would give the same result if E4 was a dimer. This latter suggestion is rather unlikely in a parthenogenetic system and although we have reported heritable changes within a strain previously[6], the system dealt with there was almost certainly an exceptional case.

The final answer awaits further work but such diet induced changes in enzyme polymorphism indicate it is important to think carefully in interpreting findings of variations in natural populations on which so much of modern evolution theory is based.

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Collagen treated with (+)-catechin becomes resistant to the action of mammalian collagenase[1]

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Summary. Treatment of radioactively labeled guinea-pig skin soluble collagen or calf skin collagen with the flavonoid (+)-catechin makes the collagen resistant to the action of mammalian collagenase but not to the action of bacterial collagenase. Complete resistance to the action of the mammalian enzyme may be achieved by incubating 0.6 mg of collagen (dry weight) with 0.1 mM (+)-catechin, followed by dialysis to remove the unbound flavonoid. Since incubation of the mammalian enzyme with (+)-catechin does not inhibit its activity, it is postulated that (+)-catechin binds tightly to collagen and modifies its structure sufficiently to make it resistant to enzyme degradation.

The flavonoid (+)-catechin has been proposed as a collagen stabilizer because of its postulated ability to form hydrogen bonds and possibly cross-links among different collagen chains[2]. Because of this claim, in the past years we have repeatedly added (+)-catechin to the culture medium of fibroblasts derived from patients with various inherited diseases considered to involve the structure and function of collagen. When the collagen synthesized by the cultured fibroblasts of those patients was excessively soluble, the addition of (+)-catechin to their culture medium decreased its abnormal solubility[3]. In other studies, administration of (+)-catechin to experimental animals treated with β-aminopropionitrile or 3′,3′-iminodipropionitrile has produced similar, beneficial changes in collagen solubility and has decreased the extent of lesions[4,5].

Despite these practical results, no evidence has been provided thus far for a physical interaction between collagen and (+)-catechin. In the present communication we demonstrate that incubation of soluble collagen with (+)-catechin results in the formation of a tightly-bound complex which becomes resistant to the action of mammalian collagenase.

Materials and methods. Human skin fibroblasts, obtained from patients by punch skin biopsies after obtaining informed consent, were cultured in minimum essential medium containing Earle's base and 10% fetal calf serum, as described previously[6]. In order to harvest the mammalian collagenase produced by these fibroblasts, the cells were cultured in serum-free medium for 2 days, as described by Bauer et al.[7]. Thereafter, the medium was dialyzed exhaustively at 4 °C against 0.01 M Tris-HCl buffer, pH 7.5, containing 0.1 mM calcium chloride. The retentate was concentrated to $^1/_{10}$ of its volume by lyophilization and aliquots of this crude enzyme preparation, containing 200 µg protein/ml, were used as described.

The activity of the enzyme was measured using either ^{14}C-acetylated collagen or [^{14}C]glycine-labeled guinea-pig skin soluble collagen. The former was prepared by treating acid-soluble collagen from calf skin with [1-^{14}C]acetic anhydride (New England Nuclear, Boston, MA) according to the method of Gisslow and McBride[8]. The ^{14}C-glycine-labeled guinea-pig skin soluble collagen was a gift of Dr M.H. Dresden of this department.

The incubation mixture consisted of acetylated collagen, 0.6 mg, 1800 cpm, in 0.3 ml of 0.01 M Tris-HCl buffer pH 7.8 containing 0.4 M sodium chloride; crude enzyme preparation, 40 µg protein, in 0.2 ml of 0.1 M Tris-HCl buffer pH 7.5; 0.5 M Tris-HCl buffer pH 7.5, 70 µl; 1.0 mM calcium chloride 70 µl and water, 60 µl, to a final volume of 0.7 ml. Incubation was performed at 37 °C for 5 h. Thereafter, 1 mg of bovine serum albumin in 0.1 ml of water was added as a carrier and proteins were precipitated with trichloroacetic acid (5% final concentration) at 4 °C for 20 min. The bulk of the precipitate was removed by centrifugation at 700 × g for 15 min and the supernatant collected was centrifuged again at 15,000 × g for 2 h at 4 °C. A 0.6-ml aliquot of the clear supernatant was added to 10 ml of Aquasol (New England Nuclear) and counted in a Packard Tri-Carb spectrometer.

When the ^{14}C-glycine-labeled substrate was employed, 0.2 mg, 3300 cpm, in 50 µl Tris-HCl buffer, were added to 0.2 ml of enzyme solution in presence of 0.1 mM calcium

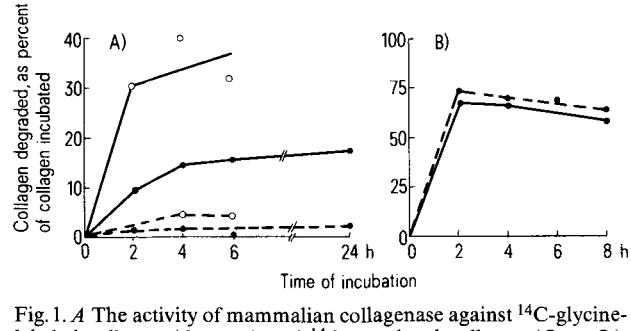

Fig. 1. *A* The activity of mammalian collagenase against ^{14}C-glycine-labeled collagen (○——○) and ^{14}C-acetylated collagen (●——●). The broken lines indicate the activity of the enzyme against the same substrates pretreated with (+)-catechin. *B* The activity of bacterial collagenase against ^{14}C-acetylated collagen (●—●) and against the same substrate pretreated with (+)-catechin (●– –●).

chloride, the final volume being brought to 0.3 ml with water. The mixture was incubated for 5 h at room temperature (25 °C) in order to prevent the substrate from forming a gel. Thereafter, the incubation mixture was kept at 37 °C overnight, so that the undegraded collagen could form a gel. This was centrifuged in a Brinkmann-Eppendorf centrifuge at 15,600× g at room temperature for 20 min and a 0.1-ml aliquot of the supernatant was used for counting radioactivity.

Bacterial collagenase from *Clostridium histolyticum*, 485 units/mg, was purchased from Worthington Biochemicals, Freehold, NJ. Its activity was measured using the ^{14}C-acetylated collagen incubated at 37 °C with 24 units of the enzyme under the conditions described, for periods ranging from 2 to 8 h. Upon termination of the incubation, the mixtures were precipitated with trichloroacetic acid and aliquots of the supernatants were employed for counting radioactivity.

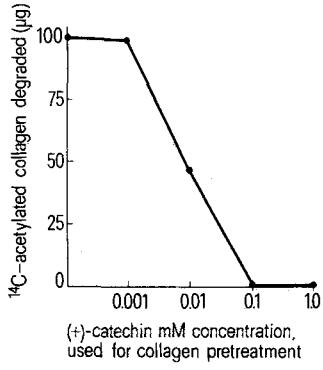

Fig. 2. The activity of mammalian collagenase against ^{14}C-acetylated collagen, pretreated with increasing concentrations of (+)-catechin.

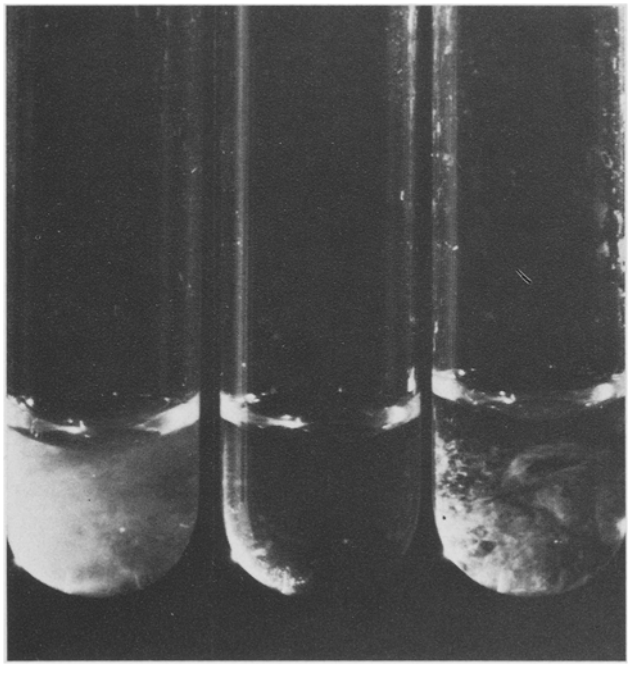

Fig. 3. *A* The gel formed by dissolving 0.4 mg of guinea-pig skin soluble collagen in 0.5 ml of 0.05 M Tris-HCl buffer pH 7.5. *B* Disappearance of the gel upon incubation with 40 µg of crude mammalian collagenase for 3 h at room temperature. *C* Persistence of the gel upon incubation with the same amount of enzyme of (+)-catechin pretreated guinea-pig skin soluble collagen.

The treatment of collagen with (+)-catechin was performed as follows. 4 mg of ^{14}C-acetylated collagen in 2 ml of 0.01 M Tris-HCl buffer pH 7.8, containing 0.4 M sodium chloride, were diluted with 0.5 M tris-HCl buffer pH 7.5 to obtain a final buffer concentration of 0.05 M. An aqueous solution of (+)-catechin was added so that the final concentration of the flavonoid would be 1 mM. The solution was incubated at 37 °C overnight and then it was dialyzed against distilled water at 4 °C to remove the unbound (+)-catechin. Thereafter, aliquots of the retentate corresponding to 1500 cpm were diluted with 0.5 M Tris-HCl buffer, to obtain a final buffer concentration of 0.05 M. This solution of modified collagen was incubated for 5 h at 37 °C with 0.2 ml of enzyme preparation in presence of 0.1 mM calcium chloride. Precipitation of the undegraded collagen with trichloroacetic acid, centrifugation and counting of the radioactivity in the supernatants were done as described.

The treatment of ^{14}C-glycine-labeled guinea-pig skin collagen with (+)-catechin was performed in the proportions stated above at room temperature for 3 h, because at 37 °C the complex formed becomes insoluble. Because of the small amount of ^{14}C-glycine-labeled collagen available, dialysis was omitted and aliquots of the mixture, containing 0.2 mg of the modified collagen (3300 cpm), were incubated with 0.2 ml of enzyme preparation for 5 h at room temperature. Thereafter, the incubation mixture was kept at 37 °C overnight and the gel formed was removed by centrifugation, as described.

In additional experiments, the treatment of ^{14}C-acetylated collagen with (+)-catechin was performed with decreasing concentrations of the flavonoid (from 1 mM to 0.001 mM). Dialysis of the complex formed was omitted. Thus, 600 µg dry weight of the modified collagen was used as substrate in presence of unbound (+)-catechin.

In order to verify whether unbound (+)-catechin could inhibit the collagenase activity, a 1-ml aliquot of the enzyme preparation was treated with 0.1 mM (+)-catechin at 4 °C for 1 h and 3 h. After removing the free catechin by dialysis against 0.01V M Tris-HCl buffer pH 7.5, containing 0.1 mM calcium chloride, the activity of the enzyme was determined by incubating a 0.2-ml aliquot with ^{14}C-acetylated collagen as described.

Results. The crude collagenase prepared from the medium of cultured human fibroblasts, grown in absence of fetal calf serum, was active and degraded the collagen substrates. The results shown in figure 1, A, indicate that ^{14}C-glycine-labeled collagen is degraded to a greater extent that the ^{14}C-acetylated collagen. When the 2 substrates had been preincubated with (+)-catechin (1 mM final concentration), the same amount of crude enzyme did not degrade them.

Under similar conditions, bacterial collagenase rapidly degraded ^{14}C-acetylated collagen, whether or not it had been pretreated with (+)-catechin (figure 1, B).

Incubation at 37 °C of constant amounts of ^{14}C-acetylated collagen with increasing amounts of (+)-catechin demonstrates that at a final concentration of 0.01 mM the flavonoid made the collagen substrate partially resistant to the action of mammalian collagenase, while it made it completely resistant at a final concentration of 0.1 mM (figure 2).

Because this latter experiment was performed without removing by dialysis the unbound (+)-catechin, additional experiments were performed to verify whether incubation of the enzyme with (+)-catechin would inhibit its activity. The results obtained indicated that incubation of the en-

zyme with 0.1 mM (+)-catechin for 1 h and 3 h at 4 °C resulted in 15 and 24% inhibition, respectively.

Figure 3, A, shows the gel formed by 0.4 mg of guinea-pig skin soluble collagen dissolved in 0.5 ml of 0.05 M Tris-HCl buffer pH 7.5 containing 0.1 mM calcium chloride; B shows the disappearance of the gel upon addition of 40 µg of crude collagenase and incubation for 3 h at room temperature; C shows the persistence of a coarse gel when the same amount of enzyme is incubated with the same amount of collagen pretreated with 0.1 mM (+)-catechin.

Discussion. Although (+)-catechin has been proposed as a stabilizer of collagen[2] and, under experimental conditions has been proven to reduce collagen solubility[3] and the severity of lesions secondary to defective formation of collagen crosslinks[4,5], evidence for its binding to collagen has not been provided.

In this study we have reasoned that a possible binding of (+)-catechin to collagen might change its conformation and alter its sensitivity to the action of specific collagenases. The results of our experiments demonstrate that incubation of 2 different types of soluble collagen with increasing amounts of (+)-catechin affords increasing resistance to degradation by mammalian collagenase but not to degradation by bacterial collagenase. Considering the different mechanism of action of the 2 types of enzymes, it is reasonable to assume that the conformation changes caused by (+)-catechin must be moderate, to the extent that they cannot prevent the activity of bacterial collagenase.

It has been postulated that the interaction between soluble collagen and (+)-catechin occurs through hydrogen bonds. From our results, it is evident that exhaustive dialysis of the complex does not remove the bound flavonoid, nor eliminates the protective effect against the activity of mammalian collagenase. Our experiments also exclude the possibility that the resistance to the action of this enzyme might be ascribed to a direct inhibition of the enzyme rather than to stabilization of the substrate. These results reinforce the rationale that (+)-catechin be tried as a therapeutic agent in human diseases considered to involve either structural abnormalities of collagen fibres or their excessive enzymic degradation. Although the safety and lack of toxicity of (+)-catechin in humans have been abundantly proven[9], the questions related to its intestinal absorption and solubility suggest that the development of suitable derivatives might help to achieve effective concentrations at the level of the peripheral tissues.

1 Acknowledgments. This work was supported by grants from the Easter Seal Research Foundation of the National Easter Seal Society for Crippled Children and Adults (R-7821), and the National Institutes of Health (HL-20447). (+)-Catechin was a generous gift from Zyma SA, CH-1260 Nyon, Switzerland.

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An enzymatic spectrophotometric assay for inosinic acid

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Summary. An enzymatic, spectrophotometric assay for inosinic acid is described. Inosinic acid is reacted with excess pyrophosphate in the presence of magnesium ions and hypoxanthine guanine phosphoribosyltransferase to form hypoxanthine which is oxidized to uric acid in the presence of xanthine oxidase. Inosinic acid concentration is measured by the increase in absorption at 293 nm at the end of the reaction.

Inosinic acid (IMP) is the first product in de novo purine biosynthesis to possess a complete purine ring system, and is an intermediate for the biosynthesis of both adenylic acid (AMP) and guanylic acid (GMP)[1]. It appears to be of widespread occurrence in nature and has been detected in microorganisms and in various animal tissues[2]. The determination of this nucleotide is of particular interest not only in metabolic studies[3] but also in the analysis of products in the foodstuff industry[4]. IMP is generally determined after purification by column or thin layer chromatography by means of the characteristic UV absorption[5,6]. In the present paper an enzymatic spectrophotometric method for the determination of IMP is described. IMP is pyrophosphorylated to hypoxanthine by hypoxanthine guanine phosphoribosyltransferase (HGPRT) in the presence of excess pyrophosphate (PP_i) and of magnesium ions. Hypoxanthine is oxidized to uric acid by xanthine oxidase (XOD).

Materials. HGPRT was purified to apparent electrophoretic homogeneity from human erythrocytes as previously described[7]. XOD and IMP were purchased from Boehringer A.G. Paper chromatography according to Gerlach et al.[8] showed no significant impurity in the commercial sample of the nucleotide. All other reagents were high-purity commercial samples from Boehringer A.G. and Merck A.G.

Methods. IMP was assayed by the following procedure: IMP was reacted with excess PP_i in the presence of magnesium ions, HGPRT, and XOD[7] to form uric acid. At the end of the reaction, uric acid concentration was measured by the increase in absorption at 293 nm.

The reaction mixture contained 0.1 M Tris HCl, pH 7.4, 0.01 M $MgCl_2$, 0.001 M PP_i, 0.04 IU/ml XOD, and IMP ranging from 0 to 3.7×10^{-5} M. The 1st spectrophotometric reading was made at 293 nm to determine the initial