Defect in Conversion of Procollagen to Collagen in a Form of Ehlers-Danlos Syndrome

Abstract. *Three patients with a form of the Ehlers-Danlos syndrome, a generalized disorder of connective tissue, have detectable amounts of procollagen in extracts of their skin and tendon. The activity of procollagen peptidase, the enzyme that converts procollagen to collagen, is reduced in cultures of fibroblasts. The clinical manifestations of this syndrome may be related to impaired enzymatic conversion of procollagen to collagen. Cultures of skin fibroblasts from these patients have an increased rate of synthesis of collagenous protein (collagen and procollagen), possibly related to the inability of these cells to convert procollagen to collagen.*

Recent evidence indicates that collagen molecules are formed from a larger biosynthetic precursor, procollagen (*1*). This conversion results from cleavage of the NH_2-terminal ends from the proα1 and proα2 chains that form the procollagen molecule by a specific enzyme, procollagen peptidase (*2*). The peptides cleaved from procollagen have a different amino acid composition than does the collagenous portion of the molecule. Some major differences in these peptides are the presence of cysteine, a lower proportion of glycine and proline residues, and the absence of hydroxyproline and hydroxylysine (*3*).

In our screening for biochemical abnormalities of collagen in tissues of patients with heritable disorders of connective tissue, we found three unrelated patients affected with a form of the Ehlers-Danlos syndrome who had an increased amount of procollagen in skin and tendon in comparison with normal controls. The clinical manifestations in these patients include short stature; stretchable, velvety skin; hypermobile joints; and multiple dislocations of joints, especially bilateral dislocations of the hips.

Skin and tendon from these patients were obtained at the time of surgery or elective skin biopsy and from the controls either at surgery or at autopsy. To obtain soluble collagenous protein, the tissues were finely ground and then extracted for 48 hours at 4°C in 0.5*M* acetic acid. The skin from two of the patients was four times more soluble in aqueous solvents than that of controls, as estimated from the amount of hydroxyproline in the extracts. A portion of the extracts was examined by acrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) (*4*) so that the collagenous components could be identified (Fig. 1).

In addition to the α chains and β components normally present in collagen, proteins with a slightly higher molecular weight than α1 and α2 were observed in extracts from the tissues of the patients but not in extracts from the control tissues. The most prominent bands (Fig. 1) migrated in the positions where proα1 and proα2 from rat procollagen appeared (*5*). Estimated molecular weights for the proα chains

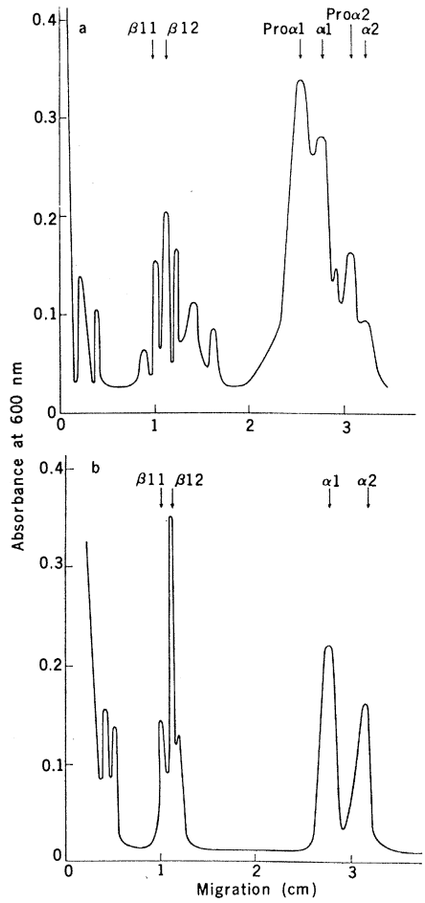


Fig. 1. Densitometric scan of an SDS-acrylamide gel of acid extracts of skin from (a) patient and (b) control. Gels were stained in a solution of 0.2 percent Coomassie blue, 50 percent methanol, and 10 percent acetic acid and destained in a solution of 7 percent acetic acid and 5 percent methanol, then scanned with a Gilford spectrophotometer at 600 nm.

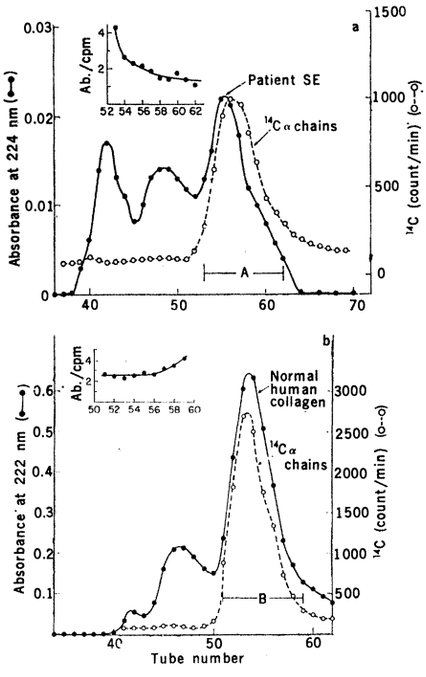
in the extracts of the tissues from the patients, with α chains and β components as references, were from 105,000 to 110,000 for the major proα1 band and 98,000 for the proα2 band (*4*). These bands of higher molecular weight were present on SDS-acrylamide gels of skin in all three patients and of tendon and round ligament from the one patient from whom this material was available. The β components of collagen were reduced in amount, especially in tendon and round ligament extracts.

For further characterization of the collagenous protein extracted from the tissues of the patients and the controls, portions of the acid extracts were applied to an 8 percent agarose column with ^{14}C-labeled α chains added as a standard (Fig. 2). Most of the protein in the extracts from the skin or from the tendons of the patients which was comparable in molecular weight to α chains eluted slightly earlier than the labeled marker and in the same position as proα chains. The comparable material from the control tissues coincided exactly with the marker chains. This difference was most evident when the specific activities (absorbance divided by counts per minute) across the peaks were compared (inset in Fig. 2). The fractions from these regions (A and B in Fig. 2) were pooled, and the protein was isolated and analyzed (*6*). The amino acid composition of the material from the extracts of the patients was similar to that of procollagen (4 to 8 half-cystines and 320 residues of glycine per 1000 residues), and the proportion of hydroxylysine was normal. The material from extracts of control tissues had amino acid compositions similar to that of collagen and lacked cysteine. These results indicate that the extracts of the patients' tissues contain procollagen in greater than normal amounts and suggest that these patients have a defect in the conversion of procollagen to collagen.

In order to determine whether this defect resulted from decreased activity of procollagen peptidase, the activity of this enzyme was assayed in medium from cultures of fibroblasts from these three patients (*7*). Medium from cultured cells normally contains procollagen peptidase activity, whereas none is detectable in the harvested cells (*2*, *8*).

Medium was collected after 4 days from confluent cultures of patients, control fibroblast strains, and mouse cell lines 3T3 and 3T6. Portions (5 ml) were concentrated tenfold by partial

Fig. 2. Molecular sieve chromatography (Bio-Gel A 1.5, 250 by 1 cm column) of acid extracts of skin from (a) patient 1, a 16-year-old Caucasian girl, and from (b) control 1, a 48-year-old Caucasian woman. Authentic ^{14}C-labeled α chains prepared from chick calvaria biosynthetically labeled in vitro (*15*) were added to the extracts of human tissue. Samples were dialyzed against a solution of 1*M* $CaCl_2$, tris(hydroxymethyl)aminomethane hydrochloride (*p*H 7.4), and 0.2*M* 2-mercaptoethanol, and applied to the column (*16*). Regions A and B indicate the fractions pooled for amino acid analysis; *Ab.*, absorbance; *cpm*, counts per minute.



lyophilization, and the concentrates were tested for activity at several protein concentrations as described (*2*). Acrylamide gel electrophoresis was used to detect the conversion of procollagen to collagen.

Enzyme activity, expressed as micrograms of procollagen converted to collagen in 12 hours per 5 ml of medium, was 131 for 3T3 mouse cells, 182 for 3T6 mouse cells, 333 for human control 1, 298 for human control 2, 53 for patient 1, 38 for patient 2, and 68 for patient 3. Thus, medium from fibroblast cultures from the patients contained significantly less procollagen peptidase activity than did medium from normal human or reference cells.

No enzymatic activity was detected in extracts of the cells from four flasks of 3T3 or 3T6. When equal aliquots of medium from patient and control cell lines were mixed, an additive increase in activity was produced.

The data reported here indicate that a clinically distinct group of patients with the Ehlers-Danlos syndrome have a biochemical defect in the conversion of procollagen to collagen resulting from reduced activity of procollagen peptidase (*9*). The clinical findings presumably arise from the accumulation of procollagen, which does not form normal collagen fibrils (*10*).

A defect in the conversion of procollagen to collagen has previously been detected in dermatosparaxic cattle (*10*) and sheep (*11*). Although these animals have a generalized involvement of collagenous tissues, easily torn skin is the most prominent aspect of the disease. In dermatosparaxic cattle the tissues contain almost no detectable procollagen peptidase activity (*2*), while the media from cultures of skin fibroblasts from these animals contain enzyme activity similar to that in media from the cultured skin fibroblasts of these patients (*12*). Our results suggest that the biochemical defect in one form of the Ehlers-Danlos syndrome is similar to that in dermatosparaxis, although the clinical manifestations differ.

Initial studies indicate that cells from the skin of the patients have a higher rate of synthesis of collagenous protein (procollagen plus collagen) than do control cultures, as measured by the proportion of peptide-bound [^{14}C]proline converted to [^{14}C]hydroxyproline (*13*) (Table 1). This difference is not due to a variation in hydroxylation, since the increased synthetic rate of collagenous protein was also found when bacterial collagenase was used to distinguish collagenous from other proteins (*14*).

Table 1. Synthesis of collagenous protein by cutaneous fibroblasts from patients and normal controls. Duplicate 250-ml Falcon flasks of confluent cultures of fibroblasts from patients and controls were incubated for 24 hours with 10 μc of uniformly labeled [^{14}C]-proline in 10 ml of Dulbecco-Vogt medium supplemented with ascorbic acid (50 μg/ml). The medium was decanted, and the cell layer was harvested, dialyzed exhaustively against distilled water at 4°C, lyophilized, and hydrolyzed in distilled 6*N* HCl. [^{14}C]Hydroxyproline and [^{14}C]proline were measured in the effluent from an automatic amino acid analyzer (*17*); Pro, proline; Hyp, hydroxyproline.

Source of fibroblast culture	[^{14}C]Hyp (count/min)	[^{14}C]Pro (count/min)	Hyp/Pro
Patient 1	100,690	488,199	0.21
Patient 2	73,290	295,820	.25
Patient 3	71,260	375,620	.19
Control 1	14,443	148,230	.10
Control 2	11,950	122,670	.10
Control 3	26,150	264,790	.10

It is our hypothesis that the increased synthesis of collagenous protein in these fibroblast cultures with reduced activity of procollagen peptidase is related to the impaired conversion of procollagen to collagen and the accumulation of procollagen. Present evidence indicates that procollagen peptidase is an endopeptidase, cleaving a peptide from the NH_2-terminal end of each of the proα chains (*12*). These peptides may act as inhibitors of collagenous protein synthesis so that cell cultures from these patients, in which lesser amounts of these peptides are produced, show increased synthesis of collagenous protein.

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13. Hydroxyproline is used as a measure of collagenous protein, since this amino acid is relatively specific for these proteins. Proline is found in all proteins, and in these studies [^{14}C]proline incorporation is used to indicate the synthesis of both collagenous and non-

collagenous protein. No correction is made here for the differential incorporation of proline into collagenous and noncollagenous protein [H. Green and B. Goldberg, *Nature* **200**, 1097 (1963)].
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18. We thank G. Hawkins for the amino acid analyses and measurements of [^{14}C]hydroxyproline and [^{14}C]proline and S. Kopits for assistance in obtaining specimens. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.
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Oxygen Affinity and Electrolyte Distribution of Human Blood: Changes Induced by Propranolol

Abstract. *Propranolol causes a massive leakage of potassium ions from red cells, which results in an alteration of the Gibbs-Donnan equilibrium across the red cell membrane. According to such a mechanism, the presence of propranolol significantly increases the hydrogen ion activity of the interior of the red cell, causing a decreased oxygen affinity of hemoglobin according to the classical Bohr effect. No release of 2,3-diphosphoglycerate which may be bound to the membrane is thus necessary to explain the effect of propranolol on the oxygen dissociation curve of blood.*

Pendleton *et al.* (*1*) showed that propranolol, added to washed human erythrocytes at a concentration of (1 to 5) $\times 10^{-4}M$, is able to shift the hemoglobin-oxygen dissociation curve. No effect of the drug was found for dialyzed hemoglobin solutions. Pendleton *et al.* could not determine the mechanism of action of propranolol, but suggested that it may be mediated by an action of the drug on the cell membrane.

The mechanism of action of propranolol has been further investigated by Oski *et al.* (*2*), who found that incubation of red cells with this drug produced no change in the total 2,3-diphosphoglycerate (DPG) content. According to Oski *et al.*, however, a certain fraction of 2,3-DPG in the red cell may usually be bound to the membrane and thus cannot interact with hemoglobin to affect its oxygen affinity. They suggested that propranolol might affect the oxygen affinity of blood by interacting with the erythrocyte membrane and releasing the bound 2,3-DPG in the interior of the red cell. Brann and Newman (*3*), however, could not demonstrate significant binding of 2,3-DPG to the red cell membrane.

It is relevant to ask whether the experiments reported by Pendleton *et al.* and Oski *et al.* were carried out, in the presence and in the absence of propranolol, at constant activity of all the ligands known to affect oxygen affinity in human blood. So far four allosteric ligands have been found to affect the oxygen affinity of hemoglobin solutions or whole blood; these are protons (*4*), carbon dioxide (*5*), organic phosphates [such as 2,3-DPG and adenosine triphosphate (*6*)], and the anions of various salts and acids (*7*). Intracellular *p*H was not measured in the experiments of Pendleton *et al.* nor those of Oski *et al.* They reported, moreover, that propranolol has a marked effect in decreasing the hematocrit value of red blood cell suspensions, which indicates substantial changes in the electrolyte distribution across the red cell membrane. Alterations in the distribution of sodium and potassium ions in red cells treated with propranolol have also been described and systematically investigated by Manninen (*8*).

We performed a series of experiments to determine whether the effect of propranolol on the hemoglobin-oxygen dissociation curve is mediated by changes in the distribution of electrolytes and hydrogen ions across the red cell membrane. Fresh human blood (about 400 ml) was collected under sterile conditions in bottles treated with heparin and was stored for no more than 8 hours at 2° to 4°C. During this time no significant changes in the distribution of 2,3-DPG, potassium, or sodium were found to occur. Values of P_{50} (the partial pressure of oxygen at which hemoglobin is 50 percent saturated) were obtained according to the technique of Brenna *et al.* (*9*).

Either sodium bicarbonate or hydrochloric acid was used to adjust the blood *p*H to the desired value. Hemolysis was routinely checked by measuring the hemoglobin content of the plasma. The sodium and potassium concentrations were determined by flame photometry both in the plasma and in total blood. All the measurements were made 30 minutes after the addition of propranolol. A mixture of *d*- and *l*-propranolol was added to the blood to give a final propranolol concentration of $5 \times 10^{-4}M$. Control experiments (without propranolol) were done by diluting the blood with an equivalent amount of saline.

Figure 1 shows the effect of propranolol on log P_{50} at various plasma *p*H values. The data agree with the results of Pendleton *et al.* (*1*) and Oski *et al.* (*2*) in showing that propranolol lowers the oxygen affinity of whole blood (at constant plasma *p*H). However, propranolol affects the oxygen affinity more at alkaline than at acid *p*H. The influence of *p*H on the effect of propranolol on oxygen affinity does

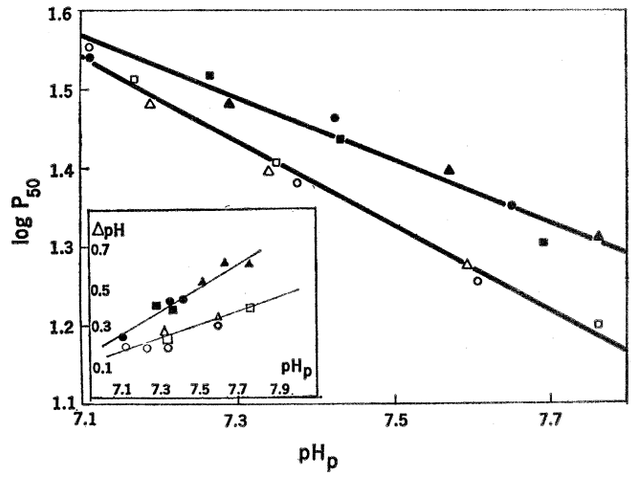

Fig. 1. Effect of propranolol on the oxygen affinity of human blood at 37°C. The different symbols refer to different subjects; (closed symbols) experiments with propranolol; (open symbols) experiments without propranolol; pH_p refers to plasma *p*H. The inset shows values of $pH_p - pH_c$ (the intracellular *p*H) as a function of pH_p.