated by $CuSO_4$. These results are consistent with the formation of irregular three-dimensional polymers of increasing molecular weights involving oxidation of (+)-catechin as proposed by Hathway and Seakins [5]. The catechin polymer that they obtained by complete autoxidation of catechin at 100° between pH 4 and pH 8 in the presence of oxygen had phenolic properties but lacked the characteristic properties of catechol and phloroglucinol. Since the colorimetric method using *p*-dimethylaminocinnamic aldehyde can only titrate catechin or phloroglucinol derivatives of a similar type [12], a solution of catechin (10^{-4} M in TNCN buffer, pH 7.5) was incubated at 35° and titrated by this method at different times. In good agreement with these authors, the phloroglucinol content of the reaction mixture decreased with increasing time of incubation. This resulted from an oxidation process as the reaction was inhibited by various anti-oxidizers ($Na_2S_2O_5$, L-cysteine, D-penicillamine, ascorbate) and stimulated by an oxidizer, cupric ions (Fig. 2).

To investigate the effects of the oxidation products of (+)-catechin on the stabilization of collagen towards collagenase, collagen-coated plates were preincubated with (+)-catechin supplemented or not with the same anti-oxidizers or oxidizers. As expected, preincubation for 2 days with 10^{-4} M catechin rendered the collagen resistant to collagenase. This protective effect disappeared, however, when the oxidation of (+)-catechin was prevented by the addition of $Na_2S_2O_5$, L-cysteine or D-penicillamine (Fig. 3). On the contrary, preincubation of the plates for 5 hr with 10^{-4} M catechin was insufficient to render their collagen

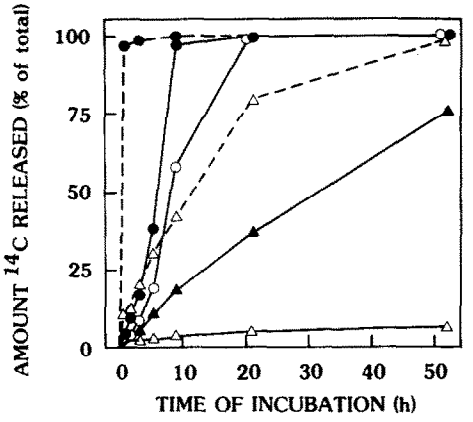

Fig. 1. Increased resistance of (+)-catechin-treated collagen against mammalian or bacterial collagenase. [^{14}C]Collagen-coated plates were preincubated for 2 days at 35°, pH 7.5, with TNCN buffer without additive (●) or with added (+)-catechin 10^{-6} M (○), 10^{-5} M (▲) or 10^{-4} M (△). They were then rinsed and further incubated for the indicated time, in a first experiment (continuous lines), with mouse collagenase, in a second (broken lines), with crude bacterial collagenase. Collagen degradation was monitored by the release of soluble ^{14}C-labelled degradation products from the plates [8], expressing it as a percentage of the total amount of ^{14}C (i.e. about 12,000 c.p.m. for 120 μg collagen) present on the plates. Each point is the mean of 3 assays.

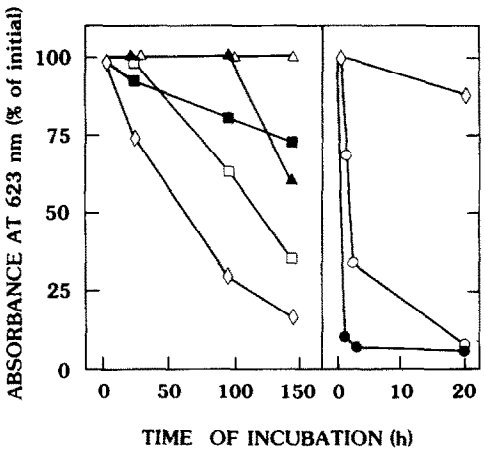

Fig. 2. Oxidation of (+)-catechin. Solutions of (+)-catechin (10^{-4} M in TNCN buffer, pH 7.5) were incubated at 35° for the indicated times after which catechin was titrated by colorimetry [12]. Without additive to the reaction mixture (◇), catechin was progressively lost. This loss was inhibited by the addition of various anti-oxidizers, either partially by 10^{-3} M ascorbic acid (■), D-penicillamine (□) or L-cysteine (▲), or completely (△) by 10^{-3} M $Na_2S_2O_5$ or 5.10^{-3} M L-cysteine or D-penicillamine. It was accelerated by an oxidizer, either 5.10^{-6} M (○) or 5.10^{-5} M (●) $CuSO_4$.

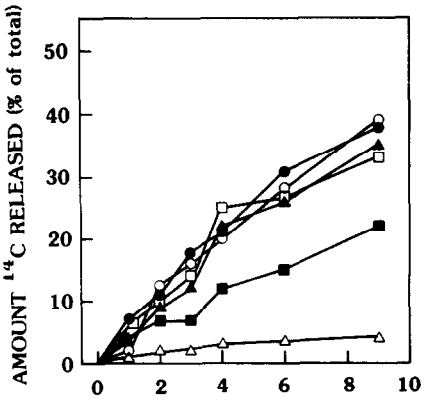

Fig. 3. Prevention of the catechin-induced stabilization of collagen by anti-oxidizers. [^{14}C]Collagen-coated plates were preincubated for 2 days at 35°, pH 7.5, with TNCN buffer without additive (●) or with added 10^{-4} M catechin (△) or 10^{-4} M catechin and 5.10^{-3} M antioxidizer, either $Na_2S_2O_5$ (○), L-cysteine (□), D-penicillamine (▲), or ascorbic acid (■). After rinsing, they were further incubated for the indicated time with mouse collagenase and collagen degradation was monitored as in Fig. 1. Control plates preincubated with TNCN containing anti-oxidizers without catechin gave results similar to controls containing TNCN only (not shown). Each point is the mean of 3 assays.

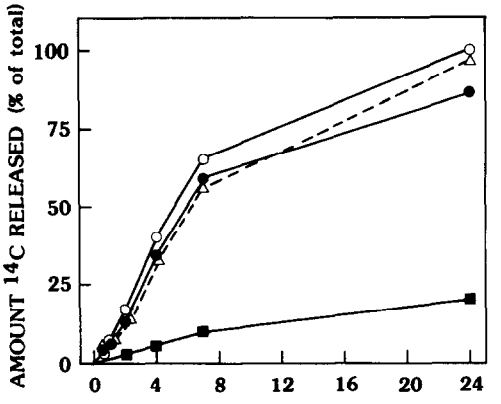

Fig. 4. Induction of the collagen-stabilizing effect of catechin by an oxidizer (Cu^{2+}). [^{14}C]Collagen-coated plates were preincubated for 5 hr only at 35°, pH 7.5, with TNCN buffer without additive (●) or with added 5.10^{-5} M $CuSO_4$ (○), 10^{-4} M catechin (△), or 10^{-4} M catechin and 5.10^{-5} M $CuSO_4$ (■). After rinsing, they were further incubated for the indicated time with mouse collagenase and collagen degradation was monitored as in Fig. 1. Each point is the mean of 3 assays.

resistant to collagenase unless cupric ions, an oxidizer, was simultaneously added to the preincubation mixture (Fig. 4).

These experiments thus indicate that products arising from the oxidation of (+)-catechin, and not intact (+)-catechin, are responsible for the stabilization of collagen towards the action of collagenase (either mammalian or bacterial) induced by this flavonoid. It is not known, however, whether other stabilizing effects exerted by catechin on collagen [4] are also due to these products. Also their mechanism of action remains to be elucidated. As the oxidation of catechin seems to involve the formation of an orthoquinone followed by condensation to form dimeric or oligomeric quinonic components [5–7], it is possible that the stabilization of collagen against the action of collagenase results from the direct addition of these quinones to free amino groups of the collagen molecules [14]. Such addition is compatible with the demonstrated binding of catechin residues (or derivatives) to collagen [4]. Moreover, when it involves dimeric or oligomeric quinonic derivatives of catechin, it could also establish bi- or multipolar cross-links between collagen chains, increasing further the stability of this substrate [15].

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