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(+)-CYANIDANOL-3 CHANGES FUNCTIONAL PROPERTIES OF COLLAGEN

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Abstract—About 6–7 (+)-cyanidanol-3 molecules are bound per collagen α-chain. The (+)-cyanidanol-3 treated collagen contains an increased number of pepsin-resistant cross-links, is less susceptible to attack by mammalian collagenase, has a higher shrinkage temp and forms unstructured aggregates. Cell and organ culture studies show that these biological systems produce less protein and collagen in the presence of (+)-cyanidanol-3 and that the newly synthesized collagen is less soluble.

(+)-Cyanidanol-3 belongs to a group of flavonoids which are commonly found in plants [1]. This drug has been used in the treatment of liver injuries in animal models and more recently in human conditions [2–4]. Furthermore, evidence was presented that (+)-cyanidanol-3 interferes with the metabolism of connective tissue proteins, in particular with the turnover of collagen. The data suggest a bifunctional action, first an inhibiting effect on collagen synthesis and second a stabilizing effect on mature collagen molecules. These somewhat controversial observations arise from the fact that (+)-cyanidanol-3 can chelate ferrous ions and thus inhibit normal hydroxylation of prolyl and lysyl residues which are required for functional collagen molecules [5, 6]. On the other hand, a stabilizing effect on collagen molecules was claimed, because (+)-cyanidanol-3 treated collagen was more resistant to collagenase attack [7, 8]. In addition (+)-cyanidanol-3 counteracted the lathyrogenic effect of β-aminopropionitril in rats when the two drugs were supplied in the diet [9, 10]. Based on this observation, the treatment of patients suffering from osteogenesis imperfecta with (+)-cyanidanol-3 was proposed [11]. It was the aim of our study to provide additional biochemical information about the interaction of (+)-cyanidanol-3 with collagen which could be helpful in deciding whether or not this drug should be used in the treatment of connective tissue disorders.

MATERIALS AND METHODS

Cell culture. Skin biopsies from healthy infants were used to establish fibroblast cultures using conventional methods [12]. Cells were grown in plastic tissue culture flasks (Falcon, Becton and Dickinson, Heidelberg, F.R.G.) using Dulbecco's modified Eagle's medium which was supplemented with sodium ascorbate (50 μg/ml), glutamine (300 μg/ml), streptomycin (50 μg/ml), penicillin (400 U/ml) and 10% fetal calf serum. The cultures were maintained in a moist atmosphere of 95% air, 5% CO_2 at 37°. Fibroblasts were passaged by trypsinization (0.05%) and medium was changed twice a week. The cells used in the experiments were between the 5th and 9th subpassage.

Metabolic labelling. After confluency, fibroblasts were preincubated in Dulbecco's modified Eagle's medium for 24 hr containing penicillin (400 U/ml), sodium ascorbate (50 μg/ml) at 37° and subsequently labelled for 24 hr in identical medium supplemented with 5 μCi/ml L-[2,3-^{3}H]proline. In some experiments β-aminopropionitril (100 μg/ml) was also added. Furthermore the effect of (+)-cyanidanol-3 (Sigma Chemie, München, F.R.G.) was studied in concns from 50 to 1000 μg/ml. Thus, cells were extracted with 1 M NaCl, 0.05 M Tris, pH 7.4 at 4°. The extract was combined with the labelled medium and dialysed against 0.1% acetic acid to remove dialisable radioactivity. The amount of the labelled collagen was calculated by determination of incorporated hydroxyproline, which was separated after acid hydrolysis as described previously [13] and counted in a liquid scintillation counter (Kontron MR 300). In parallel samples cell numbers were determined and total protein (in cells plus medium) was measured according to the procedure described by Lowry *et al.* [14].

Organ culture. Parietal bones of chicken embryos were labelled as described by Pontz *et al.* [15] with some minor modifications. The incubation time was extended to 18 hr which appeared to enhance the formation of stable cross-links. β-Aminopropionitril and (+)-cyanidanol-3, respectively, were added in varying concns. Again culture medium and calvaria extract were combined and dialysed against 0.1% acetic acid. Aliquots were treated with pepsin and/or used for further analysis by column chromatography. The newly synthesized collagen was calculated by measuring hydroxyproline as described above.

Enzymatic procedures

Pepsin treatment. Collagen or tissue homogenates

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were solubilized or suspended in 0.1% acetic acid/HCl pH 2.0 and incubated with pepsin [0.1 mg/ml, 3 × crystallized, Fa. Serva (Heidelberg, F.R.G.)] for 6 hr at 18° according to Pontz *et al.* [15]. The digested collagenous proteins were dissolved in 0.1% acetic acid and dialysed against appropriate buffers for chromatography.

Treatment with bacterial collagenase. Bacterial collagenase (1800 U/mg) was purchased from Worthington Biochemicals (Freehold, NJ) and further purified according to Peterkofsky and Diegelmann [16]. (+)-Cyanidanol-3 treated collagen and control samples were digested at an enzyme/substrate ratio of 1:60 for 30 and 60 min and 18 hr at 37° in 5 mM $CaCl_2$, pH 7.0 and subsequently analysed by slab gel electrophoresis according to Laemmli [17].

Preparation of human collagenase. Human fibroblasts were grown in Dulbecco's modified Eagle's medium as described. When cells reached confluency they were cultured in serum-free medium for 24 hr and the enzyme was prepared as described by Stricklin *et al.* [18].

Treatment with human collagenase. (+)-Cyanidanol-3 treated collagen and control samples in 5 mM $CaCl_2$, 0.17 M NaCl, 0.02 M Tris, pH 7.6 were incubated with mammalian collagenase (250 ng/ml) in an enzyme:substrate ratio of 1:50 at room temp for 24, 48 and 72 hr. The samples were analysed by slab gel electrophoresis according to Laemmli [17]. The gels were stained by Coomasie blue and scanned in a gel scanner (Fa. C. Zeiss, Oberkochen, F.R.G.) at 570 nm.

Degree of hydroxylation. Radioactivity eluting in the position of α-chains on an Agarose A5 column was dialysed against 0.1% acetic acid, lyophilized and hydrolysed (6 N HCl, 110°, 18 hr plus mercaptoethanol, under nitrogen). Hydroxyproline and proline were separated by use of an amino acid analyser (LKB Instruments), which was equipped with a fraction collector.

Molecular sieve chromatography. Samples were dialysed against 1 M $CaCl_2$, 0.05 M Tris, pH 7.4, denatured at 45° for 20 min and chromatographed on an Agarose A5 column (1.5 × 120 cm) equilibrated in this buffer according to Piez [19].

In situ *treatment of lathyritic rat skin collagen with (+)-cyanidanol-3.* Types I and III collagen isolated from the skin of lathyritic rats [20, 21] were dissolved in 0.1% acetic and dialysed against 0.2 M NaCl, 0.05 M Tris, pH 7.4. Individual samples (1.2 mg/ml collagen) were incubated with 50, 200 and 1000 μg/ml of (+)-cyanidanol-3 for 48 hr at room temp and were then shifted to 37° for an additional hr to complete fibril formation. Unbound (+)-cyanidanol-3 was removed by dialysis against 0.2 M NaCl, 0.05 M Tris, pH 7.4.

Physico-chemical evaluation

Denaturation temp m.p. The denaturation temp was determined for collagen incubated with (+)-cyanidanol-3 for 24 hr in 0.1% acetic acid at room temp. Subsequently collagen solution were gradually heated and changes in optical rotation were recorded at 405 nm.

Shrinkage temp. (+)-Cyanidanol-3 treated and precipitated collagen was heated continuously. Shrinkage point was determined when the sample gelatinized and became translucent by visual evaluation using a microscope (Fa. Zeiss, 7082 Oberkochen, F.R.G.).

RESULTS

In situ *experiments*

Fibre formation. Since the reaction of (+)-cyanidanol-3 with collagen must alter some of its biophysical properties one should be able to analyse effects such as fibril formation and shrinkage temp by incubating collagen solutions with this drug under close physiological conditions. Solutions of lathyritic rat skin collagen (1.2 mg/ml) was incubated with increasing amounts of (+)-cyanidanol-3 at room temp for 48 hr and at 37° for an additional hr. The newly formed aggregates were prepared for the electron microscope and compared with the fibrils which had formed in the absence of (+)-cyanidanol-3 (Fig. 1). At low concns of (+)-cyanidanol-3 (up to 10 μg/ml) the cross-striation pattern of the aggregates compares with that of normal fibres. As the concn of (+)-cyanidanol-3 increases the proportion of unstructured material becomes larger, suggesting that (+)-cyanidanol-3 interacts with the collagen molecules and thus interferes with the normal deposition and growth of collagen fibres (data not shown).

Thermal stability and biochemical analysis. In subsequent experiments, we separated the aggregates from the soluble fraction by centrifugation and analysed the collagen contained in the pellet and in the supernatant for their thermal stability. The characteristics of the denaturing curves from (+)-cyanidanol-3 treated soluble collagen and control samples were indistinguishable (not shown).

The shrinkage temp of the insoluble collagen aggregates was shifted to a higher temp when (+)-cyanidanol-3 treated collagen was analysed as compared to aggregates of normal collagen. While untreated fibres shrank at a temp of about 44°, the (+)-cyanidanol-3 treated collagen had a shrinkage temp which was about 10–15° higher. The difference in shrinkage temp is more prominent for type I than for type III collagen (Table 1). It should also be noted the m.p., i.e. that temp where the collagen α-chains in the triple helix assume a randomly coiled state, is considerably higher for (+)-cyanidanol-3 treated collagen. At temps up to 80° no m.p. could be noticed. It was not possible to evaluate the aggregates at higher temps since the solvent begins to evaporate. Although the soluble proportion of (+)-cyanidanol-3 treated collagen showed a normal denaturation temp (t_M), the very same fraction of type I collagen contained a much higher proportion of cross-linked collagen chains eluting in the void vol. of the column chromatography on Agarose A 5 (Fig. 2). We observed that a high proportion of the (+)-cyanidanol-3 induced cross-links are resistant to pepsin digestion (Fig. 2D).

Sensitivity to collagenase. The soluble fraction of (+)-cyanidanol-3 treated collagen and solutions of untreated lathyritic rat skin collagen (1 mg/ml) were incubated with highly purified bacterial collagenase (1800 U/mg) and subsequently analysed by slab gel polyacrylamide electrophoresis (Fig. 3). This enzyme

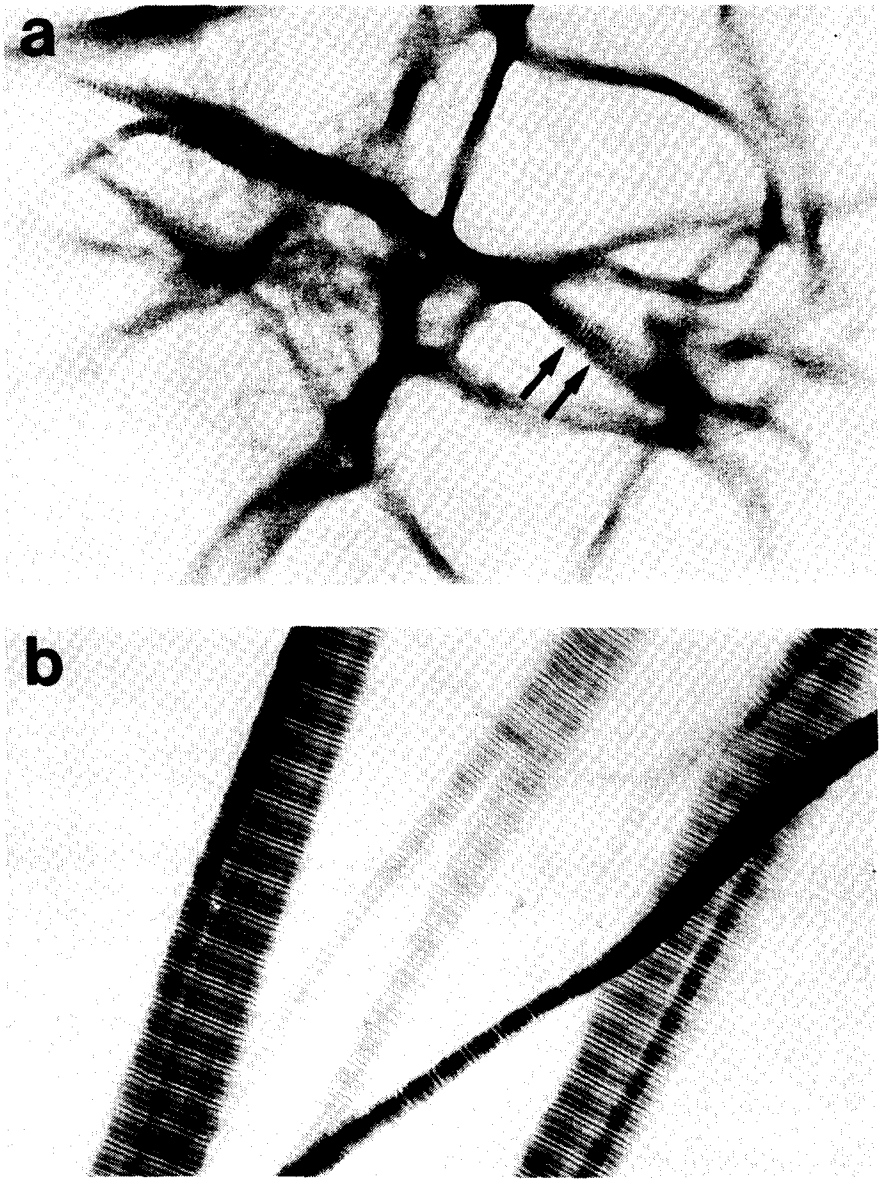

Fig. 1. Control samples and (+)-cyanidanol-3 treated collagen were positively stained with phosphotungstic acid and uranyl acetate. Samples were examined in a Siemens Elmiskop I. Magnification × 75,000. (a) Fibril network built by incubation of lathyritic rat skin collagen with (+)-cyanidanol-3 (200 μg/ml). Most of the material consisted of amorphous, irregular fibres, which have a normal cross-striation pattern in some fibrillar sections (arrows). (b) Control fibrils.

Table 1. Thermal stability of precipitated collagen in the presence of (+)-cyanidanol-3

Collagen	(+)-Cyanidanol-3 (200 μg/ml)	Dehydration/ shrinkage temp (°)	m.p. (°)
Type I	−	43.7 ± 0.6	55.6 ± 0.8
	+	59.6 ± 2.4	>80
Type III	−	52.3 ± 0.5	55.2 ± 0.7
	+	62.7 ± 2.7	>80

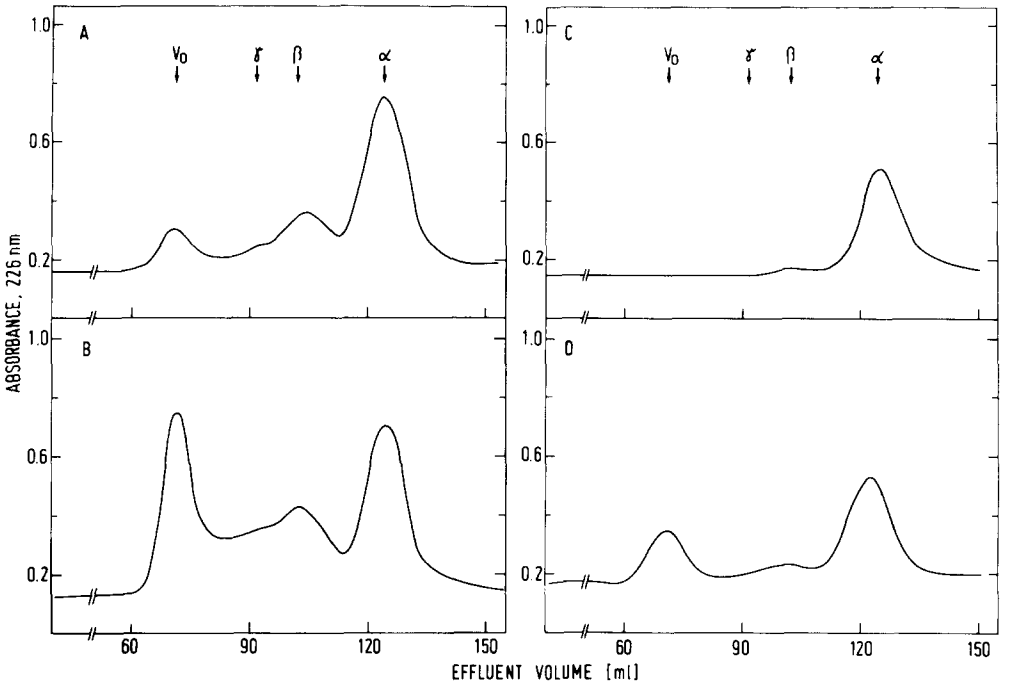

Fig. 2. Molecular sieve chromatography on Agarose A5 under denaturing conditions of the soluble part of lathyritic rat skin collagen treated with (+)-cyanidanol-3 (B); additional digestion with pepsin (D). Control of lathyritic rat skin collagen with (C) and without (A) pepsin treatment.

rapidly degrades both collagen preparations suggesting that (+)-cyanidanol-3 treated collagen is no more protected than untreated collagen. It should be mentioned, however, that (+)-cyanidanol-3 treated collagen components migrate somewhat slower than untreated material, probably due to an increase in the mol. wt (compare Figs. 3 and 4). From the R_F values and the mol. wt of (+)-cyanidanol-3 it was calculated that 7.2 residues are bound to 1000 amino acid residues of collagen components.

(+)-Cyanidanol-3 treated collagen precipitates were completely solubilized with bacterial collagenase. Measurement of the optical density at 280 nm allowed us to calculate the number of (+)-cyanidanol-3 residues covalently bound to collagen molecules. In good agreement with the estimate, which we obtained in the preceding paragraph, we found that 6.4 molecules are covalently bound per α-chain.

Mammalian collagenase in contrast to the effect of bacterial collagenase cleaves the native collagen molecule at a single specific site. This enzyme is less effective on (+)-cyanidanol-3 treated collagen than on untreated collagen (Fig. 4). About 60% of the untreated collagen is cleaved after 24 hr of incubation (Table 2). (+)-Cyanidanol-3 treated collagen, however, is cleaved by mammalian collagenase only to an extent between 20 and 30% (Table 2). It is conceivable that the cleavage site for mammalian col-

Table 2. Densitometric values of collagenase derived fragments in respect to uncleaved (+)-cyanidanol-3 treated collagen components (compare slab gel electrophoresis (Fig. 4))

Time of incubation with collagenase (hr)	(+)-Cyanidanol-3 (200 μg/ml)	Ratio of uncleaved collagen components : collagenase derived fragments (%)*			
		α1	α2	β11	β12
—	−	100	100	100	100
24	−	40	36	35	36
48	−	34	34	28	40
—	+	100	100	100	100
24	+	75	85	89	92
48	+	63	79	86	88

* These percentages were derived using densitometric determination and the fragment lengths were taken into account, i.e. the densitometric values of the collagenase derived α-chains (789 amino acid residues) were multiplied by a factor of 1.26 in order to compensate for their reduced length thus allowing a true comparison with the complete α-chain (1000 amino acid residues).

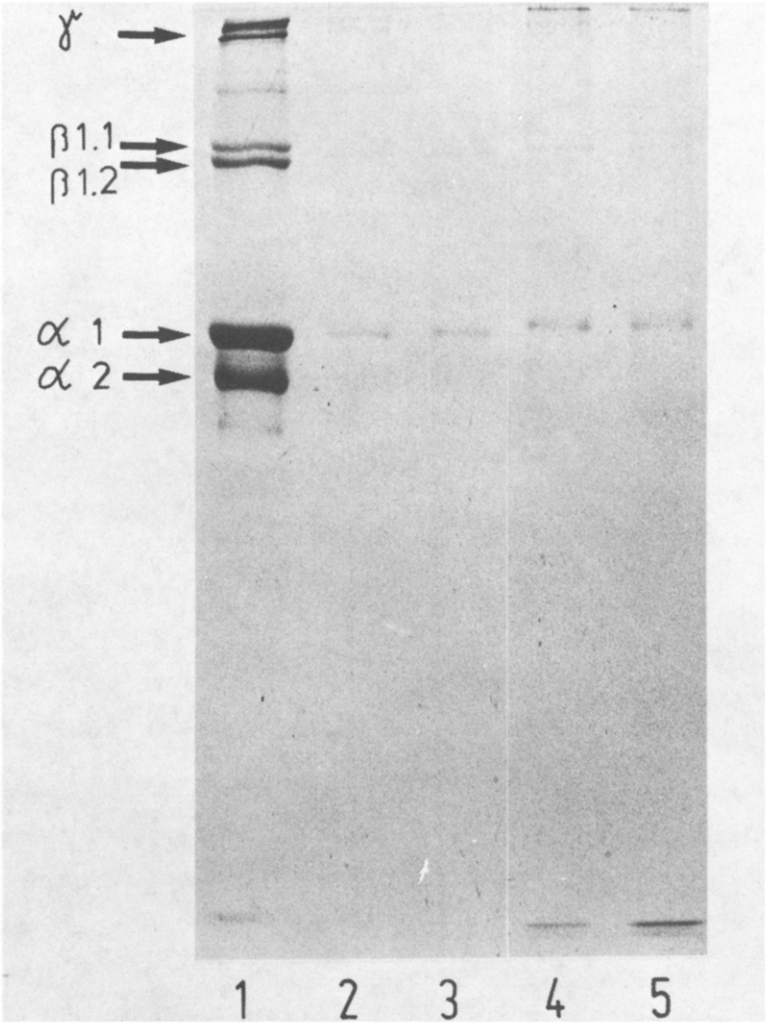

Fig. 3. Slab gel electrophoresis of lathyritic rat skin collagen before and after treatment with (+)-cyanidanol-3 and subsequent digestion by bacterial collagenase. Lane 1: control; lanes 2 and 3: digestion of untreated collagen with collagenase for 30 and 60 min respectively; lanes 4 and 5: digestion of (+)-cyanidanol-3 pretreated collagen for 30 and 60 min by collagenase. The electrophoretic mobility of the α-chains is reduced after incubation with (+)-cyanidanol-3.

lagenase is protected or altered in such a way that it can no longer serve as a substrate.

In vitro *experiments*

Synthesis of total protein and collagen in fibroblast cultures. These experiments were designed as to demonstrate the metabolic interference with the synthesis of collagenous and non-collagenous protein. Thus, monolayer cultures of fibroblasts were incubated in the presence of increasing concns of (+)-cyanidanol-3 using fully supplemented growth medium. At low concns, fibroblasts maintain their morphology and remain attached to the plastic surface. At higher concns, cells round up and begin to detach. On a quantitative scale this is seen in Fig. 5 which depicts the numbers of attached cells following incubation with various concns of (+)-cyanidanol-3 for up to 4 days. In parallel with the impaired viability of the cells, a lower content of total cell protein was found.

Furthermore, fibroblast cultures were incubated with L-[^{3}H]proline and increasing amounts of (+)-cyanidanol-3 both in the presence and absence of β-aminopropionitril as a potential cross-link inhibitor. After 24 hr medium was separated from the cell layer, which was subsequently extracted with a neutral salt solution. Both medium and salt extracts were combined and used for hydroxyproline determination. Similarly, the cell debris was hydrolysed and analysed for its content of hydroxyproline. As summarised in Table 3, the proportion of collagen which was insoluble in neutral salt ranged from 1 to 2%, the changes which were observed in the presence of (+)-cyanidanol-3 were within the experimental limitations. β-Aminopropionitril did not alter this pattern. Again, the data provide further evidence that the rate of collagen synthesis is lower in the presence of (+)-cyanidanol-3.

Organ cultures. While monolayer cultures of fibroblasts are quite useful in evaluating the drug influ-

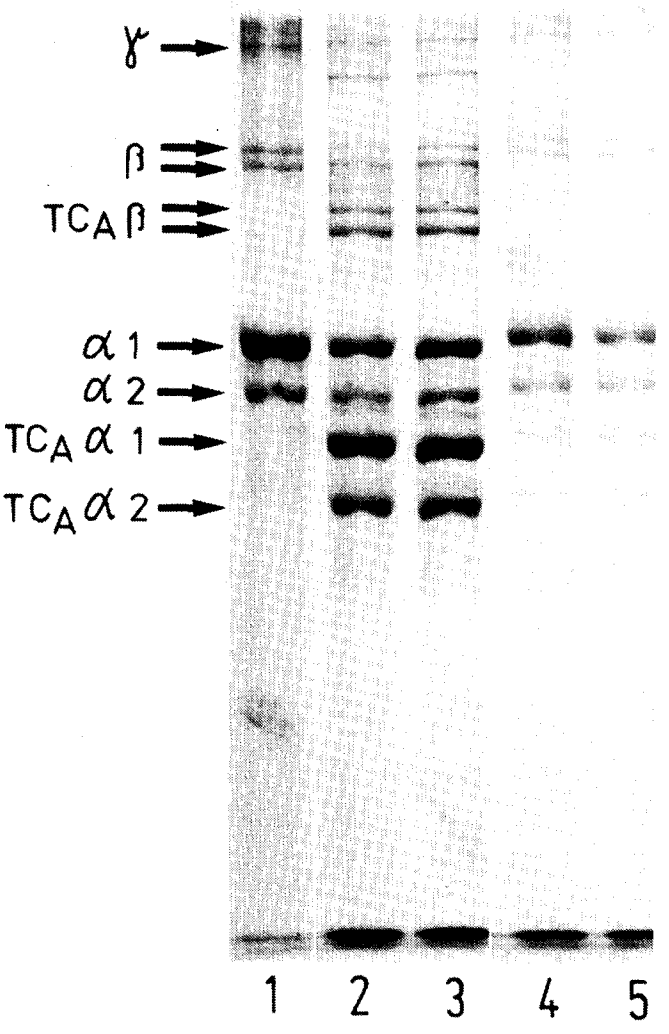

Fig. 4. Slab gel electrophoresis of lathyritic skin collagen before and after treatment with (+)-cyanidanol-3 and subsequent digestion with human collagenase isolated from fibroblasts. Lane 1: control; lanes 2 and 3: control after digestion with collagenase for 24 and 48 hr; lanes 4 and 5: before digestion with collagenase for 24 and 48 hr the collagen samples were pretreated with (+)-cyanidanol-3. The electrophoretic mobility of all fragments or collagen components is slightly reduced.

Table 3. Collagen synthesis of fibroblasts in the presence of (+)-cyanidanol-3 and β-aminopropionitril

(+)-Cyanidanol-3 (μg/ml)	β-Aminopropionitril (μg/ml)	Radioactivity (cpm × 10^{-4} [^{3}H]hydroxyproline of newly synthesized collagen) Medium and neutral soluble cell extract (a)	Insoluble residue (b)	Total synthesis (a) + (b)	Decrease of collagen synthesis per 3×10^6 cells (%)
—	—	109.8	2.2	112	100
—	50	106.8	1.1	108	96.4
50	—	72.2	0.8	73	69.4
50	50	79.1	0.9	80	76.0
200	—	54.1	0.9	55	69.5
200	50	68.8	1.2	70	88.5

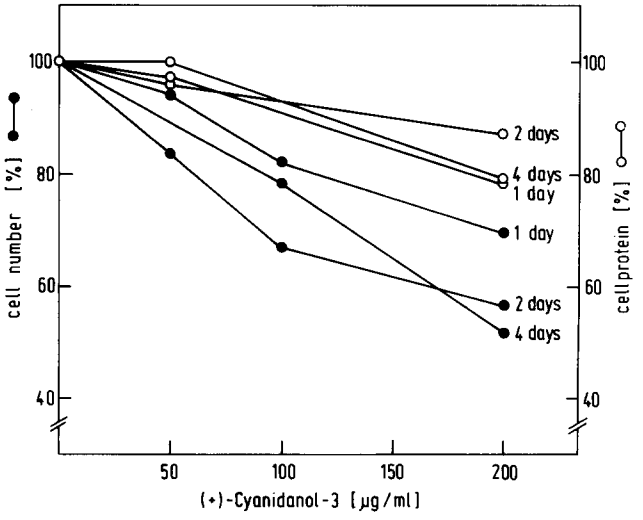

Fig. 5. Changes of cell protein and cell numbers in fibroblast cultures incubated with of (+)-cyanidanol-3 for 1, 2 and 4 days.

ence on cell viability, cross-link formation of collagen may be seen better in organ culture, since conversion of procollagen to collagen is more effective in this biological system. Mature collagen molecules, however, are required for functional covalent bonds between collagen chains. For this reason, we incubated parietal bones of embryonic chickens with L-[^{3}H]proline for 10 hr and analysed the effects of (+)-cyanidanol-3 and/or β-aminopropionitril on collagen synthesis and cross-link fomation. We pooled the medium with a neutral salt extract, treated the bones with pepsin which rendered us a pepsin-solubilized and an insoluble fraction. The control of hydroxyproline in each was used to calculate the amount of newly synthesized collagen (Table 4). The data clearly demonstrate that the collagen synthesized in the presence of (+)-cyanidanol-3 accumulates in the pepsin-solubilized and in the insoluble fraction. Much less collagen is in the combined pool of medium plus neutral salt extract which comprised the largest fraction in control cultures. It is also quite remarkable that (+)-cyanidanol-3 can overcome the cross-link inhibiting effect of β-aminopropionitril. Little impact of (+)-cyanidanol-3 on medium and neutral salt soluble collagen could be seen since the elution pattern on an Agarose A5 column did not provide evidence for larger proportion of higher mol. wt components (not shown). Furthermore, the degree of the hydroxylation of prolyl residues amounted to 48% even in the presence of 200 μg/ml (+)-cyanidanol-3. This value is well in accordance with published data on the hydroxyproline content in chicken bone [22].

Table 4. Effect of (+)-cyanidanol-3 and β-aminopropionitril on collagen synthesis in calvaria of 17-day-old chicken embryos

(+)-Cyanidanol-3 (μg/ml)	β-Aminopropionitril (μg/ml)	Distribution of [^{3}H]hydroxyproline (representative for collagen) in various compartments (%)			Relative decrease of total synthesis of collagen (%)
		Medium and neutral salt extract	Pepsin-solubilized material	Pepsin resistent material	
—	50	80	19.3	0.7	100
—	—	49	48.3	2.6	100
50	50	33.8	53.2	12.9	36.3
50	—	37.8	46.1	16.1	37.6
200	50	53.2	39.4	7.4	43.1
200	—	42.1	38.5	19.4	26.0

DISCUSSION

A number of recent reports emphasize the physiological role of (+)-cyanidanol-3 and its beneficial influence on patients suffering from liver injuries or from malfunctioning of connective tissue. The molecular target, however, of this drug, has not clearly

been identified as yet, although a number of chemical reactions have been proposed. In general it is reasoned that (+)-cyanidanol-3 among others has a stabilizing effect on membranes or on collagen fibrils. In our current study we provided evidence that (+)-cyanidanol-3 interacts with collagen molecules and promotes the formation of insoluble aggregates which do not show, or only to some extent, a cross-striation pattern. The shrinkage temperature as a measure for thermal stability of soluble aggregates is significantly higher in (+)-cyanidanol-3 treated collagen than in untreated collagen which is also to be expected for a collagen that contains increasing numbers of covalent cross-links. Similar observations have been made on the ageing rat tail tendon [23]. Even the soluble portion of (+)-cyanidanol-3 treated lathyritic rat skin collagen contains cross-links which are stable under conditions where collagen molecules have lost any helical conformation. About 30% of the (+)-cyanidanol-3 induced cross-linked material is resistant to pepsin treatment. This suggests that cross-links had formed in the helical region of the collagen molecule which is not attacked by this protease. When (+)-cyanidanol-3 treated collagen is electrophoretically separated on SDS polyacrylamide slab gels the α1- and α2-chains of type I collagen migrate slower than untreated collagen chains which is probably caused by the covalent binding of about 7 (+)-cyanidanol-3 residues per collagen α-chain. Similar binding equivalents were found by determination of the optical density of a solution of (+)-cyanidanol-3 treated collagen and digestion with bacterial collagenase. The binding of (+)-cyanidanol-3 to collagen did not change its susceptibility to bacterial collagenase but markedly reduced the cleavage by mammalian collagenase. This differential stability has been observed previously using a different assay system and is probably explained by the many cleavage sites which are available for bacterial collagenase while only one is present for the mammalian collagenase [8]. Even a detailed sequence analysis of the amino acid residues in the neighbourhood of the cleavage site for mammalian collagenase cannot at present provide additional evidence for the immediate binding site of (+)-cyanidanol-3. Evidence exists from another study that the incubation of collagen fibres in the presence of copper ions plus (+)-cyanidanol-3 ions leads to an increase of specific precursors of collagen cross-links and to an increase in reducible cross-links [24]. These authors, however, could see a similar effect of (+)-cyanidanol-3 on albumin, which would indicate a non-specific oxidation of amino groups of lysine and hydroxylysine residues by the drug. This action, however, cannot be the sole effect of (+)-cyanidanol-3 since we could clearly demonstrate that about 7 residues of (+)-cyanidanol-3 are bound to each collagen chain. Thus, we assume that (+)-cyanidanol-3 acts in itself as a bipolar cross-linking agent similar to compounds which have been used for tanning of hides [25]. Since we cannot find precipitates or polymers of albumin following incubation with (+)-cyanidanol-3, we conclude that the formation of covalent bounds is rather specific for collagen molecules.

In *in vitro* cultures of parietal bones which are known to synthesize a large proportion of collagenous protein, (+)-cyanidanol-3 causes a significant increase in the insoluble fraction which cannot be solubilized by pepsin treatment. (+)-Cyanidanol-3 can even overcome the cross-link inhibiting effect of β-aminopropionitril, which does, however, not imply that (+)-cyanidanol-3 forms bond of the nature usually found under physiological conditions.

It should be noted, however, that (+)-cyanidanol-3 reduces the rate of total protein and collagen synthesis both in the calvarial and in the fibroblast system. It is unlikely, however, that the rate of reduction is due to a chelating effect on ferrous ions, which are necessary for the hydroxylation of fully functional collagen molecules, because the degree of hydroxylation of collagen α-chains was not altered.

In spite of the fact that our observations of the influence of (+)-cyanidanol-3 on collagen turnover were made in *in vitro* systems, we see our study in the light of potential application of (+)-cyanidanol-3 in connective tissue disorders. More information, however, is required about for example the metabolic pathway of (+)-cyanidanol-3 and its effective *in vivo* concentration. Under these limitations, there is good reason to believe that this drug may be most beneficial in those instances where the formation of a normal collagen matrix is hindered. For example, certain types of osteogenesis imperfecta, Ehlers Danlos syndrome or epidermolysis bullosa, appear to be most suitable for a therapeutical trial.

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