Biochem. J. (1986) 240, 699–708 (Printed in Great Britain)

Collagen defects in lethal perinatal osteogenesis imperfecta

John F. BATEMAN,*‡ Danny CHAN,* Thomas MASCARA,* John G. ROGERS† and William G. COLE*
*Department of Paediatrics, University of Melbourne, and †Department of Genetics, Royal Children's Hospital, Melbourne, Victoria 3052, Australia

Quantitative and qualitative abnormalities of collagen were observed in tissues and fibroblast cultures from 17 consecutive cases of lethal perinatal osteogenesis imperfecta (OI). The content of type I collagen was reduced in OI dermis and bone and the content of type III collagen was also reduced in the dermis. Normal bone contained 99.3% type I and 0.7% type V collagen whereas OI bone contained a lower proportion of type I, a greater proportion of type V and a significant amount of type III collagen. The type III and V collagens appeared to be structurally normal. In contrast, abnormal type I collagen chains, which migrated slowly on electrophoresis, were observed in all babies with OI. Cultured fibroblasts from five babies produced a mixture of normal and abnormal type I collagens; the abnormal collagen was not secreted in two cases and was slowly secreted in the others. Fibroblasts from 12 babies produced only abnormal type I collagens and they were also secreted slowly. The slower electrophoretic migration of the abnormal chains was due to enzymic overmodification of the lysine residues. The distribution of the cyanogen bromide peptides containing the overmodified residues was used to localize the underlying structural abnormalities to three regions of the type I procollagen chains. These regions included the carboxy-propeptide of the proα1(I)-chain, the helical α1(I) CB7 peptide and the helical α1(I) CB8 and CB3 peptides. In one baby a basic charge mutation was observed in the α1(I) CB7 peptide and in another baby a basic charge mutation was observed in the α1(I) CB8 peptide. The primary defects in lethal perinatal OI appear to reside in the type I collagen chains. Type III and V collagens did not appear to compensate for the deficiency of type I collagen in the tissues.

INTRODUCTION

Osteogenesis imperfecta (OI) is a genetically determined disorder of the connective tissues in which a variety of metabolic and structural abnormalities of type I collagen have been reported (Prockop & Kivirikko, 1984). We previously observed abnormalities of type I collagen in cultured fibroblasts from seven babies with the lethal perinatal form of OI (Bateman *et al.*, 1984). Four of these fibroblast cultures produced both normal and abnormal type I collagen whereas the other three produced only abnormal type I collagen. In each of the cultures the abnormal type I collagen was poorly secreted and intracellular degradation of collagen was increased. The α1(I)- and α2(I)-chains of the abnormal type I collagens contained excessive amounts of hydroxylysine and glycosylated hydroxylysine and in one case a charge mutation was observed in the CNBr peptide 'map' of the α1(I)-chain.

In this paper we present a detailed analysis of the abnormal collagens of dermis and bone from these seven babies and of dermis, bone and cultured fibroblasts from a further ten babies with lethal perinatal OI.

EXPERIMENTAL

Materials

L-[5-^{3}H]Proline (30 Ci/mmol) and NaB^3H_4 (75 Ci/mmol) were obtained from Amersham Australia Ltd, Sydney, Australia. Protosol tissue solubilizer was purchased from New England Nuclear, Boston, MA, U.S.A. Trypsin (TPCK-treated) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Pepsin, β-aminopropionitrile fumarate and sodium ascorbate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-Ethylmaleimide, α,α'-dipyridyl and trifluoroacetic acid were purchased from BDH Chemicals, Poole, Dorset, U.K. and phenylmethanesulphonyl fluoride was obtained from Merck, Darmstadt, West Germany. Acetonitrile was purchased from Waters Associates, Milford, MA, U.S.A. TSK 4000 SW gel permeation h.p.l.c. columns were obtained from Toyo Soda Manufacturing Co., Tokyo, Japan, and C-18 Pep-RPC reverse-phase columns from Pharmacia Fine Chemicals, Uppsala, Sweden. Amino acid analysis standards were supplied by Pierce Chemical Co., Rockford, IL, U.S.A. The source and composition of the cell culture media have been described previously (Bateman *et al.*, 1984). All other chemicals were commercially available analytical grade reagents.

Human dermis and bone

Normal dermis and bone were obtained at autopsy from 14 babies who had died at birth from diseases that did not affect the connective tissues. Dermis and bone were also obtained at autopsy from 17 babies who died at birth from type II (lethal perinatal) OI. The babies showed the typical features of this type of OI (Sillence *et al.*, 1979). The long bones were short, broad and crumpled but in one baby (OI 26) the long bones had a more normal shape. The biopsies were obtained with the approval of the Ethics Committee of this hospital and with informed parental consent.

Abbreviation used: OI, osteogenesis imperfecta.

‡ To whom correspondence and reprint requests should be sent.

Solubility of dermal and bone collagens

Each dermal sample was milled to a powder in a freezer mill. The powdered samples were freeze-dried to constant weight and their hydroxproline contents were determined (Jamall *et al.*, 1981). The samples were extracted for 24 h at 4 °C with 0.05 M-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and the proteinase inhibitors 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride and 10 mM-EDTA. These samples were extracted for a further 24 h with 0.5 M-acetic acid at 4 °C. The residues were freeze-dried, weighed and subjected to limited digestion with pepsin (enzyme/substrate ratio 1:10) for 24 h at 4 °C (Cole *et al.*, 1984). The hydroxyproline contents of the extracts were determined.

Bone (femur) samples were washed with 0.05 M-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and proteinase inhibitors at 4 °C to remove blood and then milled to a fine powder in a freezer mill. The powdered bone was decalcified in repeated changes of 0.2 M-EDTA (pH 7.5) over 5 days. The decalcified matrix was washed with deionized water and defatted with chloroform/methanol (2:1, v/v) at 4 °C. The residue was freeze-dried, weighed and the hydroxyproline content was determined. Limited pepsin digestion was carried out twice and the amount of hydroxyproline in the solubilized collagen was determined. The types of collagen present in the dermal and bone extracts were determined by electrophoretic analysis.

Purification of α-chains

Pepsin-solubilized type I and III collagens were purified from dermis and type I collagen was also purified from bone. The pepsin-solubilized collagens were precipitated with 1.2 M-NaCl and the type I, III and V collagens were then separated at neutral pH by differential salt precipitation (Epstein, 1974; Burgeson *et al.*, 1976).

Type III collagen was purified by using the differential denaturation and renaturation procedure described by Chandrarajan (1978) and by gel-permeation chromatography of denatured samples with a TSK 4000 SW column as described by van der Rest & Fietzek (1982).

The α-, β- and γ-chains of type I collagen were separated from each other by chromatography of denatured samples using a TSK 4000 SW column (van der Rest & Fietzek, 1982). The fractions containing the α1(I)- and α2(I)-chains were pooled, dialysed against 0.1 M-acetic acid at 4 °C and freeze-dried. The dried samples were dissolved in 0.1% (v/v) trifluoroacetic acid and denatured at 50 °C for 3 min, and chromatography was performed on a C18 Pep-RPC reverse-phase column. Collagen chains were separated at room temperature in aqueous 0.1% (v/v) trifluoroacetic acid with a linear gradient of 18–31.5% (v/v) acetonitrile over 37 min. The eluent flow was 0.7 ml/min. Under these conditions the α1(I)- and α2(I)-chains were resolved completely (Bateman *et al.*, 1986).

Cyanogen bromide cleavage of dermis and bone

Freeze-dried samples of dermis and decalcified bone were suspended in 70% (v/v) formic acid containing 50 mg of CNBr/ml and cleavage was achieved by the method of Scott & Veis (1976). To study the peptides containing reducible cross-links and aldehydes, some samples of decalcified bone were reduced with NaB^3H_4 before cleavage with CNBr (Kuboki *et al.*, 1981).

Trypsin digestion of bone

The distribution of the collagens between the calcified and uncalcified parts of OI bone was determined by digesting the uncalcified matrix with trypsin (Banes *et al.*, 1983). Dried undecalcified bone powder was rehydrated in 50 mM-$(NH_4)_2CO_3$, pH 7.5. Proteins in the uncalcified matrix of the bone were denatured at 60 °C for 20 min and the specimens were then cooled to 37 °C (Kuboki *et al.*, 1981; Banes *et al.*, 1983). Trypsin was added to a final concentration of 1% (w/w) and digestion was performed for 2 h at 37 °C. The mixture was again heated to 60 °C for 20 min. The mixture was cooled to 37 °C and redigested with trypsin (0.5%, w/w) for 2 h. Under these conditions, collagen in the calcified matrix is protected from denaturation and trypsin digestion (Banes *et al.*, 1983). The supernatant, obtained by centrifugation, was freeze-dried and the hydroxyproline content determined. The pellet was suspended in 0.2 M-EDTA (pH 7.5) and dialysed against repeated changes of this same buffer over 5 days. The decalcified bone matrix was dialysed against deionized water and freeze-dried. Limited pepsin digestion was carried out at an enzyme/substrate ratio of 1:10 in 0.5 M-acetic acid for 24 h at 4 °C. The pepsin-solubilized collagens were analysed by electrophoresis.

Dermal fibroblast cultures

Dermal fibroblast cultures were established from 17 babies with lethal perinatal OI and from seven age-matched controls. The cell culture and collagen labelling procedures are described elsewhere (Bateman *et al.*, 1984). Briefly, procollagens were labelled for 18 h with L-[5-^{3}H]proline at 10 μCi/ml of medium containing 0.15 mM-sodium ascorbate and 0.1 mM-β-aminopropionitrile fumarate. Limited pepsin digestions of procollagens in the cell layer and medium were carried out and collagen secretion was determined from the distribution of L-[5-^{3}H]proline-labelled collagen chains between the cell layer and medium fractions. The distribution of the collagen types was determined after electrophoresis by the excision of the collagenous bands and scintillation counting.

SDS/polyacrylamide-gel electrophoresis

Collagen chains were analysed on 5% (w/v) acrylamide separating gels with a 3.5% (w/v) acrylamide stacking gel. The sample preparation, electrophoresis conditions, gel staining and method of quantifying the types of collagen are described elsewhere (Chan & Cole, 1984; Cole *et al.*, 1984). Radioactivity in the protein bands was determined after fluorography (Bonner & Laskey, 1974) by excision of the individual bands, mild alkaline hydrolysis in Protosol at 60 °C for 60 min and scintillation counting.

Collagen CNBr-cleavage peptides were analysed on 12.5% (w/v) acrylamide slab gels with a stacking gel of 4.5% (w/v) acrylamide. The sample preparation, conditions of electrophoresis and procedures for staining and scanning of gels are described elsewhere (Cole *et al.*, 1984).

Collagen CNBr-cleavage peptides were also analysed by two-dimensional polyacrylamide-gel electrophoresis.

Table 1. Collagen composition of OI dermis

Experimental details are given in the text. The control results are given as the means ± S.D. The number of controls are given in parentheses. Collagen solubility was determined from the proportion of the total amount of Hyp that was present in each fraction. The recovery of Hyp was close to 100% in all samples. The total concentrations of type I and III collagens were determined from their concentrations in the various extracts. Type V collagen accounted for < 0.5% of the total amount of collagen. The percentages of type III collagen in the total extracts of the dermis are given in brackets. The α1(I)/α2(I) ratios of type I collagen were determined in the pepsin-soluble fractions.

Sample	Hyp (μg/mg dry wt.)	Hyl/Hyp	Collagen solubility (% Hyp)				Type of collagen† (μg/mg dry wt.)			α1(I)/α2(I)
			NaCl	Acetic	Pepsin	Residue	Type I	Type III		
OI 24	57.6*	0.079*	0.6	5.0*	89.5	1.0	238*	146	[38]*	2.0
OI 26	14.0*	0.069	0.9	15.7	82.0	0.2	73*	34*	[32]*	2.0
OI 30	63.7*	0.082*	0.7	9.7*	90.1	1.1	321*	155	[33]*	2.0
OI 31	29.1*	0.083*	3.5	6.2*	71.8	3.5	194*	59*	[23]	2.4
OI 35	18.0*	0.128*	2.7	4.3*	71.5	4.4	120*	52*	[30]*	2.1
OI 39	40.0*	0.069	—	—	—	—	—	—	—	—
OI 45	45.7*	0.085*	2.0	6.8*	96.0*	0.8	211*	125	[37]*	2.2
OI 51	63.9*	0.092*	0.1	5.4*	100.5*	1.9	323*	149	[32]*	2.0
OI 53	60.8*	0.092*	0.7	9.6*	99.4*	0.6	297*	171	[37]*	1.8
OI 56	72.2	0.080*	1.4	9.1*	84.9	0.6	368	181	[33]*	1.9
OI 57	53.6*	0.068	—	—	—	—	—	—	—	—
OI 59	28.0*	0.098*	2.9	6.6*	98.0*	1.7	143*	70*	[33]*	1.7
OI 60	55.6*	0.066	1.2	0.8*	96.7*	1.0	218*	159	[42]*	2.2
OI 62	35.4*	0.093*	1.5	5.7*	90.9	1.1	167*	80*	[32]*	2.0
OI 74	24.4*	0.075*	3.2	8.8*	86.7	0.7	120*	50*	[29]*	2.0
Controls	91.0 ± 13.4 (14)	0.054 ± 0.008 (12)	2.5 ± 1.7 (10)	22.5 ± 6.1 (10)	77.6 ± 8.6 (10)	1.2 ± 0.6 (10)	510 ± 82 (10)	159 ± 26 (10)	[23.8 ± 2.7 (10)]	1.8 ± 0.2 (14)

* Values outside the control mean ± 2 S.D.

† A positive correlation ($r = 0.85$) was found between the concentrations of type I and III collagens in the OI samples.

Table 2. Collagen composition of OI bone

Experimental details are given in the text. The control results are given as the means ± S.D. All measurements were carried out in triplicate and the numbers of control samples are given in parentheses. The types of collagen and the α1(I)/α2(I) ratios were determined by using the pepsin-solubilized collagen fraction. ND, type III collagen was not detected in control bone.

Sample	Hyp (μg/mg dry wt.)	Hyl/Hyp	Incorporation of NaB^3H_4 (d.p.m./μg of Hyp)	Pepsin-soluble collagen (% Hyp)	Type of collagen (%)			α1(I)/α2(I)
					I	III	V	
OI 24	77.2*	0.098*	15.0*	43.4	72.3	22.5	5.2	2.3
OI 26	22.8*	0.082*	28.0*	100.0*	97.6	2.0	0.4	2.3
OI 30	92.2	0.137*	14.6*	63.8	74.7	23.3	2.0	3.2
OI 31	48.0*	0.172*	30.0*	55.0	87.3	6.5	6.2	3.1
OI 35	61.7*	0.171*	31.4*	49.9	79.5	6.0	14.4	3.1
OI 39	56.8*	0.155*	14.1*	44.7	88.3	6.5	5.2	3.0
OI 40	79.2*	0.118*	26.6*	38.8	81.0	9.7	9.3	3.2
OI 45	57.0*	0.093*	22.8*	81.8*	85.4	9.8	4.8	3.8
OI 48	96.8	0.093*	14.5*	67.7*	89.1	7.0	3.1	3.0
OI 51	79.3*	0.139*	10.1	52.7	82.0	14.0	4.0	3.7
OI 53	67.5*	0.103*	7.9	48.2	84.8	11.2	4.0	3.4
OI 56	78.9*	0.095*	8.5	37.9	70.8	18.6	10.5	3.0
OI 59	75.6*	0.132*	16.8*	40.9	79.6	9.0	11.4	3.4
OI 60	68.7*	0.115*	7.6	57.7	79.8	11.8	8.4	3.1
OI 74	27.5*	0.089*	16.5*	47.6	83.5	12.5	4.0	3.3
Controls	104.0 ± 11.6 (12)	0.068 ± 0.006 (12)	9.0 ± 1.0 (11)	49.0 ± 8.1 (10)	99.3 ± 0.3 (10)	ND	0.7 ± 0.3 (10)	4.3 ± 0.9 (10)

* Values outside the control mean ± 2 S.D.

Electrophoresis consisted of non-equilibrium pH-gradient electrophoresis in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second dimension (Cole & Chan, 1981). The second dimension gels were stained with Coomassie Brilliant Blue (Cole & Chan, 1981) and the radioactivity in peptide spots was determined by fluorography (Bonner & Laskey, 1974).

Amino acid analysis

Samples of dermis, decalcified bone and purified collagen α-chains were hydrolysed in 6 M-HCl under N_2 at 110 °C for 20 h. The dried hydrolysates were analysed by using a Waters amino acid analyser equipped with a post-column *o*-phthaldehyde derivatization system and a fluorescent detector. In calculating the amino acid compositions, correction factors were employed for losses of threonine, serine and tyrosine, and incomplete release of valine during hydrolysis (Piez *et al.*, 1960). Cysteine residues were assayed as carboxymethylcysteine after reduction and alkylation (Gollwitzer *et al.*, 1972).

RESULTS

Collagen composition of dermis

Each sample of OI dermis had an abnormal collagen composition (Table 1). The collagen concentrations, as determined by hydroxyproline analysis, were significantly reduced in 14 of the 15 samples studied. The solubility of collagen was also different as less collagen was extracted by acetic acid from 12 of the 13 OI samples analysed, but the residual collagen was completely extracted by limited pepsin digestion. The NaCl and acetic acid extracts contained type I collagen, and the pepsin digests also contained type III collagen. The concentrations of type I collagen were significantly reduced in 12 samples and the concentrations of type III collagen were also significantly reduced in six samples. However, the concentrations of type I collagen were reduced to a greater extent than the concentrations of type III collagen so that the proportion of type III collagen was slightly increased in 11 samples of OI dermis.

Type V collagen chains were detected in the pepsin digests of normal and OI dermis. In both groups the amount of type V collagen was less than 0.5% of the total collagen.

Collagen composition of bone

Each sample of OI bone also had an abnormal collagen composition (Table 2). The concentrations of hydroxyproline were significantly reduced in 13 of the 15 samples studied. With the exception of OI 26, the collagen was incompletely solubilized from normal and OI bone although the proportion of the collagen solubilized by limited pepsin digestion was significantly increased in three OI bone samples. The pepsin digests of OI bone contained type I, III and V collagens, whereas the digests from normal bone contained type I collagen and a small amount of type V collagen and no detectable type III collagen. The proportion of type V collagen was increased in the digests of OI bone.

The distribution of type I, III and V collagens between the calcified and uncalcified matrix of OI bone was determined after removal of the denatured uncalcified matrix by trypsin digestion. Trypsin solubilized 28% of the hydroxyproline from OI 56 bone, 25% from OI 53 bone and 15–19% from control samples. The calcified matrix was shown by electrophoresis to contain type I and V collagens; type III collagen was not detected (results not shown).

Table 3. Collagen types and secretion in OI fibroblasts

Cell cultures were labelled with [5-^{3}H]proline and collagens from the cell layer and medium were subjected to limited pepsin digestion (see the Experimental section for details). The resultant α-chains were analysed by electrophoresis. The proportions of type I, III and V collagens and the α1(I)/α2(I) ratios were determined by scintillation counting of the excised α-chains. Collagen secretion was determined from the distribution of the collagen between the cell layer and medium fractions. All analyses were made in triplicate and the control values are given as the means ± S.D.

Sample	Type of collagen (%)			α1(I)/α2(I)	Collagen secretion (%)
	I	III	V		
OI 24	87.7	6.6	5.7	2.4	42.0
OI 26	86.2	5.1	8.7	2.7	60.7
OI 30	88.3	6.6	5.1	2.2	69.6
OI 31	87.1	8.0	4.9	2.2	68.4
OI 35	88.5	13.5	8.0	1.9	72.6
OI 39	89.2	5.6	5.2	2.5	61.2
OI 40	87.6	3.8	8.6	1.9	48.9
OI 45	89.2	5.7	5.1	2.7	51.7
OI 48	87.3	6.2	6.5	1.9	57.1
OI 51	88.1	7.3	4.6	2.2	77.5
OI 53	83.5	12.1	4.4	1.8	63.6
OI 56	86.0	10.9	3.1	2.1	74.3
OI 57	86.4	10.0	3.6	3.0	62.2
OI 59	77.2	18.6	4.2	2.4	70.5
OI 60	90.4	6.2	3.4	1.8	61.1
OI 62	80.7	14.8	4.5	2.1	63.6
OI 74	89.2	7.0	3.8	2.2	51.4
Controls ($n = 7$)	92.3 ± 1.9	5.7 ± 2.3	2.0 ± 1.0	2.2 ± 0.1	85.4 ± 4.4

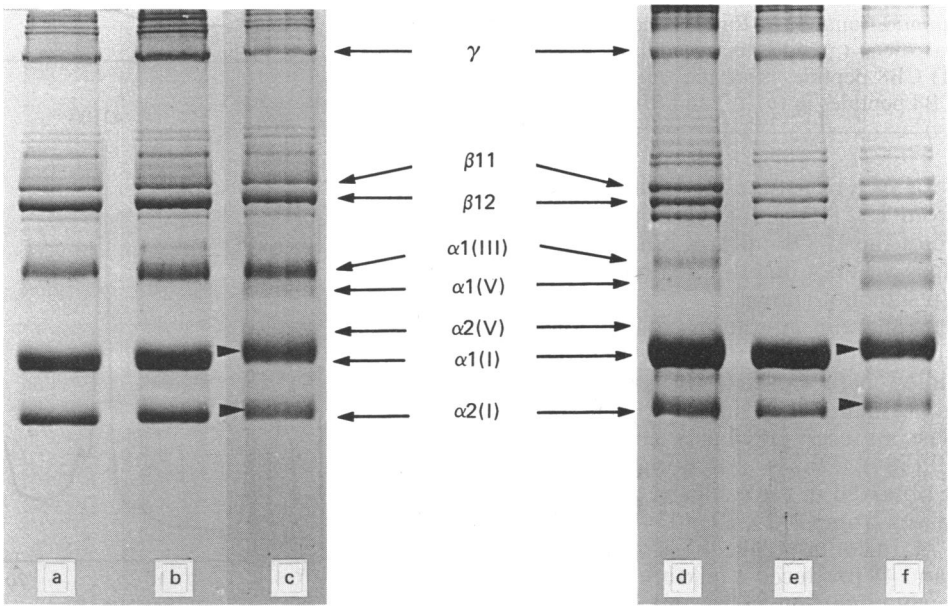

Fig. 1. Electrophoresis of pepsin-digested collagen from dermis and bone

Pepsin-digested collagens were analysed by SDS/polyacrylamide-gel electrophoresis with delayed reduction of disulphide bonds with 10% β-mercaptoethanol (Sykes *et al.*, 1976). The samples consist of dermal collagen from OI 26 (lane a), control (lane b) and OI 35 (lane c); bone collagen from OI 26 (lane d), control (lane e) and OI 35 (lane f). The migration positions of type I collagen chains [α1(I), α2(I), dimeric β11 and β12], type V collagen chains [α1(V) and α2(V)] and type III collagen chains [α1(III)] are shown. γ refers to collagen molecules containing three α-chains. ▶ indicates the slowly migrating α1(I) and α2(I) chains in OI 35.

Characterization of collagen chains from cultured fibroblasts

All of the OI and control fibroblast cultures produced type I, III and V collagens (Table 3). Most of the OI fibroblast cultures contained an increased proportion of type V collagen but this type of collagen was still present only in small amounts. The proportion of type III collagen was also increased in six of the OI cultures but the highest value was only 18.6% of the total amount of

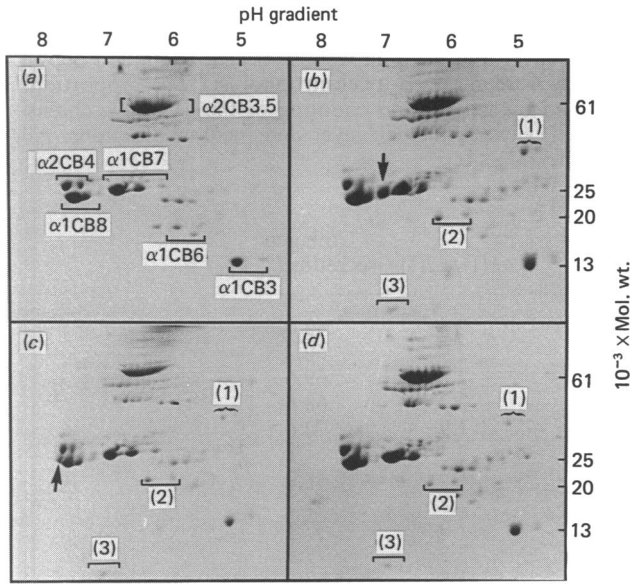

Fig. 2. Two-dimensional electrophoresis of CNBr-cleavage peptides of normal and OI bone

Samples (200 μg) of CNBr-cleavage peptides obtained from (*a*) normal bone, (*b*) OI 51, (*c*) OI 24 and (*d*) OI 53 were resolved by two-dimensional electrophoresis and stained with Coomassie Blue as described in the text. The major α1(I) and α2(I) peptides are marked in (*a*). Peptide (1) is a type V collagen marker peptide, (2) is the α1(III) CB5 peptide and (3) is the α1(III) CB8 peptide. The arrow in (*b*) indicates an additional basic form of the α1(I) CB7 peptide and the arrow in (*c*) indicates an additional basic form of the α1(I) CB8 peptide. Note the 'tailing' of the α1(I) CB3 and CB8 peptides in (*b*), (*c*) and (*d*).

collagen. The ratios of α1(I)- to α2(I)-chains in the OI samples varied from 1.8:1 to 3.0:1. Collagen secretion was reduced in 16 of the 17 OI cultures but the reduced secretion of collagen was not due to impaired hydroxylation of proline (results not shown).

Abnormal electrophoretic migrations of the collagen chains produced by OI fibroblast cultures were observed in the cases studied here (results not shown). This electrophoretic abnormality was similar to that of the seven OI fibroblast cultures previously studied by us (Bateman *et al.*, 1984). Slowly migrating α1(I)- and α2(I)-chains were observed in the cell layer and medium of cultured fibroblasts from OI 24, 39, 40, 45, 51, 53, 56, 57, 59, 60, 62 and 74. In contrast, fibroblast cultures from OI 26, 30, 31, 35 and 48 produced a mixture of normally migrating and slowly migrating α1(I)- and α2(I)-chains. Pulse-chase studies showed that the type I collagens containing the slowly migrating chains were slowly secreted when compared with the normal type I collagen. This was most marked in OI 26 and OI 48 cells, in which the abnormal type I collagens were completely retained within the cell layer (results not shown).

Characterization of collagen chains from tissues

With the exception of OI 26 and OI 48, the type I collagen chains were abnormal in all OI dermal and bone samples. The α1(I)-, α2(I)-, β11- and β12-chains migrated slowly on electrophoresis. The α1(I)- and α2(I)-chains from OI bone migrated more slowly than those from OI dermis (Fig. 1). The ratios of the α1(I): α2(I) chains from OI dermis were normal (Table 1) but the ratios in OI bone were low (Table 2) when compared with the control values. However, the ratios obtained from normal bone were higher than those from normal dermis, in agreement with the report by Fujii & Tanzer (1977). The high proportion of α1(I)-chains was not due to comigrating α1(I)-chains of α1(I)-trimer or α1(II)-chains of type II collagen. Neither of these forms of collagen was detected by electrophoresis of collagen fractions obtained by differential NaCl precipitation. In addition, two-dimensional electrophoretic 'maps' of CNBr-cleavage peptides of bone did not show any of the characteristic α1(II)-peptides (Fig. 2) (Cole & Chan, 1981).

Comparison of the collagen chains extracted from tissues with those produced by fibroblast cultures

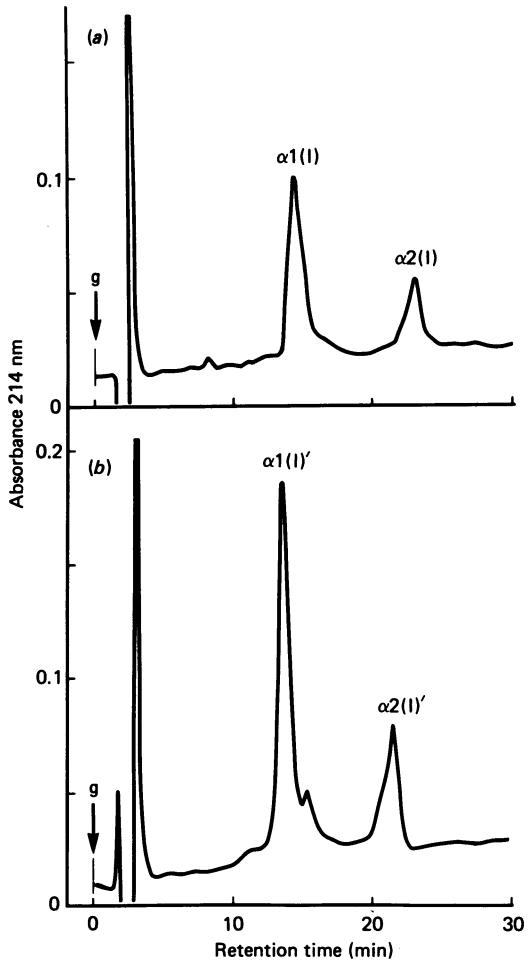

Fig. 3. Separation of α-chains from control and OI dermal collagens by reverse-phase h.p.l.c.

Pepsin-digested dermal collagen was chromatographed on a TSK 4000 SW column to separate type III collagen and cross-linked type I collagen chains from the α-chains of type I collagen (van der Rest & Fietzek, 1982). The α-chains (100 μg) were denatured and separated by using a C18 Pep-RPC column (see the Experimental section for details). (*a*) Chromatogram of α-chains from normal dermis; (*b*) chromatogram of α-chains from OI 60 dermis. The abnormal α1(I)- and α2(I)-chains from OI 60, which are designated α1(I)′ and α2(I)′, eluted before their normal control counterparts. g (arrow) indicates the start of the gradient.

Table 4. Lysine hydroxylation in purified type I and III collagen α-chains

Type I and III collagen chains were purified as described in the Experimental section. The chains were hydrolysed in 6 M-HCl under N_2 at 110 °C for 20 h. The dried hydrolysates were analysed in a Waters amino acid analyser, as described in the text. The results for hydroxylysine and lysine are listed as the number of residues/1000 total residues. The control values are given as the means ± S.D.

	Dermis						Bone			
	α1(I)		α2(I)		α1(III)		α1(I)		α2(I)	
Sample	Hyl	Lys	Hyl	Lys	Hyl	Lys	Hyl	Lys	Hyl	Lys
OI 24	8.1*	30.8	10.0*	21.5	6.1	31.3	18.6*	17.8†	12.8*	19.4
OI 26	4.9	31.9	8.4	21.2	5.9	27.1	4.7	35.6	6.6	24.5
OI 45	7.2*	31.2	8.5	21.7	7.8	28.2	13.8*	30.6	12.7*	17.9
OI 53	7.9*	31.6	8.6	21.8	6.0	25.7	17.0*	14.6†	12.8*	18.2
OI 56	7.7*	32.6	8.3	23.0	6.4	29.1	8.9*	25.3†	11.7*	19.9
OI 60	8.0*	31.4	9.0	23.0	6.4	30.2	10.9*	21.2†	12.3*	16.0
Controls	5.5±0.2	32.9±1.4	9.0±0.5	22.6±1.3	5.9±1.0	31.2±2.5	6.1±0.8	33.9±1.5	7.7±0.5	23.2±1.6

* Values more than 2 S.D. above the control mean values.
† Total Hyl and Lys values more than 2 S.D. below the control mean values.

demonstrated that slowly migrating chains were present in the tissues of babies whose fibroblasts produced only the slowly migrating α-chains. In contrast, the tissues from OI 30, 31 and 35 contained slowly migrating chains only, although the cultured fibroblasts secreted a mixture of normal and slow type I collagen chains.

The α1(I)- and α2(I)-chains from type I collagen were purified from the dermis and bone of OI 24, 45, 53, 56 and 60. In each case the chains, in particular the α1(I)-chain, showed reduced retention times when chromatographed on a reverse-phase column (Fig. 3). We have previously shown that slow electrophoretic migrations and reduced chromatographic retention times of type I collagen chains produced by cultured fibroblasts from babies with OI were due to an increased content of hydroxylysine and glycosylated hydroxylysine (Bateman *et al.*, 1984). The elevated ratios of hydroxylysine:hydroxyproline in OI dermis and bone are consistent with this proposal (Tables 1, 2).

Direct evidence for the overmodification of lysine residues was obtained by amino acid analysis of α1(I)- and α2(I)-chains that were purified from dermis and bone of OI 24, 45, 53, 56 and 60. The number of hydroxylysine residues was increased in the α1(I)-chains from dermis and bone and in the α2(I)-chains from bone (Table 4). The α1(I)-chains from OI bone had the slowest electrophoretic migrations. Although the number of hydroxylysine residues was increased the total number of hydroxylysine and lysine residues in these chains was significantly reduced (Table 4). The amino acid compositions of the α1(I)- and α2(I)-chains from OI dermis and bone were otherwise normal.

The type I collagen chains from OI 26 dermis and bone and from OI 48 bone, in contrast with the other cases of OI, migrated normally on electrophoresis. The chains from OI 26 were analysed further and shown to have normal retention times when chromatographed on a reverse-phase column and normal amino acid compositions (Fig. 1; Table 4).

The type III collagen chains obtained from all samples of OI dermis migrated normally on electrophoresis (Fig. 1). Type III collagen, purified from six samples of OI dermis, had normal amino acid compositions with normal contents of hydroxylysine (Table 4). The α1(V)-chain of type V collagen, from all samples of OI bone, also migrated normally on electrophoresis (Fig. 1). The α2(V)-chains comigrated with the slowly migrating α1(I)-chains, making analysis difficult, but were shown by using purified preparations of type V collagen to migrate normally (results not shown). Type V collagen was detected in minor amounts (< 0.5% of total collagen) in extracts of normal and OI dermis and normally migrating type V collagen chains were observed in the cell layer of control and OI cultured fibroblasts (results not shown).

Collagen CNBr-cleavage peptides from tissues and cultured fibroblasts

The two-dimensional electrophoretic 'maps' of CNBr-cleavage peptides from OI 26 dermis and bone and OI 48 bone were normal whereas the 'maps' from the other OI tissue samples were abnormal (Fig. 2). As observed previously, each CNBr-cleavage peptide contained several charged forms which were reproducible in all control samples studied and in replicate analyses of the same sample (Cole & Chan, 1981). Against this background of consistent charge heterogeneity, we observed abnormally charged peptides in two OI samples (OI 24 and 51) and peptides that migrated slowly in the second dimension were a common feature of the OI samples.

The α1(I) CB8 peptide obtained from the dermis, bone and cultured fibroblasts of OI 24 was found to migrate slowly, with 'tailing' of the proximal edge, and had an extra basic charged form (Fig. 2). However, the *C*-terminal α1(I) CB6 and CB7 peptides migrated normally. The additional basic form of the α1(I) CB8 peptide was previously observed when the hydroxylation of proline and lysine were blocked by the addition of αα′-bipyridyl to the culture medium, indicating that the extra charged form was not due to post-translational modifications (Bateman *et al.*, 1984).

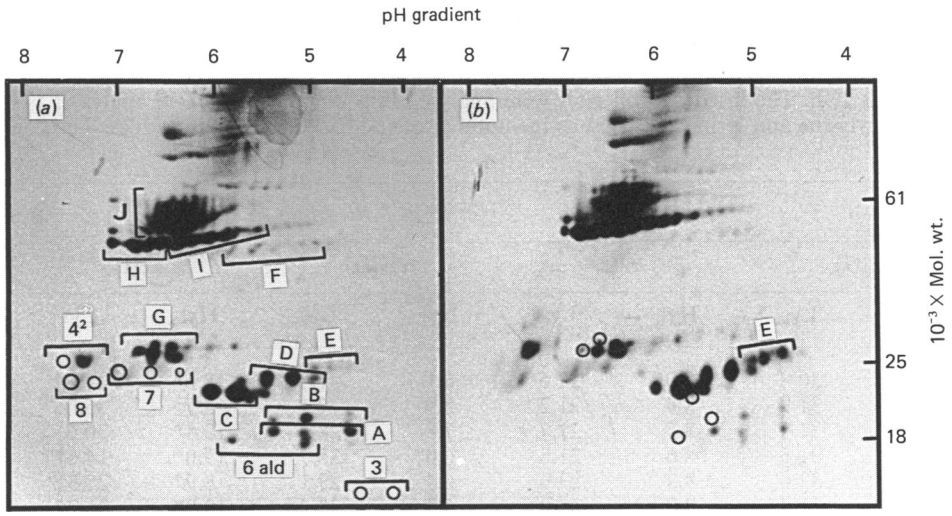

Fig. 4. Two-dimensional electrophoresis of CNBr-cleavage peptides of NaB^3H_4-reduced normal and OI bone

Reduced samples (200 μg) of CNBr-cleavage peptides obtained from (*a*) normal bone and (*b*) OI 24 bone were resolved by two-dimensional gel electrophoresis and fluorography (see the Experimental section for details). The open circles in (*a*) represent the normal positions of the α1(I) CB3 (3), α1(I) CB7 (7), α1(I) CB8 (8) and the α2(I) CB4 (4^2). The radiolabelled peptides were identified from the 'maps' of Cheung *et al.* (1981). 6 ald refers to the allysine-containing form of the α1(I) CB6 peptide. Peptides A to E represent the cross-linked α1(I) CNBr-cleavage peptides $6 \times 0,1$, $6 \times (0,1)_2$, 6×5, $6 \times 5 \times 0,1$ and $6 \times 5 \times (0,1)_2$, respectively. Peptides F and G are unassigned. Peptides H and I may represent α1(I) CB6 $\times$ α2(I) CB4 and J represents cross-links involving the α2(I) CB3.5. In (*b*) the open circles indicate radiolabelled peptides that were either absent or reduced in intensity in the OI sample. Peptide E was increased in intensity.

The α1(I) CB7 peptide obtained from dermis, bone and cultured fibroblasts of OI 51 also migrated slowly with proximal 'tailing' and an extra basic charged form (Fig. 2). The *C*-terminal α1(I) CB6 peptide migrated normally in contrast with the *N*-terminal α1(I) CB3 and CB8 peptides, which migrated slowly and with proximal 'tailing'. All of these peptides migrated normally when α,α'-dipyridyl was included in the medium but the additional basic charged form of the α1(I) CB7 peptide persisted. In both cases the localization of the charge mutation to the α1(I) CB8 and CB7 peptides, respectively, was verified by peptide map analysis of purified CNBr peptides (results not shown).

Slow migration and 'tailing' of CNBr peptides were observed in the peptide 'maps' obtained from the cell layers of cultured fibroblasts from OI 26 and 48 and from the cell layer and media obtained from all of the other cases of OI. These abnormal electrophoretic features were due to the overmodification of lysine residues. The distribution of the overmodified peptides along the α-chains was used to classify the babies into three groups. In the first group, which included OI 24, 39, 40, 45, 48, 53 and 56, the overmodification of lysine residues was confined to the α1(I) CB8 and CB3 peptides. In the second group, OI 51 and 62, the α1(I) CB8, CB3 and CB7 peptides were overmodified. However, the majority of babies (OI 26, 30, 31, 35, 57, 59, 60 and 74) were in the third group with overmodification of all the major α1(I) CNBr peptides.

The electrophoretic 'maps' of the α1(III) CNBr-cleavage peptides from OI dermis were normal, in agreement with the normal electrophoretic migrations and amino acid compositions of the α1(III)-chains (Figs. 1, 2; Table 4). The electrophoretic 'maps' of the type V collagen CNBr-cleavage peptides from OI bone were also normal.

Collagen cross-linkages in dermis and bone

Type I collagen extracted from OI dermis and bone contained β11- and β12- chains and higher molecular weight cross-linked chains as observed in controls (Fig. 1). The two-dimensional electrophoretic 'maps' of CNBr-cleavage peptides from normal and OI dermis and bone contained reduced amounts of the *C*-terminal α1(I) CB6 and α1(III) CB9 peptides which normally take part in forming intermolecular cross-linkages (Light & Bailey, 1979; Cheung *et al.*, 1983).

The incorporation of NaB^3H_4 into reducible cross-linkages and aldehydes was significantly increased in most samples of OI bone (Table 2). The two-dimensional electrophoretic 'maps' of the labelled collagen CNBr-cleavage peptides obtained from normal bone were similar to those reported by Cheung *et al.* (1981). The labelled peptides from OI bone also showed a similar pattern although there was more variability in the number and relative intensity of the spots (Fig. 4).

DISCUSSION

Quantitative and qualitative abnormalities of collagen were observed in fibroblast cultures and tissues from 17 consecutive cases of lethal perinatal OI. In every case abnormal type I collagen was produced and in most cases there was a significant reduction in the collagen content of the dermis and bone.

The reduced amount of tissue collagen may result from decreased production, increased degradation or decreased secretion of collagen by the fibroblasts and osteoblasts. Collagen production was not determined in the present study but it has been shown to be reduced in other cases of OI (Barsh *et al.*, 1982; Rowe *et al.*, 1985). Direct evidence was obtained of decreased secretion of procollagen from fibroblasts. Fibroblasts from OI 26

and OI 48 were unable to secrete the abnormal type I procollagen *in vitro* and this was also likely *in vivo*, as the tissues contained only normal type I collagen. In other apparently heterozygous cases (OI 30, 31 and 35) the abnormal type I procollagens were secreted more slowly than their normal counterparts.

Previous studies have shown increased intracellular degradation of collagen in seven of the cases included in the present study (Bateman *et al.*, 1984). It is likely that the abnormal collagen retained within the cells contained structural defects that made it more susceptible to degradation (Prockop, 1984). Increased extracellular degradation may also have occurred *in vivo* as the proportion of collagen that could be extracted from the dermis with acetic acid was reduced. This collagen fraction has been reported to contain recently synthesized collagen (Jackson & Bentley, 1960). As most of the extracted collagen was in the pepsin-soluble fraction it is likely that the dermal collagen became more resistant to degradation once it was incorporated into the collagen fibres of the extracellular matrix.

In most cases of OI there was a good correlation between the type I collagen abnormalities observed *in vitro* and *in vivo*. Cultured fibroblasts that produced overmodified type I collagen chains and CNBr-cleavage peptides also showed these abnormalities *in vivo*. The tissues contained increased ratios of hydroxylysine: hydroxyproline. The hydroxylysine content was also increased in α1(I)-chains from dermis and bone and in α2(I)-chains from bone. The higher content of hydroxylysine in the α1(I)- and α2(I)-chains from bone may be due, in part, to the fracture repair process, as the hydroxylation of lysine is increased in healing tissues (Kirsch *et al.*, 1983). The glycosylation of hydroxylysine was not determined in the present study but our previous studies, as well as studies by others, have shown that increased formation of hydroxylysine is accompanied by increased glycosylation (Bateman *et al.*, 1984; Kirsch *et al.*, 1983; Trelstad *et al.*, 1977).

The increase in lysine hydroxylation was restricted to type I collagen, and in some babies to only a proportion of the type I collagen molecules, suggesting that increased activity of lysyl hydroxylase was not the cause of the overmodification. An alternative explanation, as proposed by Bateman *et al.* (1984) and Bonadio *et al.* (1985), is that type I procollagen molecules are overmodified in OI because of structural defects of the pro α-chains which delay the initiation and propagation of the triple helix and so increase the time available for enzymatic modification of lysine residues. They also proposed that the distribution of these overmodified lysine residues along the chains could be used to localize the site of the structural defect.

We localized the structural defects, from the distribution of the overmodified peptides, to three regions of the α1(I)-chain. In one group of babies we localized the structural defects to the *C*-terminal propeptides as all of the CNBr peptides in the helical region of the α-chains were overmodified. In another group, the overmodifications involved the α1(I) CB8, CB3 and CB7 peptides but not the *C*-terminal CB6 peptide. In this group the structural defects were probably in the CB7 peptide. This proposal is supported by our observation that one baby (OI 51) from this group had a charge mutation in the CB7 peptide. In the third group only the *N*-terminal α1(I) CB8 and CB3 peptides were overmodified, which suggested that the defects were in the *C*-terminal part of this region of the chain. This proposal is also supported by our finding of a charge mutation of the α1(I) CB8 peptide in one of these babies (OI 24).

In OI 30, 31 and 35 there was an apparent discrepancy between the type I collagen abnormalities observed *in vitro* and *in vivo*. Fibroblasts from these babies produced a mixture of normal and abnormal type I procollagens and the tissues contained only the apparently abnormal type I collagen. The reasons for this discrepancy were not determined but it may be due to differences between the extent of lysine hydroxylation *in vitro* and *in vivo*.

Cultured fibroblasts from OI 26 and OI 48 also produced a mixture of normal and abnormal type I collagen molecules. However, the tissues from these babies, in contrast with OI 30, 31 and 35, contained only normal type I collagen molecules. The tissues from OI 26 contained about a fifth of the normal amount of collagen. The severity of the type I collagen deficiency in this baby is consistent with the 'protein suicide' model proposed by Prockop (1984). According to this model half of the chains produced from the normal α1(I)-allele would be wasted in type I procollagen molecules which also contain a mutant pro α1(I)-chain. As a consequence, the proportion of normal type I procollagen molecules produced would be reduced to a quarter as three-quarters of the molecules would contain either one or two mutant pro α1(I)-chains. If the abnormal chains were degraded then only one-quarter of the normal amount of collagen would be available to form the extracellular matrix, and this reduction is similar to that observed in the tissues of OI 26. However, we do not have any direct evidence of a primary structural defect of the abnormal pro α1(I)-chain in this baby although it is likely, because of the overmodification of all of its CNBr peptides, to reside in the *C*-terminal propeptide.

We found that type III collagen was qualitatively normal but quantitatively abnormal in OI dermis and bone. There was a reduced amount of type III collagen in dermis and there was a positive correlation betwen the concentrations of type I and III collagens in this tissue. The explanation for this finding was not determined but it may be due to secondary effects of the type I collagen abnormalities on type III collagen production, transport, secretion or matrix formation.

Our finding of type III collagen in all OI bone samples and its absence from normal bone agrees with the findings of Pope *et al.* (1980). We localized the type III collagen to the uncalcified matrix of OI bone. It is likely that the type III collagen was produced as part of the fracture repair process. The absence of type III collagen from the calcified matrix indicated that this collagen was not able to compensate for the reduced amount of type I collagen in the mineralized matrix.

The proportion of type V collagen in extracts of OI bone was increased, in agreement with the findings of Pope *et al.* (1980). It was present in the calcified and uncalcified matrix. As this form of collagen is often found around cells, it is likely that the increased proportion of this collagen was due to the increased number of osteoblasts that have been observed in OI bone (Ornoy & Kim, 1977). Type V collagen was present in only small amounts in normal and OI dermis. As a result, it is unlikely that there was a primary abnormality of this collagen in our patients.

The altered solubility pattern of OI dermal collagen was not due to an inability to form cross-linkages as the extracted collagens contained apparently normal amounts of cross-linked chains. The CNBr-cleavage peptides also contained reduced amounts of the *C*-terminal α1(I) CB6 and α1(III) CB9 peptides that are normally consumed in forming intermolecular cross-linkages (Cheung *et al.*, 1981).

Some abnormalities of cross-linking were observed in OI bone. The incorporation of NaB^3H_4 into reducible cross-linkages and aldehydes of OI bone was increased, as observed previously in milder forms of OI (Fujii & Tanzer, 1977). This finding may be due to delayed maturation of cross-linkages of OI bone collagen and may reflect diminished stability of the collagen during bone development (Fujii & Tanzer, 1977). Two-dimensional electrophoretic 'maps' of CNBr-cleavage peptides from reduced OI bone contained most of the labelled peptides observed in normal bone by Cheung *et al.* (1981). By reference to their normal 'maps' we concluded that the cross-linked peptides in OI bone included the α1(I) CB0,1, CB5, CB6 and α2CB3.5 peptides. These peptides contain the normal sites of donor aldehyde and recipient lysine or hydroxylysine residues involved in the formation of covalent cross-linkages. There were, however, some differences in the relative proportions of the labelled peptides in the OI bone.

We found that the total number of lysine and hydroxylysine residues was reduced in α1(I)-chains purified from OI bone but not in α1(I)-chains from dermis or α2(I)-chains from dermis and bone. Petrovic & Miller (1984) also observed similar changes in OI dermal α1(I)- and α2(I)-chains as well as increased amounts of α-aminoadipic acid residues in these chains. They proposed that helical lysine and hydroxylysine residues may have been oxidatively deaminated to reactive aldehydes in OI tissues. Petrovic & Miller (1984) also observed similar changes in type III and V collagens from dermis but the total content of hydroxylysine and lysine in dermal type III collagen was normal in the present study.

This is the first report in which collagen abnormalities have been studied *in vitro* and *in vivo* from a large consecutive series of babies with lethal perinatal OI. The severity of the disease was related to both quantitative and qualitative abnormalities of the collagens in the tissues. The level of post-translational modification of lysine was increased in the abnormal type I collagen and we propose that this is due to underlying structural defects of the type I collagen chains. In two cases charge mutations were observed in the α1(I)-chain and these abnormalities probably represent the mutations accounting for the disease in these children. Type III and V collagens did not appear to compensate for the deficiency of type I collagen in the tissues examined.

We thank Professor D. Danks, Dr. A. Bankier, Dr. P. Campbell and Professor D. Sillence for their assistance. The work was supported by grants from the National Health and Medical Research Council of Australia, the Utah Foundation, the University of Melbourne and the Royal Children's Hospital Research Foundation.

REFERENCES

Banes, A. J., Yamauchi, M. & Mechanic, G. L. (1983) Biochem. Biophys. Res. Commun. **113**, 975–981
Barsh, G. S., David, K. E. & Byers, P. H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3838–3842
Bateman, J. F., Mascara, T., Chan, D. & Cole, W. G. (1984) Biochem. J. **217**, 103–115
Bateman, J. F., Mascara, T., Chan, D. & Cole, W. G. (1986) Anal. Biochem. **154**, 338–344
Bonadio, J., Holbrook, K. A., Gelinas, R. E., Jacob, J. & Byers, P. H. (1985) J. Biol. Chem. **260**, 1734–1742
Bonner, W. M. & Laskey, R. A. (1974) Eur. J. Biochem. **46**, 83–88
Burgeson, R. E., El Adli, F. A., Kaitila, I. I. & Hollister, D. W. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2579–2583
Chan, D. & Cole, W. G. (1984) Anal. Biochem. **139**, 322–328
Chandrarajan, J. (1978) Biochem. Biophys. Res. Commun. **83**, 180–186
Cheung, D. T., Benya, P. D., Hofer, D. F. & Nimni, M. E. (1981) Collagen Rel. Res. **1**, 247–256
Cheung, D. T., DiCesare, P., Benya, P. D., Libaw, E. & Nimni, M. E. (1983) J. Biol. Chem. **258**, 7774–7778
Cole, W. G. & Chan, D. (1981) Biochem. J. **197**, 377–383
Cole, W. G., Chan, D., Hickey, A. J. & Wilcken, D. E. L. (1984) Biochem. J. **219**, 451–460
Epstein, E. H., Jr. (1974) J. Biol. Chem. **249**, 3225–3231
Fujii, K. & Tanzer, M. L. (1977) Clin. Orthop. Rel. Res. **124**, 271–277
Gollwitzer, R., Timpl, R., Becker, U. & Furthmayr, H. (1972) Eur. J. Biochem. **28**, 497–506
Jackson, D. S. & Bentley, J. P. (1960) J. Biophys. Biochem. Cytol. **7**, 37–42
Jamall, I. S., Finelli, V. N. & Que Hee, S. S. (1981) Anal. Biochem. **12**, 70–75
Kirsch, E., Glanville, R. W., Krieg, T. & Müller, P. (1983) Biochem. J. **211**, 599–603
Kuboki, Y., Tsuzaki, M., Sasaki, S., Liu, C. F. & Mechanic, G. L. (1981) Biochem. Biophys. Res. Commun. **102**, 119–126
Light, N. D. & Bailey, A. J. (1979) FEBS Lett. **97**, 183–188
Ornoy, A. & Kim, O. J. (1977) Isr. J. Med. Sci. **13**, 26–32
Petrovic, O. M. & Miller, E. J. (1984) J. Clin. Invest. **73**, 1569–1575
Piez, K. A., Weiss, E. & Lewis, M. S. (1960) J. Biol. Chem. **235**, 1987–1991
Pope, F. M., Nicholls, A. C., Eggleton, C., Narcissi, P., Hey, E. & Parkin, J. M. (1980) J. Clin. Pathol. **33**, 534–538
Prockop, D. J. (1984) Am. J. Human Genet. **36**, 499–505
Prockop, D. J. & Kivirikko, K. I. (1984) New Engl. J. Med. **311**, 376–384
Rowe, D. W., Shapiro, J. R., Poirer, M. & Schlesinger, S. (1985) J. Clin. Invest. **76**, 604–611
Scott, P. G. & Veis, A. (1976) Connect. Tissue Res. **4**, 107–116
Sillence, D. O., Senn, A. & Danks, D. M. (1979) J. Med. Genet. **16**, 101–116
Sykes, B., Puddle, B., Francis, M. & Smith, R. (1976) Biochem. Biophys. Res. Commun. **72**, 1472–1480
Trelstad, R. L., Rubin, D. & Gross, J. (1977) Lab. Invest. **36**, 501–508
van der Rest, M. & Fietzek, P. P. (1982) Eur. J. Biochem. **125**, 491–496

Received 21 April 1986/7 July 1986; accepted 20 August 1986