Biochem. J. (1979) **182**, 337–345
Printed in Great Britain

Biosynthesis of Skin Collagens in Normal and Diabetic Mice

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(*Received 5 January* 1979)

Synthesis of collagens *in vitro* was studied on minced mouse skins incubated with [^{3}H]-proline in organ-culture conditions. A comparative study was carried out on genetically diabetic mice (KK strain) and control mice (Swiss strain). After incubation, neutral-salt-soluble and acid-soluble collagens were extracted. The insoluble dermis was digested by pepsin and type I and type III collagens separated by differential precipitation in neutral salt solutions. Type I and type III collagens were characterized by ion-exchange and molecular-sieve chromatography, amino acid analysis and by the characterization of CNBr peptides. In diabetic-mouse skin, the relative proportion of type III collagen was significantly higher than in control-mouse skin. The incorporation of radioactively labelled proline into hydroxyproline of type III collagen was significantly faster in diabetic-mouse skin than in control-mouse skin. No significant modifications in the total collagen content of the skin or of their rates of synthesis were observed between the two strains. Alteration in the ratio of type III to type I collagen in the diabetic-mouse skin can be interpreted as a sign of alteration of the regulation of collagen biosynthesis and may be related to the structural alterations observed in the diabetic intercellular matrix.

It is now well recognized that diabetes mellitus is accompanied by defects in various connective and vascular tissues (Berenson *et al.*, 1972; Ramamurthy *et al.*, 1972; Kern *et al.*, 1972, 1976*a*; Spiro, 1973; Kefalides, 1974; Francis *et al.*, 1974; Westberg, 1976). Special attention has been focused on capillary basement-membrane thickening and biochemical modifications of these structures in diabetes with controversial results (Spiro, 1973; Kefalides, 1974; Westberg, 1976; Beisswenger, 1976; Ristelli *et al.*, 1976).

We have postulated that alterations of collagen and/or glycoprotein components of the basement membranes would be only one aspect of a general disturbance of the regulation of intercellular-matrix biosynthesis in diabetes (Robert, 1971; Kern *et al.*, 1972). Several authors and ourselves have shown qualitative and quantitative alterations at the levels of collagens (Berenson *et al.*, 1972; Ramamurthy *et al.*, 1972; Francis *et al.*, 1974; Kern *et al.*, 1976*a*), glycosaminoglycans and glycoproteins (Berenson *et al.*, 1972; Kern *et al.*, 1972; Malathy & Kurup, 1972; Robert *et al.*, 1972; Telner & Kalant, 1974; Kern *et al.*, 1976*b*) in several connective tissues and in various types of diabetes.

The diabetic KK mice are of particular interest for the study of the hereditary aspects of the disease. This strain of mice exhibits human-like diabetic vasculopathy (Kern *et al.*, 1972; Opperman *et al.*, 1973; Duhault *et al.*, 1974). Metabolic disorders of KK mice have been described as chemical diabetes showing impaired tolerance to glucose (without hyperglycaemia in the fed state), marked hyperinsulinaemia (Opperman *et al.*, 1973, 1975; Duhault *et al.*, 1974; Nakamura & Yamada, 1967) with a decrease in insulin binding to liver plasma membranes (Kern *et al.*, 1975).

We had previously found connective-tissue alterations in the conjunctiva of these diabetic mice, including thickening of the capillary basement membranes accompanied by the appearance of collagen-like cross-striated fibrils in the width of the basement membranes, increase of the incorporation of [^{14}C]proline in polymeric collagen and a decrease in the sulphated-glycosaminoglycan content of the tissue (Kern *et al.*, 1972, 1976*b*).

Skin was reported to contain two species of collagens, types I and III (Miller *et al.*, 1971; Chung & Miller, 1974; Epstein, 1974; Shuttleworth & Forrest, 1975; Meigel *et al.*, 1974; Pope *et al.*, 1975; Pentinnen *et al.*, 1975; Bailey *et al.*, 1975; Miller, 1976).

In the present paper, we report analytical and metabolic studies of the skin collagens of control and

diabetic mice. Modifications in the relative rates of synthesis and in the ratio of type I and type III collagens are described in diabetic animals.

Materials and Methods

Animal used

Non-obese genetically determined spontaneously diabetic KK mice were obtained from the laboratory of Professor J. P. Levy, Service d'Hématologie, Hôpital Cochin, 75014 Paris, France, who received them directly from Hoechst (Japan).

In the present study all mice were males (7–9 weeks old) raised from the FS generation derived from the initial stock. Age-matched male Swiss albino (SW) mice, kept under identical laboratory conditions, served as controls and were fed Purina rat chow *ad libitum*.

Physiological parameters (blood sugar, plasma insulin) were determined by standard methods (Kern *et al.*, 1972).

Preparations of skin for incubation in vitro

The animals were killed by decapitation and shaved. The dorsal skin was removed, and dissected free of subcutaneous muscle and fat. The weighed tissue was finely cut into approx. 1 mm^2 pieces in sterile conditions.

Incubations were carried out in 20ml flasks for short-period incubations (up to 4h) or in 5cm diameter Petri dishes for longer periods. The amount of tissue (150mg wet wt. per flask or Petri dish) varied maximally by $\pm 10\%$ and was incubated in 5ml of Eagle's minimum essential medium in Earle's salt buffer (MEM buffer), supplemented by 10% foetal calf serum, 100μg of streptomycin/ml, 100μg of penicillin/ml and 100 units of mycostatin/ml (all products obtained from Gibco, Paisley, Scotland, U.K.).

After a preincubation period in this medium (30min), 10μCi of L-[3,4-^{3}H]proline/ml (sp. radioactivity 55Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the medium.

Incubations were carried out at 37°C in a metabolic shaker for the flasks (up to 4h of incubation) and in a 37°C incubator gassed with air/CO_2 (19:1, v/v) as the atmosphere for the Petri dishes. The incubation was terminated by cooling the mixture quickly to 0°C. The medium was discarded and the tissue washed at 4°C with a large excess of MEM buffer containing 1% proline.

Extraction procedure

The extraction procedure used was essentially the one recommended by Chung & Miller (1974).

All subsequent manipulations were carried out at 4°C. The minced skins were ground in 10 vol. of 1M-NaCl, 0.05M-Tris/HCl buffer, pH7.4, and homogenized 5 times for 10s in an Ultra-Turrax homogenizer. The extraction procedure was repeated twice. Collagen was salted out of the pooled extracts by adding NaCl to the solution to 17%. The precipitated collagen was redissolved in 0.1M-acetic acid dialysed against the Tris buffer, and reprecipitated by adding NaCl to 17%. This precipitate was solubilized in 0.1M-acetic acid, dialysed against 0.1M-acetic acid and was referred to as neutral-salt-soluble collagen. Because of the low amount of material in this fraction, no attempt was made to separate procollagen or collagen type I and type III in this fraction (Byers *et al.*, 1974; Anesey *et al.*, 1975; Lenaers & Lapière, 1975).

To remove acid-soluble collagen, the 1M-NaCl-extracted skin residue was extracted 3 times overnight with 0.5M-acetic acid. Pooled extracts were dialysed against water and the precipitate formed was collected by centrifugation at 20000*g* for 30min, redissolved in 0.5M-acetic acid and freeze-dried. The remaining tissue was solubilized by pepsin treatment as described by Chung & Miller (1974) and Barnes *et al.* (1976).

Briefly, pepsin [EC 3.4.23.1; Worthington Biochemicals, Freehold, NJ, U.S.A.; twice recrystallized; 2700units as defined by Anson (1938)/mg] was added to a suspension of the insoluble residue in 0.5M-acetic acid with a pepsin/collagen ratio of 1:10. The mixture was stirred for 24h at 4°C and centrifuged at 30000*g* for 1h at 4°C. This digestion step was repeated once more with additional pepsin. The pepsin-insoluble residue was analysed for the presence of hydroxyproline. The pepsin-treated material was precipitated by addition of NaCl up to a concentration of 0.9M. After centrifugation the precipitate was suspended by stirring in 1.0M-NaCl, pH7.5, dialysed against the same solution, and the resulting suspension was centrifuged (30000*g* for 1h).

The pellet containing the 1M-NaCl-insoluble fraction was solubilized in 0.5M-acetic acid and freeze-dried (Barnes *et al.*, 1976). The supernatant was subjected to fractional precipitation with NaCl to permit the separation of collagen type I and type III (Chung & Miller, 1974). By this fractionation, the collagen was divided in fractions precipitating at pH7.5 at 1.5M-NaCl and 2.5M-NaCl.

These fractions, as well as the 1M-NaCl-insoluble fraction, were solubilized in 0.5M-acetic acid and freeze-dried. For each fraction, portions were analysed for hydroxyproline before freeze-drying to control losses during further treatments.

Ion-exchange chromatography

The denatured-collagen samples were chromatographed at 42°C on a column (1cm×12cm) of carboxymethyl-cellulose (Whatman CM52), which was equilibrated with a 0.02M-sodium acetate buffer at pH4.8 containing 1.0M-urea (freshly deionized). Elution was carried out with a linear gradient from

0 to 0.12M-NaCl dissolved in the starting buffer (Herbage *et al.*, 1977).

Molecular-sieve chromatography

Chromatograms were obtained on a column (1.6cm×100cm) of Bio-Gel A-15m (200–400 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) equilibrated and eluted with 0.1M-sodium phosphate buffer, pH7.4, containing 0.1% sodium dodecyl sulphate at a flow rate of 6.5ml/h. Collagen samples were dissolved and dialysed against the same buffer containing 1% sodium dodecyl sulphate, heated to 45°C for 15min and applied to the column (Mahieu & Winand, 1973).

Disc electrophoresis

Electrophoresis in sodium dodecyl sulphate/polyacrylamide gel was performed as described by Furthmayer & Timpl (1971). In some cases, samples were reduced before electrophoresis with 2-mercaptoethanol or dithiothreitol (Fuji & Kuhn, 1975).

After staining with Coomassie Blue R 250 (Scott & Veis, 1976) the gels were scanned at 560nm on a spectrophotometer fitted with a linear transporter. Peak areas were determined by planimetry.

Amino acid analysis

For amino acid analysis on a Technicon analyser, samples were oxidized by performic acid to determine cysteine content as cysteic acid and hydrolysed in 6M-HCl for 24h at 105°C under N_2.

Digestion with CNBr

A sample of collagen was dissolved in deaerated formic acid (70%, v/v) at a concentration of 6–8mg/ml (Scott & Veis, 1976). The CNBr concentration (12mg/ml) was the same for all digestions.

The reaction (for 4h at 26°C) was terminated by 10-fold dilution with water and freeze-drying. The peptides were redissolved in 0.1M-acetic acid and freeze-dried again.

The peptides produced from CNBr treatment (CNBr peptides) were studied on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the procedure of Furthmayer & Timpl (1971) modified by Scott & Veis (1976), by using 7.5% acrylamide-gel concentrations.

Standard individual CNBr peptides from type I calf skin collagen were obtained by CM-cellulose and Bio-Gel chromatography (Herbage *et al.*, 1977).

The identification of the CNBr peptides from type III mouse skin collagen was made by comparison with a CNBr digest of purified type III collagen from calf skin isolated as previously described (Fuji & Kuhn, 1975).

Assay of proline and hydroxyproline radioactivity

Specific radioactivity of [^{3}H]proline and hydroxy-[^{3}H]proline were determined in the HCl hydrolysate by the method of Rojkind & Gonzales (1974).

DNA

This was determined by the method of Burton (1956) with portions of dried tissues.

Results

Physiological parameters

The young KK mice studied (8 weeks old) showed a moderate, but significantly increased, plasma insulin concentration. Other parameters were not significantly different from control values (Table 1).

Characterization of collagen fractions

Total collagen present in whole skin was not significantly different in the two strains: SW, 7.5±0.8 (mean±S.E.M.) μg of hydroxyproline/μg of DNA; KK, 8.4±0.9 (mean±S.E.M.) μg of hydroxyproline/μg of DNA (Table 2). The yield from the extraction procedure is indicated in Table 2. The total quantity of collagen recovered in the different fractions represented more than 78% of the initial amount of dermal collagen.

Table 1. *Physiological parameters in 8 week-old KK and control mice*

The values are determined on fed mice and are the means (±S.E.M.) for ten determinations.

Mice	Blood sugar (mg%)	Plasma insulin (μ-units/ml)	Body wt. (g)
Swiss	140±20	24±4	30±3
KK	150±20	43±6*	25±4

* $P<0.01$.

Table 2. *Distribution of collagens in different fractions of Swiss and diabetic-KK mouse skin*

For details of the extraction procedure see the Materials and Methods section. The results are expressed as percentages of peptide-bound hydroxyproline recovered in each fraction, compared with the total amount of skin hydroxyproline. All values are the means for three determinations in each of six extraction procedures described in the Materials and Methods section. S.E.M. is indicated for total collagen only.

	Distribution of collagen (%)	
Strain of mouse ...	SW	KK
Neutral-salt-soluble collagen	0.6	0.7
Acid-soluble collagen	3.2	3.6
Total pepsin-soluble collagen	76.4	73.7
Hydroxyproline recovered (%)	80.2	78.0
Total collagen (μg of hydroxyproline/μg of DNA)	7.8±0.9	8.4±0.9

The pepsin-insoluble residue contained less than 1% of the total skin hydroxyproline and can probably be attributed to skin elastin.

Pepsin-soluble collagen that represented more than 90% of total recovered collagen was subjected to fractional precipitation with NaCl. A minor fraction (the 1.5M-NaCl-precipitated fraction) that contained approx. 90% of type III collagen as estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (results not shown) was not extensively analysed.

The major fractions obtained were examined on ion-exchange and Agarose-gel chromatography. Fig. 1 shows the CM-cellulose elution pattern of the main fractions after denaturation.

The 1M-NaCl-insoluble fraction was eluted as a single component in a position coincident with α_2-chains (Fig. 1*a*). This elution pattern corresponds to the α_1 (III)-chain in rodents (Byers *et al.*, 1974). The 2.5M-NaCl-precipitated fraction exhibited the typical features of type I collagen (Fig. 1*b*).

Sodium dodecyl sulphate/agarose chromatograms of the same fraction (Fig. 2) confirmed the presence of type III collagen in the 1M-NaCl-insoluble fraction (Fig. 2*b*).

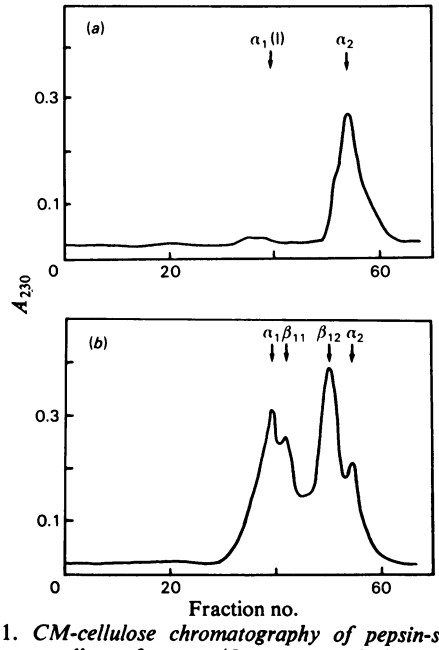

Fig. 1. *CM-cellulose chromatography of pepsin-soluble collagen fractions (Swiss mouse skin)*

(*a*) 1.0M-NaCl-insoluble fraction; (*b*) 2.5M-NaCl-precipitated fraction. The ion-exchange chromatography was performed as described in the Materials and Methods section. The arrows show the elution position of subunits from calf skin acid-soluble collagen as indicated.

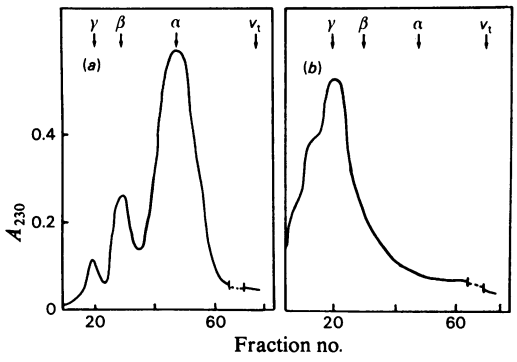

Fig. 2. *Gel filtration on sodium dodecyl sulphate/agarose Bio-Gel A-15m (200–400 mesh) of pepsin-soluble collagen fraction (Swiss-mouse skin)*

Sample treatment and conditions of chromatography are described in the text. The agarose column was calibrated with α, β and γ components of purified acid-soluble collagen of mouse skin as indicated. V_t is the total volume, as determined by the elution position of ^3H_2O. The chromatogram of 2.5M-NaCl-precipitated fraction is shown in (*a*). The chromatogram of the 1.0M-NaCl-insoluble fraction is shown in (*b*).

In non-reducing conditions, the majority of the material contained in these fractions was eluted at the level of γ components and in some cases at positions corresponding to even higher polymeric levels (Fig. 2). After reduction with 2-mercaptoethanol, they were quantitatively recovered as α-chains (mol.wt. 95000) (results not shown). The 2.5M-NaCl-precipitated fractions exhibited a chromatographic pattern similar to the one obtained with standard (acid-soluble rat skin) type I collagen (Fig. 2*a*).

Essentially similar results were obtained with diabetic-mouse skin. Amino acid analysis of these fractions revealed compositions similar to those reported previously (Chung & Miller, 1974; Epstein, 1974; Fuji & Kuhn, 1975; Table 3). The presence of cysteine residues in the 1M-NaCl-insoluble and in the 1.5M-NaCl-precipitated fractions can be observed and also a higher hydroxyproline/proline ratio (1.15) than in the 2.5M-NaCl-precipitated fraction (0.80). This ratio was slightly decreased in the 1.5M-NaCl-precipitated fraction, compared with the 1M-NaCl-insoluble fraction.

These results confirm the slight contamination of the minor 1.5M-NaCl fraction by type I collagen. In some cases, amino acid analysis has been performed on α_1-chains, isolated from CM-cellulose-chromatographed material, of 1M-NaCl-insoluble and 2.5M-NaCl-precipitated fractions. Essentially identical results were obtained with these isolated α-chains as with the whole fractions.

Fig. 3 shows sodium dodecyl sulphate/polyacryl-

Table 3. *Amino acid composition of pepsin-soluble collagen fractions (SW mouse skin)*

Amino acid	Amino acid content (residues/1000 residues)		
Fraction ...	1M-NaCl-insoluble	1.5M-NaCl-precipitated	2.5M-NaCl-precipitated
Hyp	113	100	95
Asp	56	56	45
Thr	13	15	18
Ser	43	44	42
Glu	78	78	76
Pro	98	102	120
Gly	358	346	329
Ala	82	90	114
½CyS	2	2	—
Val	17	18	21
Met	8	7	8
Ile	15	14	7
Leu	16	17	19
Tyr	2	2	2
Phe	6	9	13
Hyl	5	5	4
Lys	34	33	30
His	11	9	5
Arg	43	45	48

amide-gel electrophoresis of CNBr peptides from the 2.5M-NaCl-precipitated fraction (Fig. 3*a*) and from the 1M-NaCl-insoluble fraction (Fig. 3*b*).

A tentative assignment of certain peptides could be made, on the basis of the relative mobility of known CNBr peptides (shown by arrows on Fig. 3).

The CNBr peptides shown in Fig. 3(*a*) exhibited the typical pattern of type I collagen CNBr peptides (Scott & Veis, 1976). The absence of α_1(III)-chain CNBr8 peptide specific for type III collagen is noteworthy.

In Fig. 3(*b*), the absence of a peak corresponding to α_1(I)-chain CNBr6 peptide and the general pattern of the CNBr peptides are in good agreement with the results previously described for type III collagen CNBr peptides in the non-reduced form (Scott & Veis, 1976).

Similar results were obtained with diabetic-mouse skin. However, the percentage of each fraction varied in the two strains (Table 4). These results clearly indicate a higher percentage of type III collagen in diabetic-KK-mouse skin. This finding is noteworthy because of the good recovery of total pepsin-soluble collagen in each fraction; the total amount recovered represents more than 85% of the initial pepsin-soluble collagen. These results have been confirmed by analysis of the CNBr-peptide patterns of the total pepsin digests of the KK- and control-mouse skins.

The clear separation of α_1(III)-chain CNBr8 peptide from other CNBr peptides (Figs. 3 and 4) enables a good comparison of the relative proportion of type III collagen in both types of mouse skin.

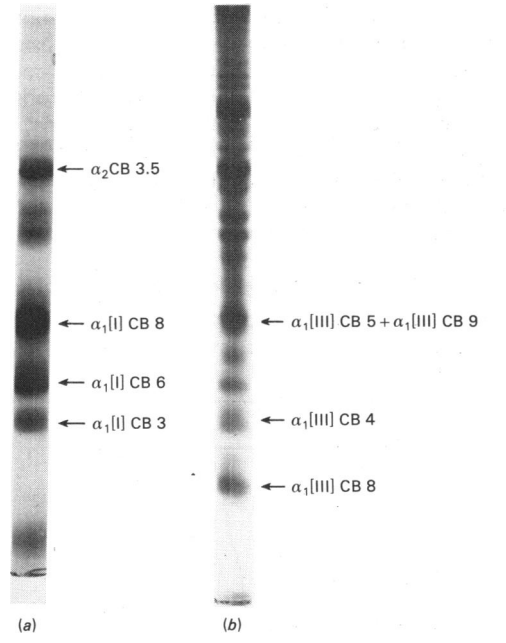

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of CNBr peptides of type I collagen (a) and of type III collagen (b) from Swiss mouse skin*

The arrows indicate the relative mobility of standard CNBr peptides calculated as described in the text. In the abbreviated notation for the standard CNBr peptides, CB indicates CNBr, α_1 and α_2 indicate the subunits of collagen, and [I] and [III] indicate the type of collagen.

Table 4. *Distribution of collagens in pepsin-soluble fractions of Swiss and diabetic-KK mouse skin*

For details of the extraction procedure see the Materials and Methods section. The results are expressed as percentages of the total peptide-bound hydroxyproline recovered. All values are means ±S.E.M. for three determinations in each of six extraction procedures.

Fraction	Distribution of collagens (% of total peptide-bound hydroxyproline recovered)	
Strain of mouse ...	SW	KK
1M-NaCl-insoluble (type III)	18±2	28±3*
1.5M-NaCl-precipitate (type III)	4±0.5	7±1*
2.5M-NaCl-precipitate (type I)	78±6	65±6

* A significant difference between SW and KK mice ($P<0.01$)

This CNBr peptide estimated by planimetry represents 1.7% of the total peptide material observed on the gel for control skin and 3.1% for KK-mouse skin respectively (Fig. 4). In purified type III collagen fractions obtained from both mouse strains, this CNBr peptide represents 10% of the total peptides. These data lead to the conclusion that approx. 17% of the insoluble skin collagen is of type III in control mice and 31% in KK mice.

Incorporation of [^{3}H]proline in the collagen fractions

Radioactive-isotope kinetic studies of these fractions confirmed the observed differences.

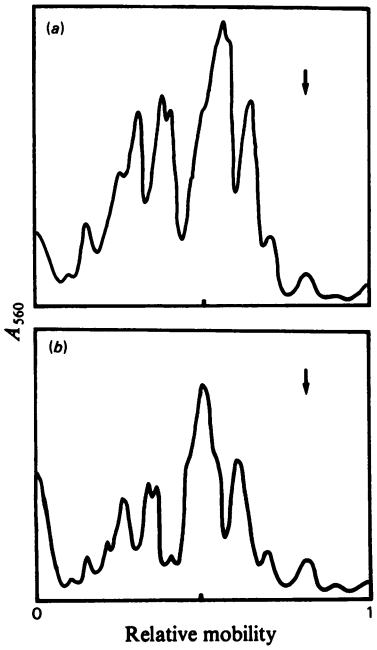

Fig. 4. *Scan of CNBr peptides from total pepsin digests of KK-mouse skin (a) and control-mouse skin (b)*
The arrows indicate the relative mobility of α_1(III) CNBr8 peptide.

To study incorporation of [^{3}H]proline in type III collagen, we have focused our attention on the 1 M-NaCl-insoluble fraction, which does not contain

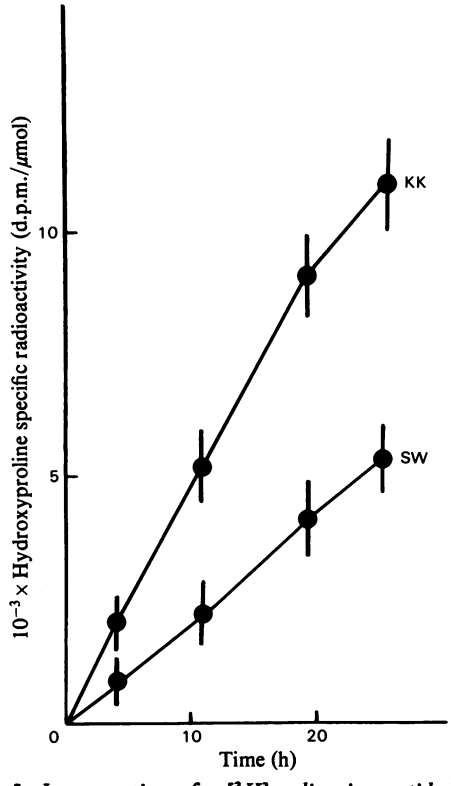

Fig. 5. *Incorporation of L-[^{3}H]proline in peptide-bound hydroxy[^{3}H]proline of type III collagen (1.0 M-NaCl-insoluble fraction) from control- and diabetic-mouse skins*
Tissues were incubated for various time intervals and sequentially extracted as described in the text. The 1.0 M-NaCl fraction characterized as type III collagen was isolated and hydrolysed to determine specific radioactivity of hydroxyproline as described in the Materials and Methods section. Each point is the mean ± S.E.M. for three determinations in five independent series of incubations. The strain of mouse is indicated for each curve.

Table 5. *Incorporation of L-[^{3}H]proline in peptide-bound hydroxy[^{3}H]proline of type I collagen (2.5 M-NaCl-precipitated fraction) from control (Swiss) and diabetic (KK) mouse skins*
Treatment of the tissues and determinations of radioactivity are described in the legend to Fig. 3 and in the text. Results are expressed as specific radioactivity (d.p.m./μmol of hydroxyproline). Each value is the mean ± S.E.M. for three determinations in four independent series of experiments. Abbreviation: N.D., not detectable.

		Specific radioactivity (d.p.m./μmol of hydroxyproline)			
Mice	Incubation time (h) ...	4	11	15	25
Swiss		N.D.	80±10	160±20	250±30
KK		N.D.	70±10	130±10	220±30

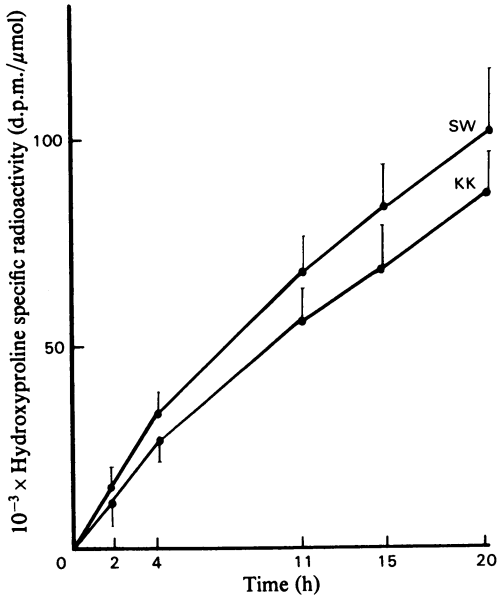

Fig. 6. *Incorporation of* L-[^3H]*proline in peptide-bound hydroxy*[^3H]*proline of neutral-salt-soluble collagens from control- and diabetic-mice skin*

Treatment of the tissues and determination of radioactivity are detailed in the legend to Fig. 3 and in the text. Each point is the mean ± S.E.M. for three determinations in four independent series of experiments. The strain of mouse is indicated for each curve.

any detectable amount of type I collagen and represents the major part of type III collagen-containing fractions. Fig. 5 indicates a linear incorporation of radioactivity into peptide-bound hydroxyproline of type III collagen for the two strains of mice. This incorporation is, however, significantly faster in the diabetic-KK-mouse skin.

We have examined incoporation of radioactivity in peptide-bound hydroxyproline of the 2.5M-NaCl-precipitated fraction that contains polymeric type I collagen (see Table 5). No significant differences were observed between the control and diabetic mice.

The newly synthesized type I collagen is present essentially in the neutral-salt-soluble collagen fraction. This fraction was reported to contain also newly synthesized type III collagen (Byers *et al.*, 1974; Anesey *et al.*, 1975; Lenaers & Lapière, 1975). Radioactivity was linearly incorporated in this fraction into peptide-bound hydroxyproline over the first 20h of incubation (Fig. 6). This result is in agreement with previous reports of collagen synthesis *in vitro* in human skin (Craig *et al.*, 1975). The results obtained showed a slight, but not significant, increase of incorporation in collagen of control mice, compared with KK mice.

Discussion

The present results demonstrate an alteration in the proportions of type I and type III collagens in diabetic-KK-mouse skin. The major pepsin-soluble collagen fractions obtained are the 1.0M-NaCl-insoluble fraction and the 2.5M-NaCl-precipitated fractions. A minor fraction precipitates at 1.5M-NaCl.

CM-cellulose and sodium dodecyl sulphate/agarose chromatograms of the 1M-NaCl-insoluble fraction indicate typical patterns of type III collagen with no detectable or very small traces of type I collagen. Amino acid analysis confirmed a composition compatible with type III collagen. The 2.5M-NaCl-precipitated fraction consists essentially of type I collagen indicated by the analytical data. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of CNBr digests of these two fractions give patterns in agreement with the expected distribution of collagen types in each fraction (Fig. 3). Similar distributions of collagen types have recently been reported in guinea-pig skin (Barnes *et al.*, 1976).

Quantitative analysis of collagen fractions revealed a good recovery of the initial amount of pepsin-soluble skin collagen (more than 80%), and also confirmed the satisfactory reproducibility of the fractionation procedure (Table 2).

The results enabled us to estimate the relative proportions of the different collagen types in control- and diabetic-mouse skins (Table 4).

The relative amount of type III collagen in control-mouse skin is in agreement with the values obtained with rat skin of approximately the same age (Klein & Chandrarajan, 1977). A significant increase in the percentage of type III collagen is found in the diabetic-mouse skin.

Another independant estimation of type III collagen was performed (Fig. 4). Analysis of the CNBr-peptide patterns of the total pepsin digests of KK- and control-mouse skin gave results very similar to those shown in Table 4.

These analytical data are confirmed by the incorporation studies (Fig. 5). There was a faster incorporation of radioactivity labelled proline in type III collagen of diabetic-mouse skin than in control-mouse skin. In addition, we observed that the ratio between the specific radioactivities of hydroxyproline in type III collagen in diabetic-mouse skin compared with control-mouse skin (1.8) correlated favourably with the ratio between the analytically determined amounts of type III collagen in diabetic- and control-mouse skins (1.6).

The other parameters obtained with the other collagen fractions did not exhibit significant differences between control and diabetic mice. It is noteworthy that the incorporation of proline in newly synthesized collagen (neutral-salt-soluble

fraction) is not significantly different in control- and diabetic-mouse skins (Fig. 6). These results indicate a modified regulation of the synthesis of the different types of collagens without an important change in the rate of total collagen synthesis.

The higher specific activity of the type III collagen in the insoluble collagen fraction of the skin could be due to a higher turnover of this type of molecule in the diabetic strain. It probably reflects an increase in the rate of conversion of freshly synthesized type III collagen to its insoluble form. An increased rate of incorporation of radioactive proline in insoluble collagen could be demonstrated in the diabetic state in KK-mouse conjunctiva (Kern *et al.*, 1972), in diabetic-human conjunctiva (Kern *et al.*, 1976*a*), in diabetic-human skin and gingiva biopsies (Robert *et al.*, 1979; Lagrue *et al.*, 1979). Similar conclusions were reached by using a different approach (Hamlin *et al.*, 1975). Further studies on the soluble form of type I and type III collagen are required to elucidate this problem.

Preliminary results on KK mice, reversed to the non-diabetic state by inbreeding in our laboratory, indicate that the type III to type I skin collagen ratio reverted also to the value found in Swiss-mouse skin (P. Kern, unpublished results). Determinations were carried out also on the skin collagens of the C57 Bl/67 strain, which gave rise by mutation to the KK-diabetic strain. The ratio of type III to type I collagen was similar to that found in the Swiss-mouse skin. The relative rates of incorporation of radioactive proline in these collagens determined as above was also similar to that found for the Swiss strain (P. Kern, unpublished work). It appears therefore that the increase in the ratio of type III collagen to type I collagen is not due to strain differences, but to the diabetic state of KK mice.

Disturbances in the intercellular matrix have been described in various types of diabetes at the level of glycosaminoglycans (Berenson *et al.*, 1972; Malathy & Kurup, 1972; Telner & Kalant, 1974; Kern *et al.*, 1976*b*), glycoproteins (Kern *et al.*, 1972, 1976*a*; Robert *et al.*, 1972), collagens (Berenson *et al.*, 1972; Ramamurthy *et al.*, 1972; Kern *et al.*, 1972, 1976*a*; Francis *et al.*, 1974) and basement membranes (Spiro, 1973; Kefalides, 1974; Westberg, 1976; Beisswenger, 1976; Ristelli *et al.*, 1976). Alterations of the cells synthesizing these macromolecules have also been demonstrated in diabetic subjects (Goldstein *et al.*, 1969; Vracko & Benditt, 1975). In previous studies on conjunctival tissue of diabetic-KK mice, we have shown an increase of proline incorporation into total polymeric collagen (Kern *et al.*, 1972). Similar results were obtained in human diabetic subjects (Kern *et al.*, 1976*a*). Other investigators also indicated an increased 'stabilization' of human diabetic skin collagen in this disease (Hamlin *et al.*, 1975). The discovery of genetically different types of collagens now justifies a more precise investigation of collagen synthesis in various tissues and in several pathological states. The present report gives more detailed information on the disturbance of collagen synthesis in diabetic-KK mice.

The electron-microscopic study of type III collagen, usually present as thin fibres (Lapière *et al.*, 1977), and its particular location in the papillary dermis (Lapière, 1976; Meigel *et al.*, 1977), suggested a possible distinction between these two types of collagens. An increased proportion of type III collagen in intercellular matrix from diabetic mice could be related to the reported ultrastructural alterations of diabetic-mouse skin (Bouissou *et al.*, 1973), and also to the appearance of cross-striated collagen-like thin fibrils in the thickened basement membranes of diabetic-mouse capillaries (Kern *et al.*, 1972, 1976*a*).

This work was supported by the Centre National de la Recherche Scientifique (Groupe de Recherches no. 40), Délégation Générale à la Recherche Scientifique et Technique (Contrat no. 76-07-0986), Institut National de la Santé et de la Recherche Médicale (Contrat no. 14-75-37), Direction de l'Enseignement Supérieur and Fondation pour la Recherche Médicale Française.

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