HUMAN SKIN COLLAGEN FROM DIFFERENT AGE GROUPS BEFORE AND AFTER COLLAGENASE DIGESTION

AN ELECTRON MICROSCOPIC STUDY*

BY

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That some alteration in collagen occurs with age has been indicated by various workers. Young rat tendon (Neuberger and others, 1951) shows a greater radioactive glycine turnover than that of adult animals. Similar findings were reported in the skin of rats (Neuberger and Slack, 1953) and rabbits (Harkness and Neuberger, 1952). Under the electron microscope the collagen fibres in newborn rat skin were shown to be narrower than in older rats (Gross, 1950). Human skin, tendon, and dura collagen was found to vary in extractability with dilute acid according to age, being more soluble below the age of one year (Banfield, 1952). The excretion of hydroxyproline in children was significantly greater than in adults (Ziff and others, 1954), indicating the presence of an increased pool of hydroxyproline peptide for collagen synthesis in the growing individual. The authors found no difference between patients with or without collagen disease. Previous chemical studies (Keech, 1954a) showed a significant difference in digestion between the skin collagen of infants and adults following incubation with collagenase, but again no difference between patients with or without collagen disease.

It appeared to be of interest to find out whether collagen from different age groups differs in its response to collagenase, and whether there is any correlation with different types of disease, particularly "collagen disease". This paper describes the effect (macroscopic and electron microscopic) of collagenase on extracted human skin collagen from

sixty individuals of all ages dying from a variety of causes. In addition, some changes in the untreated skin of non-incubated controls are included, as these may lead to a better understanding of the structure of collagen fibrils.

Materials and Methods

I. INCUBATED CONTROLS AND COLLAGEN INCUBATED WITH COLLAGENASE

Collagenase obtained from *Cl. histolyticum* was kindly supplied by Dr. J. D. MacLennan, the same batch being used throughout. It contains a very small quantity of proteinase, but is the purest preparation available, as a crystalline form has yet to be made.

Unfixed, frozen autopsy abdominal skin from the left upper quadrant was thawed, the dermis minutely dissected out and the collagen extracted by a shortened form of the method of Neuman (1949a, b), as previously described (Keech, 1954a). 20 mg. (dry weight) were incubated with collagenase (0.1 ml. of a 0.5 per cent. solution or 0.1 mg. enzyme nitrogen) and 0.05 ml. penicillin and streptomycin mixture in a total volume of 5 ml. phosphate buffer (pH 7.3) for 24 hrs at 37° C. Control tubes were treated in the same way, including the antibiotic mixture but without enzyme (Fig. 1, overleaf).

The first two cases were examined at 10 min. and $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, and 24 hrs, and showed a gradual transition to the 24-hr picture. After this standard intervals of 10 or 60 min., 3 and 24 hrs were chosen as giving representative information for all age groups. The approximate macroscopic digestion as compared with the control tube was recorded, and drops of the suspension after shaking were taken for electron microscopic examination. The drops were placed on collodion-covered 200 mesh Cu-Ni grids, allowed to dry, washed for 15 min. in de-mineralized distilled water, shadowed with palladium, and examined in the RCA Model EMU 2 A electron microscope.

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Fig. 1.—Extracted skin collagen after incubation with collagenase at 37° C. for 24 hrs. Tubes 2, 4, and 6 are controls.

Tube 1.—From a 17-year-old patient dying of lymphocytic lymphoma, showing complete digestion.

Tube 3.—From a 43-year-old patient dying of uraemia; about 50 per cent. digested.

Tube 5.—From an 18-year-old patient with congenital heart disease showing no digestion.

On the extreme right is a sample of extracted skin collagen. Before incubation each tube contained 20 mg. dry weight of substrate in 5 ml. phosphate buffer at pH 7.3.

II. UNTREATED COLLAGEN

The early stages of collagen extraction (*vide supra*) entailed homogenizing the dissected, unfixed dermis in de-mineralized distilled water in a Waring blender in the cold room for 15 min. The final temperature of the solution was 30° C. or less in each case. Drops of this suspension were examined under the electron microscope as a routine in addition to the incubated collagen-in-buffer controls already described.

Results

I. INCUBATED CONTROLS AND COLLAGEN INCUBATED WITH COLLAGENASE

Normally collagen fibrils are long, passing right across the microscopic field, and it is unusual to see the fibril ends. After homogenization in a Waring blender, scanty, blunt, or torn ends are seen (Fig. 2, opposite).

Collagen incubated in buffer without enzyme presented this unaltered appearance.

A. Macroscopic Digestion.—There was a marked difference with respect both to age and to incubation time. The collagen from infants and children digested more rapidly than that of adults (Fig. 3, overleaf).

100 per cent. digestion was considered to have

occurred when no solid collagen was visible at the bottom of the test tube (Fig. 1). At 3 hrs the average digestion per age group showed a steady decline with increase in age. At 24 hrs 80 to 90 per cent. of the collagen had disappeared from individuals under 40 years of age. Above this age there was a significant fall, an average of only 19 per cent. digesting in the same time. The readings in each group were remarkably consistent apart from the few exceptions discussed below. In eight cases, examination after 6 hrs' incubation revealed practically the same macroscopic and microscopic picture as at 3 hrs, except in one man aged 52 dying from carcinoma of the lung. About 5 per cent. of this collagen had disappeared by 3 hrs, 70 per cent. at 6 hrs, and 90 per cent. at 24 hrs, with a corresponding change in microscopic components.

EXCEPTIONS (excluded from Fig. 3, overleaf)

(a) *Absent Digestion.*—In eight individuals the collagen did not digest (Table, overleaf, and Fig. 1).

Throughout this study batches of four to eight cases were incubated together, common buffer and enzyme solutions being used for all tubes. At 24 hrs the occasional exception was prominent compared with the partially or completely digested contents of adjacent

Fig. 2.—Control picture of skin collagen from a 9-year-old boy dying from dermatomyositis, after homogenizing in de-mineralized, distilled water. Note variation in fibril size, the fibrils passing right across the field, with scanty, blunt ends produced by the Waring blender. Some amorphous material is present. $\times 14,800$

Fig. 2(a).—Collagen from same case as Fig. 2 to show true twisting of the fibrils (cf. Figs 28 and 29). $\times 7,400$.

NOTE: All the electron micrographs are shadowed with palladium.

Fig. 3.—Per cent. macroscopic digestion of abdominal skin collagen from 51 cases with and without "collagen disease" after incubation with collagenase at 37° C.

KEY TO FIGURE 3.

Graph	Age Group	No. of Cases	Incubation Time (hrs)					
			3		6		24	
			Average	Range	Average	Range	Average	Range
A	NB: EF*	12	55	20-80			85	70-95
B	1-6 mths	4	30	20-60			84	85-90
C	1-10 yrs	17	17	5-40			88	80-95
D	11-20 yrs	7	7	0-10			90	85-95
E	21-30 yrs	5	0	0			82	50-95
F	31-40 yrs	2	0	0	25	20-30	85	80-90
G	41-50 yrs	4	0	0	4	0-10	19	10-25

* NB: EF newborn: erythroblastosis.

tubes. Even though the activity of the solutions was without question, a large amount of collagenase was added to one such tube and incubated for a further 46 hrs, but the collagen remained unaltered.

(b) *Excessive Digestion*.—This occurred in the case of a 20-year-old patient with fulminating acute rheumatic carditis, and in a 52-year-old man with carcinoma of the lung who had received intravenous nitrogen mustard

for the 4 days before his death. Both these gave readings comparable to those in children under one year old.

Cases of Collagen Disease.—Included in the series forming the basis of this report were eight cases of collagen disease. These gave the following results:

Dermatomyositis.—The macroscopically normal abdominal skin from one 9-year-old patient digested normally, whereas rash-bearing axillary skin from the same patient and the normal abdominal skin from a 5-year-old did not digest at all (Table).

TABLE
EXCEPTIONS EXCLUDED FROM FIG. 3

Age (yrs)	Diagnosis	Macroscopic Digestion (24 hrs)
1½	Acute leukaemia	Nil
5	*Dermatomyositis	Nil
9	†Dermatomyositis	Nil
9½	Mental deficiency, Sepsis	Nil
13	Juvenile rheumatoid arthritis	Nil
18	Congenital heart disease, Pulmonary vascular sclerosis	Nil
43	Diabetes mellitus for 15 yrs, Kimmelstiel-Wilson's disease and glaucoma	Nil
51	Hypertension, Subarachnoid haemorrhage	Nil
20	Acute rheumatic carditis	3 hrs (60 per cent.) 24 hrs (90 per cent.)
52	Carcinoma of lung	6 hrs (70 per cent.) 24 hrs (90 per cent.)

* Abdominal skin.

† Rash-bearing axillary skin. Abdominal skin from the same case digested normally.

Juvenile Rheumatoid Arthritis.—In one patient with typical widespread deformities, who had received large doses of cortisone for 3 months before death, collagen digestion was absent (Table).

Rheumatic Heart Disease.—The excessive digestion of one fulminating case is described above. One patient with subacute bacterial endocarditis super-imposed on rheumatic heart disease and one dying of chronic rheumatic heart failure conformed both macro- and microscopically to their age groups.

Disseminated Lupus Erythematosus.—Two cases exhibited the average digestion for their age groups.

Polyarteritis Nodosa.—One case exhibited the average digestion for the age group.

B. Electron Microscopic Findings.—Collagen incubated in buffer without enzyme (controls) remained unaltered. The crystalline residue from buffer alone and collagenase in buffer (following the usual

Fig. 4.—Phosphate buffer alone after 3 hrs incubation at 37° C. $\times 28,000$.

Fig. 5.—Collagenase in buffer after 3 hrs at 37° C. There are many short fibres believed to represent the enzyme protein. $\times 24,000$.

preparation for electron microscopic examination) are shown in Figs 4 and 5. The latter contains short rods, termed "enzyme fibres", presumed to be enzyme protein and analogous to the fibrous structures arising from solutions of purified, crystallized trypsin (Gross, 1951). These can be easily distinguished from the beaded fibrils and granular

debris described below (Fig. 6, overleaf).

In collagen incubated with collagenase, three classes of structure were seen as well as minute beaded fibrils and "beads". All these elements may be present together or separately in the same grid or field, the proportions varying according to age and length of incubation.

(1) "*Standard*" *Collagenase Change*.—This denotes the alterations described by Gross (1953) in cow-hide corium collagen, and by Keech (1954b) in abdominal skin from a 73-year-old adult, *i.e.* separation of the bundle fibrils, tapering of the fibril ends, and localized narrowings in the fibre

Fig. 6.—Skin collagen from a 15-year-old patient dying of a fractured skull. Preparation incubated with collagenase for 1 hr. The enzyme fibres can be clearly distinguished from the beaded fibrils and granular debris. $\times 22,200$.

width with separation to form tactoids (short lengths of striated collagen tapered at both ends). There is no observable distortion of axial periodicity and no swelling of the fibrils (Fig. 7; also Fig. 10, overleaf).

Fig. 7.—Skin collagen from a 52-year-old man dying of carcinoma of the lung. Preparation incubated for 6 hrs with collagenase. Striated fibres showing "standard" collagenase change, some of which appear to be disintegrating into finely beaded material. This illustrates localized, through-the-bundle points of enzyme action, producing narrowings in the fibril width and tactoids. Note that there is no observable distortion of the axial periodicity and no swelling of the fibrils. $\times 12,700$.

Fig. 8.—Skin collagen from a 3-year-old child dying with pulmonary arteriolosclerosis. Preparation incubated for 3 hrs with collagenase. This shows finely striated (210 Å) fibrils mixed in with others bearing the usual 640 Å band. $\times 26,000$.

Thin, finely striated collagen fibrils (210 Å) were seen to be mixed in with larger fibrils bearing the usual 640 Å band (Figs 8 and 9).

Some fibrils bore 640 Å periods in their wider part and 210 Å in their long, narrow terminations

Fig. 9.—Skin collagen from a 7-year-old child dying from Hodgkin's disease. Preparation incubated with collagenase for 1 hr. The fibril illustrated has 640 Å banding in its wider part and 210 Å striations in its narrower termination. $\times 28,000$.

(Fig. 9). Wyckoff (1949) noted that this phenomenon occurred occasionally in the Achilles tendon in animals. Finely striated fibrils with an axial periodicity one-third of the usual period have been described in animal collagen (paramuscular connective tissue from a mature rabbit; adult dog's heart tendon) as well as in re-precipitated collagen (Vanamee and Porter, 1951; Wyckoff, 1952).

Fig. 10.—Skin collagen from a 28-year-old patient dying from coronary thrombosis. Preparation incubated with collagenase for 1 hr. Striated collagen showing “standard” collagenase change, *i.e.* separation of the bundle fibrils, tapering of the fibril ends, and localized narrowings in fibril width with separation to form tactoids. Tactoids are short lengths of striated collagen tapered at both ends. $\times 6,900$.

(2) “Moth-eaten” Fibres.—Large, very dense bundles of collagen presented a striking moth-eaten appearance on the microscope screen (Figs 11-17,

overleaf). These dense bundles were segmented, the non-opaque areas frequently showing striated fibrous “links” indicating the true origin of the structure.

Fig. 11.—Skin collagen from an 11-year-old child dying from polyarteritis nodosa. Preparation incubated with collagenase for 24 hrs. Typical "moth-eaten" fibre consisting of a dense bundle of swollen, segmented collagen, the segments being joined by fibrous "links" indicating the true origin of the structure. $\times 22,500$.

Fig. 12.—Enlargement of a striated fibrous "link" from a 9-year-old mentally deficient child. The collagen had been incubated with collagenase for 24 hrs. $\times 23,000$.

Fig. 13.—Collagen from macroscopically normal skin from a 12-year-old child dying from disseminated lupus erythematosus. Preparation incubated with collagenase for 24 hrs. "Moth-eaten" fibre apparently disintegrating into clouds of "beads".
× 15,000.

Fig 14.—Skin collagen from a 5-year-old child dying from a nasopharyngeal tumour. Preparation incubated with collagenase for 3 hrs. A group of "moth-eaten" fibres apparently disintegrating into masses of long beaded fibrils and "beads". $\times 10,000$.

Fig. 15.—Skin collagen from a 5-year-old child dying from a nasopharyngeal tumour. Preparation incubated with collagenase for 3 hrs. "Moth-eaten" fibre showing basic fibrous structure. Note several long-beaded fibrils. $\times 11,600$.

Fig. 16.—Skin collagen from a leukaemic 4-year-old child. Preparation incubated with collagenase for 3 hrs. Early stage of a "moth-eaten" fibre, where some striated fibrils still remain. $\times 20,500$.

Fig. 16 shows an early stage where some striated fibrils still remain. Fig. 17 (overleaf) shows another fibre apparently disintegrating into its constituent "beads".

Fig. 18.—Skin collagen from a newborn infant dying from erythroblastosis foetalis. Preparation incubated with collagenase for 30 min. Typical "granular degenerating" fibre. $\times 6,500$.

Fig. 17.—Skin collagen from a 2-year-old child dying from α - γ -globulinaemia and bronchopneumonia. Preparation incubated with collagenase for 3 hrs. "Moth-eaten" fibre apparently disintegrating into constituent "beads". $\times 12,500$.

Fig. 19.—Same as Fig. 18. $\times 16,500$.

(3) "*Granular Degenerating*" *Fibres*.—These were characteristically seen in child's or infant's enzyme-treated collagen, the microscopic fields being filled with thick granular bundles bearing none of the

features of striated collagen. The bundles were so thick that their granular nature could only be observed at the edge, or in thinner, flatter bundles (Figs 18 and 19).

Fig. 20.—Collagen from macroscopically normal skin from a 9 year-old child dying of dermatomyositis. Preparation incubated with collagenase for 24 hrs. Disintegrating "moth-eaten" fibres, some granular debris, "beads", and long beaded fibrils. Most of the latter are lined up in register to form a skeleton fibre. $\times 16,400$.

Fig. 21.—Skin collagen from a 5-year-old child dying from a nasopharyngeal tumour. Preparation incubated with collagenase for 3 hrs. "Moth-eaten" fibres, numerous long-beaded fibrils, and "beads". The axial periodicity of the beaded fibrils "fit in" with the banding of the two striated collagen fibrils present. $\times 18,800$.

Fig. 22.—Collagen from macroscopically normal skin from a 9-year-old child dying of dermatomyositis. Preparation incubated with collagenase for 3 hrs. Transformation of striated collagen into an early "granular degenerating" fibre. $\times 16,200$.

However, early stages were found (Fig. 22) into these well-defined "bands" or "ribbons" illustrating the conversion of striated collagen of granular material. Later in the incubation

Fig. 23.—Skin collagen from a premature infant (birth weight, 1,030 g.). Preparation incubated with collagenase for 3 hrs. Back ground network of tiny tactoids and beaded fibrils. $\times 15,600$.

process these ribbons disintegrated into granular debris and “beads”.

Fig. 24.—Skin collagen from a 24-year-old patient with subacute bacterial endocarditis. Preparation incubated with collagenase for 24 hrs. shows three structures in the same field: striated collagen, a “granular degenerating” fibre, and a “moth-eaten” fibre. $\times 18,400$.

(4) *Beaded Fibrils and “Beads”*.—Numerous small, spherical, dense structures termed “beads” were observed either lying separately or linked together to form beaded fibrils. The “beads” measured about 250 Å in diameter, whereas the

beaded fibril axial periodicity varied from 455 to 610 Å, according as the component beads were widely spaced or close together. Their number were directly proportional to length of incubation and at 24 hrs “granular degenerating” and “moth-

Fig. 25.—Skin collagen from a 27-year-old patient dying of acute pancreatitis. Preparation incubated with collagenase for 24 hrs, shows masses of “beads”, about 250 Å in diameter. $\times 35,000$.

eaten” fibres were seen disintegrating into “clouds” of “beads” (Figs 13, 14, 21, and 25). In some areas the beaded fibrils were “lined-up in register” to form skeleton fibres (Fig. 20).

Fig. 26 (overleaf) shows the proportion of these various forms of collagen at different incubation times in the different age groups. The axillary (non-abdominal) skin of one patient with dermatomyositis and of two others aged 51 and 52 years who gave atypical results were excluded (see Table). Collagen from one infant examined at 2 and 7 min. showed fibre-change too scanty to be useful. 6-hr incubations were examined in eight cases and all except one presented practically the same picture as that seen at 3 hrs. The exception was the collagen from the 52-year-old patient with carcinoma of lung (noted in the Table) which digested excessively for his age.

All the seven cases of erythroblastosis foetalis looked exactly the same, both macroscopically and microscopically, at any given time. Collagen showing “standard” collagenase change was not seen after the 10-min. examination, and moth-eaten fibres were absent throughout, except in one case at 24 hrs. Each of the five newborn infants presented identical microscopic pictures at any given time, but they all differed from those with erythroblastosis in having more collagen showing the standard change at both the 10-min. and the 3-hr incubations. This difference could be related to age; the erythroblastotics were mostly premature deliveries. The 1- to 6-month group showed a similar picture, except for a smaller amount of “granular degenerating” fibres at 3 hrs. Thus the predominant feature of the sixteen individuals aged 0-6 months was the early absence of striated collagen showing the standard enzyme change, the fields being filled with masses of granular degenerating fibres, numerous, long-beaded fibrils, and “clouds” of beads. “Moth-eaten”

fibres were not seen, except in one case, at 24 hrs.

In specimens from patients over 2 years old the bundles of striated collagen were noted to be larger and more “compact” at 3 hrs, while moth-eaten fibres figured prominently from 1 to 24 hrs. Granular

Age Group	No. of Cases	Incubation Time			
		10 min.	1 hr	3 hrs	24 hrs
Erythroblastosis foetalis	7		BF +	BF + B ++	BF ++ B +++
Newborn	5		NOT DONE	BF + B +++	BF + B +++
1—6 mths	4		BF +	BF + B +	BF + B +++
1—5 yrs	11		BF + B ++	BF + B ++	BF + B +++
6—10 yrs	9		BF + B +	BF ++ B ++	BF ++ B +++
11—15 yrs	5		BF + B +	BF + B +	B ++
16—20 yrs	5		BF + B +	BF +	BF + B ++
21—30 yrs	5		BF +	BF + B +	BF + B ++
31—40 yrs	2	NOT DONE		B ++	BF + B +
41—50 yrs	5	NOT DONE			B ++

“Standard” Change

“Moth-Eaten” Fibres

“Granular Degenerating” Fibres

BF

Beaded Fibril

B

“Beads”

Fig 26. Analysis of the abdominal skin collagen from 58 cases with and without “collagen disease” during incubation with collagenase at 37C°

elements did not appear until after 3 hrs incubation. Many beaded fibrils and beads were seen at 1 hr, the number of beads increasing with the length of incubation.

Analysis of the first two decades revealed a progressive diminution of granular degenerating fibres with a corresponding decrease in the number of “beads”. Moth-eaten fibres still figured prominently and a fair amount of striated collagen showing “standard” change remained at 3 hrs. At 24 hrs this standard change was absent in cases under the age of 15, but above this age it was seen more and more often, so that in cases over 30 years

old it was the predominant finding. The collagen from individuals in the third and fourth decades contained a diminishing number of moth-eaten fibres and granular elements, until, over the age of 40, only relatively small quantities of striated collagen showing standard change were seen, with scanty moth-eaten fibres and some beads at 24 hrs.

Thus the striated collagen showing “standard” collagenase change was seen only during early incubation in babies and young children; after 3 hrs the fields contained masses of granular, and disintegrating granular degenerating fibres accompanied by numerous beaded fibrils and “beads”. In adults the striated collagen was seen at all incubation times, dominating the picture over the age of 30. “Granular degenerating” fibres were present at 10 min. in the erythroblastotics, but did not appear until 3 hrs incubation in patients over 5 years old, and not until 24 hrs in patients over 20 years old; in patients over 40 they were completely absent. “Moth-eaten” fibres figured prominently between the ages of 1 and 20, but during the third to fifth decades their numbers steadily decreased.

The youngest individual examined was a 6- to 7-month gestation premature infant, weighing 1,030 g. A profuse background network of tiny tactoids and long beaded fibrils was present in all material prepared from tubes incubated for 3

and 24 hrs (Fig. 23). This was far more pronounced than in two older premature infants and in the full-term babies examined, where, although scattered beaded fibrils and beads were numerous, they were insufficient to form a background network. The eight cases that did not digest (Table) showed only very scanty striated collagen throughout incubation, which was either unaltered or underwent “standard” collagenase change; four presented occasional moth-eaten fibres and a few beads at 24 hrs, but granular elements were entirely absent. The two cases exhibiting excessive macroscopic digestion for their age (Table) showed an electron microscopic picture

throughout incubation comparable to that of a child under 5 years old.

Cases of Collagen Disease.—The three cases of collagen disease that did not digest (Table) had very scanty striated collagen throughout, either unaltered or showing "standard" change. The microscopic picture of the three cases of rheumatic heart disease conformed with that from the other members of their respective age groups. One patient with polyarteritis nodosa (aged 11 years) and two with disseminated lupus erythematosus (aged 12 and 19 years) showed less than the average breakdown for their respective age groups at the various incubation times.

II. UNTREATED COLLAGEN

The majority of skin samples, representing all age groups and a variety of diseases, showed the usual picture of homogenized collagen as described above (Fig. 2). However, five showed collagen fibrils composed of short lengths tapered at both ends (tactoids) dove-tailed together to make a uniform fibril (Figs 27-31). Where the two ends dove-tailed, the axial repeating period of each component remained in register. Some of the fibres appeared flattened and surrounded by finely-beaded material composed of particles of uniform size, which were easily distinguishable from the background grain (Fig. 31). Others presented a rope-like appearance (Figs 27-29). Measurement of 42 tactoids indicated a basic unit of 6,000-9,000 Å in length, the longer tactoids being twice, three, and four times this size.

The five cases showing the above changes were four newborn infants dying from erythroblastosis foetalis and a bed-ridden 9-year-old child with congenital hydrocephalus, marked emaciation, spasticity of all extremities, multiple decubiti ulcers, terminal pneumonia, and a mental age of 3 years. The remaining skin samples presented the usual appearance of unaltered collagen after homogenization, *i.e.* long, striated fibrils with square or torn ends cut by the blender and an occasional tapered end.

Discussion

I. INCUBATED CONTROLS AND COLLAGEN INCUBATED WITH COLLAGENASE

(A) Macroscopic Digestion.—The significant difference between collagen digestion in babies and young children and in adults (Fig. 3) was previously noted from the chemical standpoint (Keech, 1954a). Collagen from infants produced nearly twice as much, and from children aged 1 to 10, half again as much soluble nitrogen in a given time as adults over

the age of thirty. But these readings were taken at 3 hrs for the reasons stated (Keech, 1954a), whereas in the present study incubation was extended to 24 hrs. The fact that at 24 hrs the digestion in individuals below the age of 40 was $3\frac{1}{2}$ times greater than that found above this age may indicate a relative insusceptibility to collagenase in the older age group.

The reason for the lack of digestion in eight patients aged 1 to 52 years (Table) remains obscure. All the collagen samples were satisfactory and well extracted. Therapy cannot be incriminated. Collagen from seven other children with acute leukaemia and receiving the same multiple treatments (blood transfusion, antibiotics, A-methopterin, cortisone, and ACTH) digested normally for their age group. The cases of dermatomyositis and juvenile rheumatoid arthritis had received heavy doses of the steroid hormones, but so also had other individuals who conformed to the general response-pattern. In fact, the patient with fulminating rheumatic carditis who exhibited excessive collagen digestion (Table) had received large doses of cortisone for 12 days before death.

Some of the unused extracted collagen samples prepared for the chemical study already mentioned (Keech, 1954a) were used for this investigation, and the same exceptions in collagen disappearance were noted, *e.g.* the lack of digestion associated with negligible nitrogen recovery in the 5-year-old child with dermatomyositis and in the 13-year-old child with rheumatoid arthritis. The excessive collagen digestion of the 52-year-old patient with carcinoma of the lung was again seen; it may or may not be related to his terminal treatment with intravenous nitrogen mustard. The absence of digestion of collagen in rash-bearing skin and the normal digestion in abdominal skin from the other case of dermatomyositis in a 9-year-old child is unexplained.

(B) Electron Microscopic Findings.—Gross (1953) pointed out that electron microscopy has its limitations as a method of studying collagen in that it relies for identification on the characteristic morphological "fingerprint" of axial periodicity. He produced a wide variation in structure by a few known agents, and concluded that:

a systematic stepwise investigation of controlled alterations, progressing from purified components to systems of increasing complexity, will not only lead to a better understanding of the chemistry involved in physiological and pathological processes but may give more specific meaning to the observed histological changes.

He noted that:

in several preparations of intact human skin, rat-tail tendon, and purified cow-hide that had been digested with collagenase, moderate numbers of long filaments resembling strings of beads with very regular periodicity of about 600-650 Å.

He was unable to determine their origin. Similar beaded fibrils were also found by Gross (Matoltsy and others, 1951) in the vitreous humour of cattle eyes following fragmentation in a Waring blender for 5-10 min. at 0-5° C. The axial periodicity varied between 500-850 Å, averaging 610 Å. No enzyme was used.

From the extensive observations made in the present study on purified collagen from all age groups, it is very difficult to escape the conclusion that both the beaded fibrils and "beads" are the smallest visible breakdown products of collagen. Fig. 26 shows that these fibrils occur after 1 to 24 hrs' incubation, but only after 24 hrs over the age of 30. In the presence of striated collagen these fibrils would always "fit in" with the axial periodicity of the larger fibril had they been aligned beside it (Fig. 21). The component "beads", however, are the most constant and numerous end-product. It is believed that they represent the smallest collagen macromolecule that it is now possible to photograph, although more powerful instruments may visualize even smaller particles in the future.

Recent work by Watson, Rothbard, and Vanamee (1954) demonstrated re-precipitation of rat tendon collagen in fibrous form when normal rabbit serum was added to the acetic acid collagen solution. However, when the solvated collagen was added to homologous antiserum a globular precipitate occurred. The authors interpret this as representing the macromolecules of collagen coated with antibody, the antibody preventing fibre formation. Their electron micrographs illustrate clumps of ill-defined, globular material which may well be the same basic collagen component described in the present paper, the "beads" being the collagen macromolecules minus the antibody coating.

Wyckoff (1949) illustrated collagen fibres from animal Achilles tendon after immersion in weak acid solution. His Fig. IX, 31, 32, and 34 shows that these consist of:

a bundle of filamentous macromolecules of exceedingly small cross-section . . . the cross-bands persist, however, in the remains of a ruptured fiber.

Fig. 27.—Untreated skin collagen from a 9-year-old child dying from hydrocephalus. Preparation fragmented in de-mineralized water in Waring blender in the cold room. The fibrils are composed of short lengths of collagen tapered at both ends (tactoids), dove-tailed together to make a uniform structure. Where the two ends dove-tail, the axial-repeating period of each component remains in register. Some parts of the fibrils appear flattened and surrounded by a finely beaded material continuous with the substance of the fibril. $\times 18,500$.

Fig. 28.—Untreated skin collagen from a newborn infant dying of erythroblastosis foetalis. Same description as for Fig. 1. The rope-like structures have a uniform, narrow palladium shadow surrounding each cigar-shaped component instead of a series of humps. This is believed to indicate a separation of fibril tactoids and not a true twisting (compare Fig. 2*a*). $\times 19,000$.

Fig. 29.—Same as Fig. 28. $\times 16,000$.

Fig. 30.—Untreated skin collagen from another newborn infant dying of erythroblastosis foetalis showing multiple dove-tailing. $\times 18,500$.

Fig. 31.—Untreated skin collagen from a 9-year-old child dying from hydrocephalus. Same description as for Fig. 27. The continuity of the finely beaded material with the fibril substance is well shown. $\times 18,500$.

These filaments are aligned longitudinally and strongly resemble the beaded fibrils described above.

Gross (1953) illustrated the effect of 0.1 N HCl on cow-hide corium collagen. His Fig. 4 shows swollen, flattened degenerated fibres after immersion for 3 hrs at room temperature, rather resembling the "granular degenerating" fibres described in the present study. Vanamee and Porter (1951, Fig. 2) illustrate somewhat similar structures obtained by partially dissolving rat-tail tendon with acetic acid. However, both these occurred in an acid solution, whereas the break-down products described in the present investigation were formed in a solution buffered at pH 7.3, this pH being unchanged after incubation.

Rich and others (1953) described altered collagen fibrils from local anaphylactic lesions in rabbit skin, and correlated the histological and electron microscopical appearances. Affected fibrils showed loss of axial periodicity, hyaline transformation, variation in density, irregularities of the margins, and evidences of swelling and fragmentation. Even though the descriptions and illustrations do not resemble the collagenase break-down products discussed in the present work, it is considered important to mention that structures bearing no resemblance to the usual striated collagen have been convincingly demonstrated as stemming from this source.

It is hoped that the above description of the effect of collagenase on known collagen substrates from different age groups may help in defining altered collagen in pathological tissue where the characteristic morphological "finger-print" of periodicity may be lost.

II. UNTREATED COLLAGEN

Gross (1953) illustrated similar structures after immersion of purified (extracted) cow-hide corium collagen fibrils in 0.1 N HCl at room temperature for a few minutes. Within 10 min. many fibrils were flattened and distorted along their length. After several hours there was general disintegration of axial structure with marked swelling and flattening along the whole length of most of the fibrils. As described above, the material under discussion in this investigation was simply unfixed human abdominal skin collagen homogenized in de-mineralized distilled water in the cold room and kept well below body temperature. No extraction had been performed and no chemical or enzyme added. Gross (1953) stated that excessive blending might cause fragmentation resembling that illustrated in his Fig. 2, but, in the present study, all 61 skin samples were blended for the same length of time (15 min.) and these changes were only noted in five cases. The majority of the rope-like fibres seen (Figs 27-29) were *not* considered to be twisted, as evidenced by the shape of the palladium shadow. Instead of a series of "humps", each cigar-shaped component was outlined by a narrow shadow quite unlike that cast by the rope-like re-precipitated collagen illustrated by Noda and Wyckoff (1951) and by Wyckoff (1952). This was taken to indicate a *separation* of fibril tactoid components and *not* a true twisting. The latter is illustrated in Fig. 2(a).

Erythroblastosis foetalis is a common cause of foetal maceration, but the skin used was macroscopically normal with no evidence of autolysis. However, it is possible that such a generalized antigen-antibody disturbance continued throughout gestation could render the collagen less stable and more susceptible to disintegration by the mechanical action of blending. Coons and others (1951) and Kaplan and others (1950) demonstrated the presence of a large amount of antigen-antibody precipitate on dermal collagen after intravenous injection of various fluorescein-labelled antibodies into mice. Pneumococcal polysaccharides showed a remarkable persistence on collagenous fibres, and the connective tissue of the dermis following injection of egg albumin, bovine albumin, and human γ -globulin was so brilliantly fluorescent that the dermal cells were obscured. The authors state:

It would appear that the antigen present in the tissue fluids had become adsorbed on to the collagenous fibers. The epithelial cells of the epidermis and hair follicles were negative. The human γ -globulin persisted six times as long as the two albumins.

Another possible explanation should be men-

tioned. The heat generated during homogenization may have affected the unstable erythroblastotic collagen, as electromicrographs of heat-treated collagen show a somewhat similar picture (Reed and Wood, 1954). Although the temperature of the suspension at the end of blending was always well below body temperature, the temperature at each particle-fluid interface may have been higher. Or the damaged erythroblastotic collagen may have become altered at a lower temperature than the stable substrate from the other individuals.

The findings described above suggest that at least some collagen fibrils may be composed of tactoids, the tapered ends being closely dove-tailed and, under normal conditions, invisible. On partial solution with dilute HCl (Gross, 1953), after digestion with collagenase (Gross, 1953; Keech, 1954b) in re-precipitated collagen (Vanamee and Porter, 1951), or in rare instances after mechanical blending, these bi-tapered building blocks are revealed.

Hypothetical Structure of Collagen.—This dove-tailing (Figs 27-31) suggests that collagen fibrils are composed of a chain of tactoids, the dove-tailed sections possibly providing weak points for enzyme separation. In fact, Fig. 7 suggests a localized through-the-bundle point of action by collagenase. The cigar-shaped macro-fibrils occurring in fibrous proteins and consisting of dove-tailed microfibrils is discussed by Keech (1954b). The flattened fibril areas shown in Figs 27-31 are surrounded with finely-beaded material. Careful examination suggests that this material is composed of beaded fibrils which maintain continuity with the parent fibril, rather like the effect produced by weak acid illustrated by Wyckoff (1949, Fig. IX, 32). As described above, collagenase breakdown produces beaded fibrils with a periodicity equivalent to that of their parent fibres (Fig. 21). Fig. 20 reveals that these beaded fibrils are aligned longitudinally with the competent beads in register. Fig. 32 (over-leaf) illustrates a hypothetical intra-tactoid structure compatible with these findings.

Summary

The effect of collagenase on extracted human abdominal skin collagen from sixty individuals of all ages, dying from a variety of causes, was studied under the electron microscope. Three different types of breakdown structure were found, each ultimately disintegrating to the same end product, or "bead", believed to be the smallest collagen component yet demonstrated.

The proportion of these three elements varied

Fig. 32(a).—Skin collagen from a 28-year-old patient dying from a coronary thrombosis. Preparation incubated with collagenase for 1 hr. Fibres composed of many dove-tailed tactoids. $\times 15,300$

Fig. 32(b).—Diagrammatic drawing of area marked with arrows in (a) to show dove-tailing.

Fig. 32(c).—Diagrammatic representation of fibre structure as found in untreated collagen (Figs 27-31).

Fig. 32(d).—Same fibre bundle as shown in (c) after hypothetical enzyme action. The individual tactoids have become elongated. If the components marked 1, 2, and 3 in (c) are removed in the process, localized through-the-bundle fibre narrowings result as illustrated in Fig. 7.

Fig. 32(e).—Hypothetical intra-tactoid structure. This longitudinal arrangement of beaded fibrils in register is supported by:

- (i) the skeleton fibre illustrated in Fig. 20;
- (ii) the fact that in the presence of striated collagen, the beaded fibrils would always "fit in" longitudinally with the axial periodicity of the larger fibril had they been aligned beside it (Fig. 21);
- (iii) the flattened fibril areas shown in Figs 27-31. Careful examination of the surrounding finely-beaded material suggests that it is composed of beaded fibrils which maintain continuity with the parent fibril.

markedly with age and length of enzyme-incubation. At any given time there was a characteristic picture for different age groups. No definite correlation could be established between different diseases or treatments.

A significant difference was found in macroscopic digestion between different age groups, collagen from babies and children disappearing more rapidly than that from adults. The 3-hr digestions showed a steady decline with increase in age, and the 24-hr digestions from patients under the age of 40 were $3\frac{1}{2}$ times greater than those from individuals in the fifth decade.

Collagen digestion did not occur in eight cases. This is so far unexplained.

The untreated skin of non-incubated controls showed changes suggesting that at least some collagen fibrils have a multiple-tactoid structure.

In pathological tissue, collagen may be so altered as to pass unrecognized. It is hoped that this paper, by describing the effects of collagenase on known substrates from different age groups, may serve

as a line of reference in attempts to define altered collagen in pathological tissue, when the characteristic morphological "finger-print" of axial periodicity may have been lost.

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**Collagène de la peau humaine de différents groupes d'âge
avant et après la digestion par la collagénase
Etude au microscope électronique**

RÉSUMÉ

On a étudié au microscope électronique l'effet de la collagénase sur le collagène extrait de la peau abdominale de soixante sujets de tout âge morts de causes variées. On a trouvé trois types différents d'écroulement de la structure, tous aboutissant à la formation d'un seul produit de désintégration finale, la "perle", le plus petit composant connu du collagène.

La proportion de ces trois éléments variait considérablement selon l'âge et le temps d'incubation enzymatique. A un temps donné on voyait un tableau caractéristique pour un groupe déterminé d'âge. Il n'a pas été possible de définir un rapport avec une maladie ou un traitement quelconque.

On a trouvé une différence significative dans la digestion macroscopique selon l'âge: le collagène des nourrissons et des enfants disparaissait plus rapidement que celui des adultes. La digestion de 3 heures diminuait régulièrement avec l'avance de l'âge et la digestion de 24 heures pour les sujets de moins de 40 ans était $3\frac{1}{2}$ fois plus prononcée que celle pour les sujets âgés de 40 à 50 ans.

La digestion du collagène ne s'est pas produite dans huit cas. Pour le moment on ne peut pas expliquer ce phénomène.

La peau qui n'a pas été traitée ni incubée présentait des altérations suggérant que tout au moins certaines fibrilles collagènes ont une structure tactoïde multiple.

Dans le tissu pathologique le collagène peut être si altéré qu'il devient méconnaissable. On espère que cet article, qui décrit les effets de la collagénase sur des extraits déterminés provenant de sujets d'âge différent, offre des critères permettant de définir le collagène altéré dans un tissu pathologique quand l'"empreinte digitale" morphologique et caractéristique de la périodicité axiale se perd.

**Colageno de la piel humana de diferentes grupos de edad
antes y después de la digestión por la collagenasa
Estudio al microscopio electrónico**

SUMARIO

Se estudió al microscopio electrónico el efecto de la collagenasa sobre el colageno extraído de la piel abdominal de sesenta individuos de todas edades, muertos de causas variadas. Encontráronse tres tipos diferentes de desintegración de la estructura, acabando todos con formar un solo producto final, "la cuenta de rosario", el más pequeño componente de colageno que se conozca.

La proporción de estos tres elementos fué muy variable según la edad y el tiempo de incubación enzimática. En tiempos determinados se pudo ver el cuadro característico para cada grupo de edad. No se pudo definir correlación alguna con varias enfermedades o tratamientos.

Encontráronse diferencias significativas en la digestión macroscópica entre varios grupos de edad, el colageno de los infantes y niños desapareciendo más pronto que el de los adultos. La digestión de 3 horas bajaba uniformemente con el avance de la edad y la digestión de 24 horas para los sujetos de menos de 40 años fué tres veces y medio superior a la para los sujetos en la quinta decena de vida.

La digestión del colageno no se produjo en ocho casos; fenómeno que no se puede explicar todavía.

La piel que no fué tratada ni incubada presentó alteraciones sugiriendo que por lo menos ciertas fibrillas collagenas tienen una estructura tactoide multiple.

En el tejido patológico el colageno puede sufrir alteraciones a punto de pasar inadvertido. Se espera que este artículo, al describir los efectos de la collagenasa sobre extractos determinados procedentes de sujetos de edad diferente, sirva de criterio para definir el colageno alterado en un tejido patológico en caso de perderse la "huella digital" morfológica, característica de la periodicidad axil.