Machine Translated by Google

Arch Orthop Trauma Surg (1992) 111:259–264

Archives of Orthopaedic and Trauma Surgery
© Springer-Verlag 1992

Collagen stabilization induced by natural humic substances

U. N. Riede[1], I. Jonas[2], B. Kirn[3], U. H. Usener[1], W. Kreutz[3], and W. Schlickewey[4]

[1] Department of Pathology, Medical School, [2] Department of Orthodontics, School of Dentistry,
[3] Department of Biophysics and Radiation Biology, Medical School, and [4] Department of Traumatology, Medical School,
University of Freiburg, Freiburg/Br., Federal Republic of Germany

Summary. Humic substances are polyphenolic compounds. They have antiviral as well as desmutagenic effects and react with biopolymers such as collagen; thereby they have no toxic side effects by oral administration. In vitro incubation with humic substances raises the breaking point of the tail tendon of the rat by about 75%. The chemical resistance of the collagen fibres in tail tendon collagen is also increased by in vitro incubation with humic substances, at least insofar as the ultrastructurally and biophysically measurable destruction of the collagen fibres by 4 *M* guanidinium chloride is inhibited. As humic substances increase the mechanical and chemical resistance of collagen fibres and promote their "maturity", it seems likely that this effect of humic substances depends upon their interaction with the hydrogen bonding and covalent bonding of the collagen fibres. Such a conclusion is confirmed by the results of X-ray diffraction analysis.

Naturally occurring humic substances are particular chemical compounds that are found in the humus. They are formed during the process known as humification. Starting compounds of humification are phenols, which appear naturally following the hydrolysation of lignins, as well as phenolic groups from plant flavonoids. It is possible to reconstruct a model of humification, and it has been described mathematically as a sequence of interrelated Markoff processes. The synthesis of such a model humic substance is initiated by the autoxidation of multivalent phenols. As the result of the electrophilic attack on a displaceable electron by an electron acceptor, usually molecular oxygen, highly reactive radicals are formed which possess a single unpaired electron. The instability of such an oxygen radical is in part reduced by intramolecular mesomery. This leads to the appearance of, for instance, *p*-benzo-semiquinone anions as O- or C-radicals. As a result of the everincreasing intramolecular mesomery, two C-radicals may become united by a -C-C- bond, or a C- and an O-radical by an -O-bond.

Correspondence to: Prof. Dr. U. N. Riede, Pathologisches Institut der Universität, Albertstrasse 19, W-7800 Freiburg/Br., FRG

Finally, the building up of side chains may be brought about by nucleophilic substitution. In this way large molecular complexes can originate from the binding together of individual chemical building blocks to produce a complex end structure [11, 14]. The molecular bonds resulting from electron transfer between molecules are stabilized, resulting in intermolecular mesomery: some molecules act as electron acceptors, thereby building up charge transfer complexes [14]. The final stage consists of the appearance of carboxyl groups, which are brought into existence by the oxidation of CH_3 groups or by the opening of rings with subsequent oxidation. Humic acids (humates) therefore consist of numerous phenol and quinone rings (Fig. 1), brought together by -O-, -C- and -C-C- bonds to form complex building block which cannot be defined in classical terms [11, 14]. These building blocks can bind themselves to amino acids and peptides, as well as to carbohydrates and steroids, by means of covalent bonding, hydrogen bonding or charge transfer complex bonding [14, 15].

These physicochemical properties of humic acids seem to be responsible for some reactions occurring in animal tissues [3], including antiviral [10] and desmutagenic effects [8]. Furthermore, humates act on human granulocytes as proinflammatoric stimuli [6] and take part in the mitochondrial electron transport [12].

The synthesis and molecular characteristics of the humic substances described above suggest that they would probably react with some biopolymers such as collagen, as described for the plant flavonoid (+)-catechin.

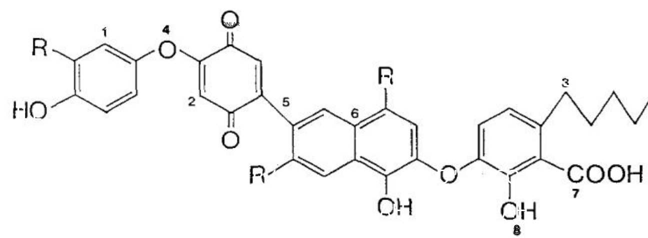

Fig. 1. Important structural characteristics of the humate model. *1*, aromatic ring; *2*, chinoid ring; *3*, aliphatic side chain produced by breaking up of ring structure; *4*, O bond; *5*, C bond; *6*, C-C bond; *7*, carboxyl group; *8*, phenolic OH-group

Machine Translated by Google

Catechin in its form of an oxydized polymer has a stabilizing effect on collagen [1, 13]. Due to the fact that the flavonoids concern the starting compounds of the humic substances [4], the stabilizing effect of (+)-catechin on collagen may be result of a "humate effect". These observations give rise to the question: are humic substances able to alter the physicochemical properties of type 1 collagen sufficiently to make it resistant to unphysiological stress?

Material and methods

Humic substance

A highly purified natural potassium humate (FL-70) obtained from Farberde Kasseler-Braun, with an average molecular weight of 3 kDa (0.5–5 kDa) was prepared according to Mach (1980) by Weyl Chemicals (Mannheim, FRG).

Analysis of the chemical resistance of collagen fibres

The human periodontal ligament (consisting mainly of type 1 collagen) was dissected away from the freshly extracted teeth of two otherwise untreated patients (ages 10 and 11 years) and immersed in a 4 *M* guanidinium chloride solution buffered with 0.05 *M* Tris-HC (pH 7.5) at 20°C for 36 h. Part of the material had previously been incubated in natural humate (FL-70; 40 µg/ml) for 30 min. Fixation, embedding and staining for electron microscopy followed in accordance with the method already described.

Analysis of the mechanical resistance of collagen fibres

The breaking point of the collagen fibres (consisting mainly of type 1 collagen) of young adult animals was analysed in ten male Wistar rats (breed: Ivanovas, Kisslegg) with an average body weight of 225 (± 15) g. We dissected 30–75 fibres from the tail tendons (more accurately, fibre bundles), with a diameter of 0.5 (± 0.1) mm and 5 cm in length (determined with the aid of a magnifiying glass × 10) from each animal.
At the beginning of each experiment the fibres were placed in Ringer solution at 20°C. Some of these served as controls, while the remaining fibres were examined in vitro at 20°C as follows:

- Incubation in humate (FL-70; 40 µg/ml)
- Incubation in 4 *M* guanidinium chloride (maintained at pH 7.5 with a Tris-HCl buffer)
- Incubation in guanidinium chloride and then in humate (FL-70)
- Incubation in humate (FL-70) and then in guanidinium chloride
- Incubation in FL-70 and then in Ringer solution.

Each collagen fibre with a predetermined diameter (Table 1) was stretched at a constant force of 500 mp (millipond) and then clamped in a technically designed tension-assessing apparatus (Textechno, Mönchengladbach, FRG) and stretched at a constant extension acceleration ($V_L = 7.5$ mm/min) until complete solution of continuity of the fibre occurred. The free fibre length between the ends of the clamps (L_O) was 10 mm, and the speed of advance of the paper (V_t) during constant tension was 7.5 or 37.5 mm/min. Maximum breaking point for each fibre was obtained from the strength/change in length diagram, and the tensile strength calculated as stress (force per unit area) at the breaking point. Statistical analysis included determination of mean values, standard deviations and standard errors, and also analysis of significance with the *F* test.

Small angle X-ray diffraction analysis of the fibres

For this analysis, five collagen fibres from five male Wistar rats (body weight 250 g) were used. Fibres – untreated or treated with humate (FL-70, 30 µg/ml, 30 min, 20°C) – were placed still wet in Mark capillaries (wall thickness 1/1000 mm, internal diameter 1 mm), gently stretched and sealed to avoid drying. A Kraty camera was used to obtain a small-angle X-ray diagram from these specimens. The construction of the diagrams was performed in a region-sensitive X-ray detector, the source of the beam coming from a X-ray anode generator. Five measurements were taken with a point focus in each experimental run, and the measurement of each fibre took 80000 s.

Results

Chemical resistance of collagen fibres

If the collagen fibres are left in humate (FL-70; 40 µg/ml) for 2 h, no swelling of the collagen fibrils is observed, and the 67-mm periodicity remains unaltered. The collagen fibrils swell to approximately double their original thickness under the influence of a guanidinium chloride solution (4 *M*) buffered with Tris-HCl, and the characteristic longitudinal and transverse ultrastructural appearance, with a periodicity of 67 mm, vanishes. Instead of a compact fibril, filamentous units (microfibrils), showing a helicoidal pattern along the longitudinal axis, become visible. In transverse section, this disintegration attacks the fibrils both peripherally and in the centre (Fig. 2). If the collagen fibres were treated with humate prior to the guanidinium chloride treatment, the twisting of the fibrils induced by guanidinium chloride does not occur (Fig. 3).

Table 1. Experimental design for testing collagen breaking point

Incubation solution	Concentration	Incubation time	Average weight of animals	Number of animals	Diameter of collagen fibres	Number of fibres per animal
Control: Ringer solution	–	20 min	225 g	10	0.5 mm	75
Humate	40 µg/ml	20 min	225 g	10	0.5 mm	75
Guanidinium chloride	4 *M*	5, 10, 15 s	225 g	5	0.5 mm	35
Humate + Guanidinium chloride	40 µg/ml 4 *M*	20 min 15 s	225 g	5	0.5 mm	35
Guanidinium chloride + Humate	4 *M* 40 µg/ml	15 s 20 min	225 g	5	0.5 mm	35
Humate + Ringer solution	40 µg/ml	20 min 20 min	225 g	5	0.5 mm	35

Machine Translated by Google

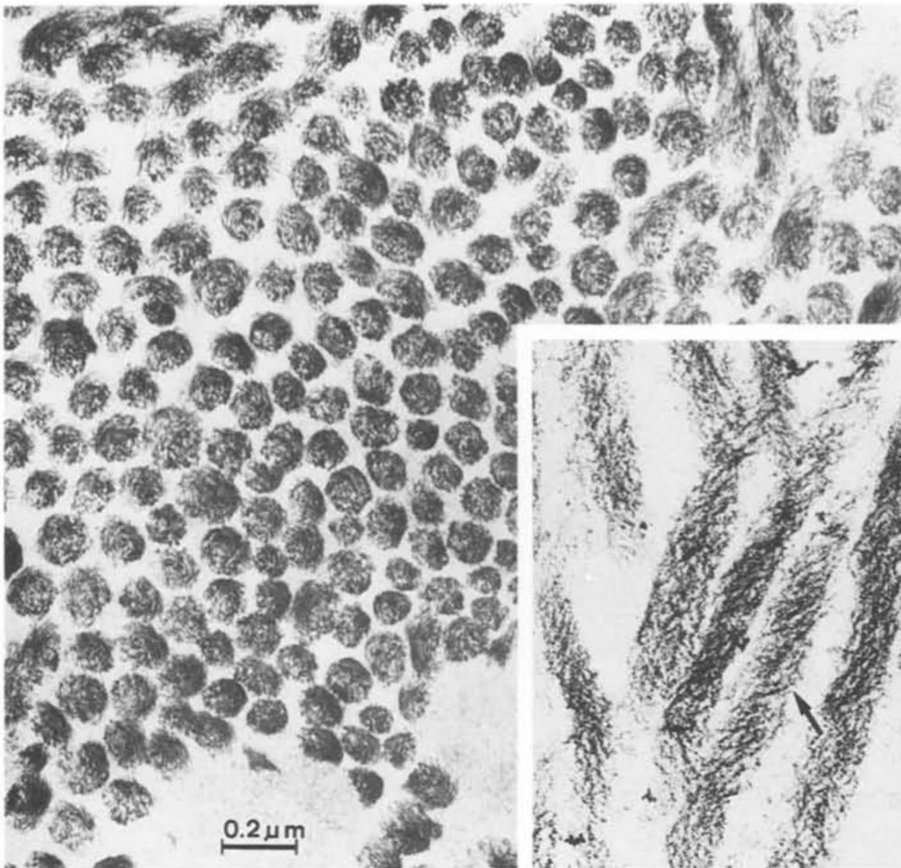

Fig. 2. Ultrastructure of the type 1 collagen fibres after incubation in guanidinium chloride in vitro (36 h). The collagen fibrils are untwisted and irregulary widened. (Staining: uranyl acetate and lead citrate) × 68000

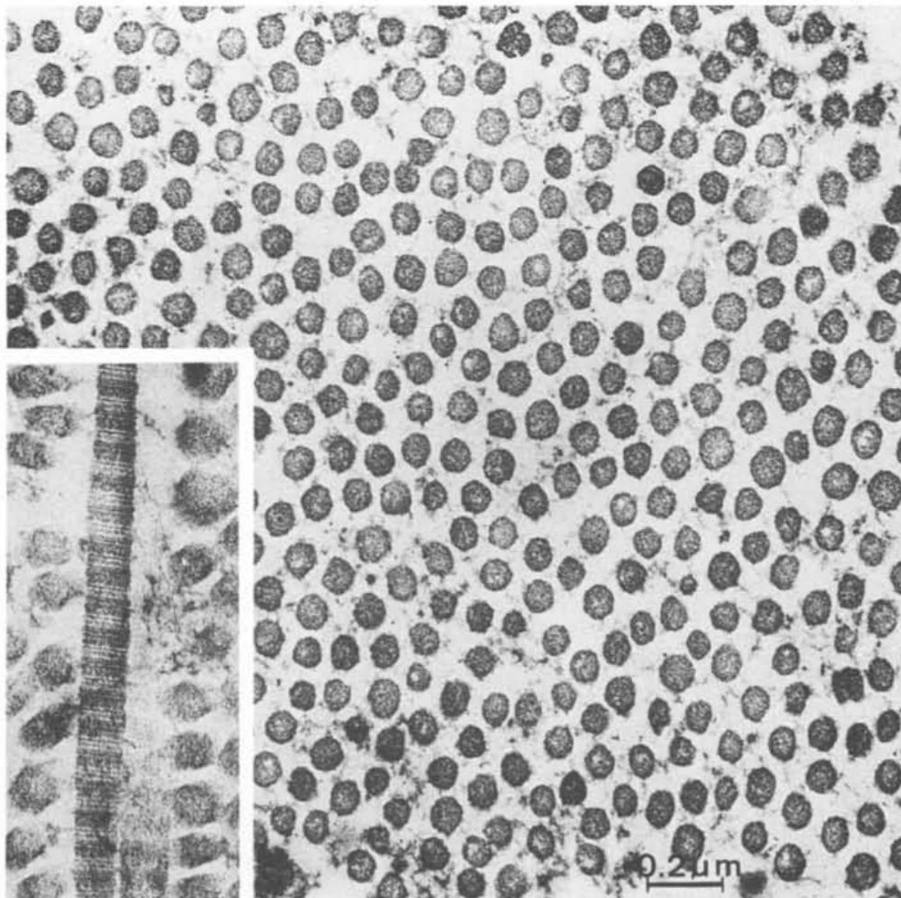

Fig. 3. Ultrastructure of the type 1 collagen fibres after incubation in guanidinium chloride in vitro (36 h), following treatment with humate (FL-70; 40 µg/ml for 30 min). (Staining: uranyl acetate and lead citrate) × 68000

Machine Translated by Google

Mechanical resistance of collagen fibres

The uniaxial test of strain, carried out with a constant speed of tension, produces a change in length per unit force curve which is characteristic for connective tissue fibres composed of parallel strands. The graphic representation begins as a non-linear curve, giving place to a nearly strict proportional segment. This curve reaches a maximum value near the point of greatest traction (tensile strength), runs steeply downwards as the fibres tear and virtually reaches the abscissa when they break completely (Table 2). The average tensile strength measured in terms of tensile stress in collagen from the untreated tails of rats is around 12 N/mm². In contrast to this, the tendon fibres after treatment with humate do not break until 21 N/mm² has been reached, which represents an increase in the average tensile stress of about 75% ($P < 0.001$).

During incubation of the type 1 collagen fibres in a solution of guanidinium chloride they become transparent. As they swell, these fibres shrink by approximately one third of their original length and become elastic. In comparison with controls, the fibres retain 65% ($P < 0.01$) of their original mechanical stength after 5 s, 40% ($P < 0.0025$) after 10 s and only 15% ($P < 0.0005$) after 15 s. If the collagen fibres are first treated for 30 min with humate (FL-70; 40 µg/ml) and finally incubated in guanidinium chloride for 15 s, the change in length per unit force curve shows the tensile strength of the tendon to have reached a maximum value lying between that of the untreated material and that of material treated with humate ($P < 0.001$). If, on the other hand, these collagen fibres are first placed in guanidinium chloride and then treated with humate (FL-70), the process of distortion observed during the incubation in guanidinium chloride is reversed ($P < 0.001$). In other words, the mechanical resistence of the collagen fibres lost during the guanidinium treatment is restored.

Table 2. Tensile strength of collagen fibres in different experiments (comp. Table 1), calculated as force per unit area

Experimental design	N/mm² (Mean)	± SD
Controls	12.1	1.3
Humate	21.3	4.1
GuCl 5 s	0.04	2.3
GuCl 10 s	1.74	0.7
GuCl 15 s	1.86	0.03
Humate + GuCl	11.8	2.4
GuCl + Humate	11.2	3.1
Humate + Ringer	20.8	6.2

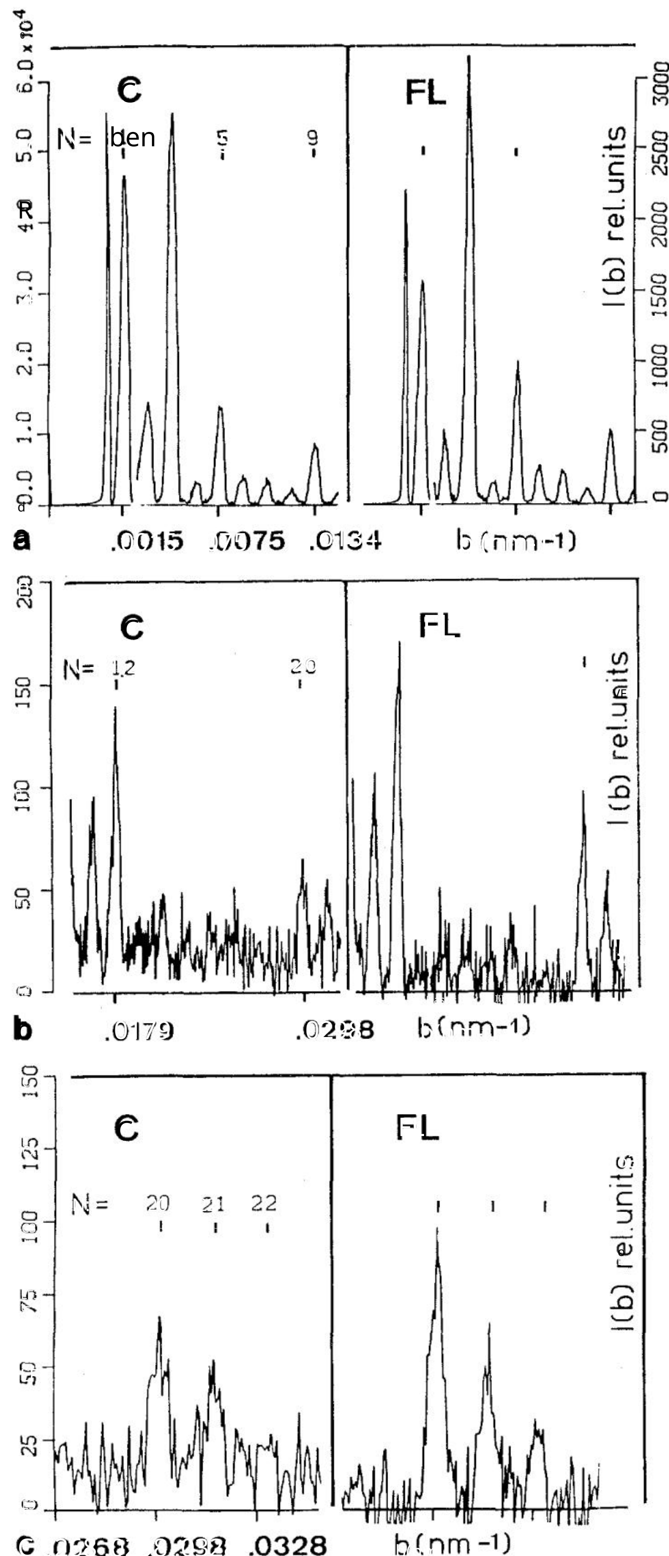

Fig. 4a–c. Graphic representation of the periodic length of the adapted X-ray diffraction diagram (500–650 nm) of collagen fibres. The relative intensity distribution of the reflections of the control collagen fibres from rat tail tendon *(C)* are different from those treated with humate *(FL)*. The periodic lengths are identical: $I(b)$ = intensity values, and $b = (2\sin\theta)/L = N/D$, where $(2\sin\theta)$ = angle of dispersion, L = wavelength of X-ray, N = ordinal number of reflection, and D = periodic length in nanometres. **a** X-ray diffraction diagram in the region of 1st–9th orders of reflections. The reflections from 2nd–9th orders of reflections are shown again with the scale increased in the ratio 1:10. There is an increase in intensity of the 5th band. **b** X-ray diffraction diagram in the region of 11th–21st orders of reflections. Relative displacements between 12th and 20th orders of reflections. **c** X-ray diffraction diagram in the region of 20th–22th orders of reflections

Machine Translated by Google

X-ray diffraction analysis

The X-ray diffraction diagrams of collagen type 1 fibres revealed a longitudinal periodicity of 67 mm with 27 bands, which corresponds to a resolution of structure of 3.05 nm. The analysis clearly shows that treatment with humate does not produce any displacement of the periodicity. Only changes in the relative intensity were found, which may be attributed to alterations in the distribution of electron density in the collagen. A relative decrease in the first, third and tenth bands was found after treatment with humate (FL-70); the fourth and eighth bands remained the same, and served as control peaks. All the other bands showed an increase in intensity in comparison with the untreated collagen fibres. In addition to this, relative changes in the intensity were seen in the individual diagrams (Fig. 4).

Discussion

The experiments carried out in vitro on collagen revealed a highly significant increase in the resistance to tearing (tensile stress) of about 75%. This result suggests changes in extracellular processing during fibrillogenesis. This view is based on the following facts:

- The biomechanical stability of the collagen depends (i) upon intermolecular and intersubfibrillar covalent bonding, (ii) upon the electrostatic interaction of groups of differently charged atoms in the form of intramolecular and intermolecular hydrogen bonds and (iii) upon hydrophobic interaction [5, 10]. Frictional forces between collagen fibres and fibrils are also involved, as well as physicochemical interaction with components of the extracellular matrix, such as proteoglycans.

- During ontogenesis of an individuum, collagen undergoes a process of "maturation" as it becomes more and more resistant to mechanical stress, heat and the action of acids. This is reflected in the ultrastructure of the fibrils, which thicken progressively. This property of "mature" collagen is due particularly to an increasing network of covalent bonds and the molecular interaction [5]. As in the previous experiments, the ultrastructural counterpart of this process is to be found in an increase in the diameter of the fibril. The increase in resistance to breaking, associated with the application of humate, is also probably due to an increase in the covalent cross-links.

The stabilizing changes which take place in the collagen fibrils after treatment with humate are also seen in the experiments with guanidinium chloride. In fact, this polar solution is supposed to increase the hydrogen bonds, but not covalent bonds; although even in weak solution it untwists the collagen fibrils and decreases their quaternary structure [4, 7]. The mechanical resistance of the collagenous tissue to tensile stress is therefore reduced, and the multifibrillar strands slide apart without showing particular tearing points. After chemical treatment with humate (FL-70) the collagen fibres are able to resist the destabilizing effect of guanidinium chloride and retain their tensile strength and quaternary structure. The stabilizing effects of humic acids on collagen can be explained on the basis of a tanning process, as in the case of the flavonoid (+)-catechin [9].

The question also arises of whether the administration of humate changes the tertiary structure of the collagen fibrils. The ultrastructural investigations carried out to answer this question, together with small-angle X-ray diffraction analysis, have established that the collagen fibrils treated with humate (FL-70) retain their periodicity of 67 mm, together with the usual cross-banding pattern associated with type 1 collagen. The X-ray analysis also showed that within the 67 mm periodicity of a collagen molecule there appears a change in the distribution of electron density. The cause may well lie in the numerous phenolic OH-groups of humate, which can raise both the number of stable covalent intermolecular cross-links and that of hydrogen bonds. The available bonding positions between the collagen fibrils can obviously reach saturation. This is related to the observation that the tensile strength of the collagen fibres continually increases in the manner of a dosage curve as the humate concentration rises to 40 μg/ml. This phenomenon appears to be more evident in young animals than in adults. If one considers that the aldehyde groups of the type 1 collagen responsible for the existence of stable intermolecular cross-links does not fall off with increasing age [2], and that the number of covalent cross-links increases, the suggestion that the collagen stabilization due to the humate depends upon an increase in the hydrogen bonding is confirmed, especially since it inhibits the loosening of the hydrogen bonds brought by guanidinium chloride. This is rather similar to the observation that the subfibrillar structure of a collagen fibre treated with humate does not tear bit by bit when its breaking point has been exceeded, but comes apart all at once – a fact which is reflected in the change in length per unit force. A similar but weaker effect can be achieved with D_2O (deuterium oxide), which replaces the hydrogen bonds of collagen by the thermodynamically more stable D bonds. These observations suggest that the humate employed react with collagen in vitro and make it resistent to mechanical and chemical alterations without denaturing them. The importance of this in traumatology is apparent, in that newly formed collagen fibres are not, during tissue repair, at first completely resistant to mechanical stress. For this they must first "mature" – a process bound up with the increase in cross-links.

Acknowledgements. The authors like to thank Prof. Dr. W. Ziechmann, Institut für Bodenwissenschaften, Universität Göttingen, Germany, and B. Seubert, Weyl-Chemicals, Mannheim, Germany, for gift samples of potassium-humate for this work.

References

1. Cetta G, Gerzeli G, Quarteri A, Castellani AA (1971) Protective effect of flavonoids on the collagen of lathyritic rats. Experientia 27:1046
2. Deshmuk KA (1974) A comparative study of the crosslinking precursors present in rat skin and tail tendon collagen. Connect Tissue Res 2:49–53

Machine Translated by Google

3. Jonas I, Riede UN (1979) Der Einfluß von Flavichromin (FL-70) auf das orthodontisch stimulierte Parodontium. Fortschr Kieferorthop 40:70–78
4. Lillie JH, MacCallum DK, Scaletta LJ, Occhino JC (1977) Collagen structure: evidence for a helical organization of the collagen fibril. J Ultrastruct Res 58:134–143
5. Miller EJ (1984) The structure of collagen. In: Wagner BM, Fleischmeyer R, Kaufman N (eds) Connective tissue diseases. Williams & Wilkins, Baltimore, pp 4–15
6. Riede UN, Zeck-Kapp G, Freudenberg N, Keller HU, Seubert B (1991) Humate induced activation of human granulocytes. Virchows Arch [B] 60:27–34
7. Ruggeri AF, Benazzo F, Reale E (1979) Collagen fibrils with straight and helicoidal microfibrils. A freeze-fracture and thin-section study. J Ultrastruct Res 68:101–108
8. Sato T, Ose Y, Nagase H (1986) Desmutagenic effects of humic acid. Mutat Res 162:173–178
9. Schlebusch H, Kern D (1972) Stabilization of collagen by polyphenols. Angiologica 9:116–124
10. Schneider J, Riede UN, Seubert B (1991) Huminatinduzierte Membranfusionshemmung bei der HIV-Infektion. Verh Dtsch Ges Pathol 75:505
11. Thurman EM, Malcolm RL (1983) Structural study of humic substances: In: Christman RF, Jessing ET (eds) Aquatic and terrestrial humic materials. Ann Arbor, 91 Ann Arbor Science, p 78
12. Visser SA (1987) Effect of humic substances on mitochondrial respiration and oxidative phosphorylation. Sci Total Environ 62:347–354
13. Wauters P, Eckhout Y, Vaes G (1986) Oxidation products are responsible for the resistance to the action of collagen by (+)-catechin. Biochem Pharmacol 35:2971–2973
14. Ziechmann W (1988) Evolution of structural models from consideration of physical and chemical properties. In: Frimmel FH, Christmann RF (eds) Humic substances and their role in the environment. Wiley, New York, pp 113–132
15. Ziechmann W (1989) Huminstoffe und ihre Wirkungen in pedogenen Funktionseinheiten. Ökologie 5:49–61