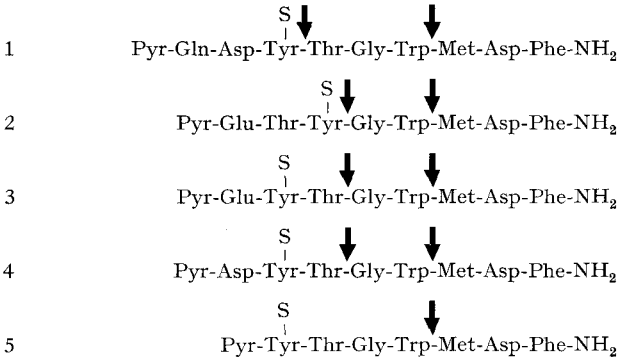
of action was not the same for the 2 peptides and this led initially to a misinterpretation of the structure of phyllocaerulein. The results of the subtilisin digestion are shown in Figure 2.

Degradation by subtilisin of caerulein and analogs



Caerulein (structure 1) was broken into 3 fragments, 2 tripeptides and an N-terminal tetrapeptide which did not contain threonine. By submitting phyllocaerulein (structure 3) to the same subtilisin digestion, an N-terminal fragment was obtained which also contained 4 residues one of which was threonine. By analogy with caerulein, it was initially thought that the bond broken was the tyrosine sulphate bond and that the threonine residue was in the next position, as in structure 2.

Further experiments showed however that this structure was not correct. Digestion with carboxypeptidase of the desulphated N-terminal tetrapeptide gave doubtful results, since the threonine and tyrosine residues were released at approximately the same rate; however, by submitting the same fragment to hydrazinolysis, free threonine was obtained and this proved that the sequence of phyllocaerulein was that of peptide 3. Furthermore, the activity spectrum of peptide 2, which was subsequently synthesized as a caerulein analog, was found to be different from that of phyllocaerulein.

Shortly afterwards it was possible to explain the discrepancy between the subtilisin degradation of caerulein and phyllocaerulein, since it was possible to examine the behaviour of 2 synthetic analogs: des-glutamine-caerulein (structure 4) and des-glutamine, des-aspartic acid-caerulein (structure 5). Peptide No. 4 was hydrolyzed at the carboxyl side of the threonine residue, giving rise to an N-terminal fragment containing threonine in the C-terminal position, while no cleavage of peptide No. 5 could be observed either at the tyrosine sulphate or at the threonine bond.

The subtilisin behaviour was also examined on the desulphated peptides and the same result was found. It was therefore evident that both the tyrosine (or tyrosine sulphate) and the threonine bonds were susceptible to the action of subtilisin but that their cleavage depended on their position in the chain. Tyrosine was preferred when it held the fourth position from the N-terminus. When tyrosine was replaced by threonine in the fourth place, the bond broken was still the fourth; but, when both threonine and tyrosine occupied a position closer than the fourth to the N-end, the enzyme could not exert any action on the bonds formed by these 2 amino acids.

Chymotrypsin has no action on tyrosine sulphate but is very specific for the tyrosine bonds. By digesting the desulphated peptides with chymotrypsin, it was found that all the tyrosine bonds were normally split, even when in the second position.

Riassunto. Vengono riportate e discusse alcune differenze osservate durante la degradazione con subtilisina dei 2 peptidi naturali ceruleina e phylloceruleina.

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Calcium as Stabilizing Factor of the Collagen Macromolecule

It is known that the stability of the collagen molecule increases with age[1], and that also the Ca content of several organs increases[2]. It has been discussed whether some relationship exists between these age changes of collagen and the increase of Ca content.

To study this we performed 2 types of experiments on rats under conditions in which an increased calcium content exists. In the first series we used artificial calcification, and in the second we studied the skin collagen (corium) of old animals. In both series we tested the stability of collagen before and after extraction of calcium. For this we estimated the total and the 'labile' hydroxyprolin (hypro) content[3].

Methods. We used Wistar-rats from this Institute's old age colony. Skin was taken immediately after death, cleaned from hair and s.c. material, defatted with ether and cut in small pieces of about 1 mm³.

Collagen was estimated as hypro after Stegemann[4], as modified by Woessner[5].

Total collagen was measured after hydrolysis with 6 *N* HCl at 110 °C for 12 h, expressed as g hypro in 100 g skin, dry weight.

'Labile' (soluble) collagen is the part which is dissolved after denaturation at 65 °C for 10 min in Ringer's solution at pH = 7.4. After hydrolysis, it is estimated as hypro and expressed as % of the total. It is known that the 'labile' (soluble) collagen decreases with aging in the skin (corium)[1] and that the decrease is larger in the dorsal than in the ventral skin[6].

Calcium was estimated after ashing about 1 g skin at 500 °C. The ash was dissolved in 2 ml 0.01 *N* HCl and calcium precipitated with 3 ml ammonium oxalate-buffer pH 5.5. The precipitate is then centrifuged and washed with H_2O. The calcium oxalate is dissolved in 0.05 *N* perchloric acid and Ca estimated in the flame photometer.

[1] F. Verzár, Gerontologia *4*, 104 (1960).
[2] V. Freydberg-Lucas and F. Verzár, Gerontologia *1*, 195 (1957).
[3] A. Meyer and F. Verzár, Gerontologia *3*, 184 (1959); Gerontologia *5*, 163 (1961).
[4] H. Stegemann, Hoppe-Seylers Z. physiol. Chem. *311*, 41 (1958).
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The extraction of calcium of skin was performed with ethylendiamintetra acetic Na (EDTA) for 40 h at room temperature. In a special series of experiments, we tested the quantity of calcium which can be extracted by our treatment of the skin with 1% EDTA in 40 h. This decreased from 49.3 ± 3 in the normal to 29.0 ± 4.5 mg/100 g wet skin in the EDTA treated.

Results. Experiments on calcified skin. The first series of experiments was done on 2-month-old rats in which a calcification of the dorsal skin was evolved by the calciphylaxis method of SELYE[7]. Dehydrotachysterol (DHT, 1 mg) was given per os. Next day 2 ml of a solution containing 0.5 mg Fe as $FeCl_3$ dissolved in 12% dextran was injected under the dorsal skin. 10 days later the skin on the place of injection was calcified. The animal was killed and in the dorsal skin total and 'labile' collagen was estimated in the different regions. As in former papers of this laboratory[8,9], we sign as A, B, the calcified central parts of skin and C, D, E, the more peripheral, normal skin. From each part of the skin 1 piece was analysed in the normal state, another after extraction of calcium with EDTA.

Table I shows these results. Total and 'labile' collagen (hypro) were measured. Total collagen decreased in the central calcified part while it remained normal in the surroundings (C, D, E), as was described in our former paper[8]. The decrease in the calcified centre was explained[8] in our former paper as the result of a 'sterile inflammation', caused by the injection of ferrichloride in dextran. It is well known from morphological pathology that inflammation destroys the collagen fibres of tissues. The liberation of hydrolases (Kathepsin) from the lysosomes of leucocytes seems to be the mechanism with which collagen is destroyed[10].

An aliquot part of this skin was placed in 1% EDTA for 40 h at room temperature for decalcification. For the content of total collagen, no significant differences were found either in the calcified (A, B) or in the farther (C, D, E) normal skin regions.

'Labile' collagen, however, behaved in a different way. According to former studies in the skin of 2-month-old rats, 'labile' collagen is about 50%, and this was found also in these animals in the uncalcified peripheral (normal) skin regions C, D, E.

Treatment of the normal parts (C, D, E) with EDTA, in the same way as mentioned above, increased the quantity of 'labile' collagen in each of 11 cases (from 12) by about 10–20%. This result is highly significant.

The calcified central region (A, B) contains less 'labile' collagen than the peripheral normal skin. Thus it changed in the direction of old age. This difference is present in each case, with mean values of 34.3% against 50.9%.

If these parts of the skin were treated with EDTA in the way mentioned, and 'labile' collagen measured, this gave a considerable increase after Ca extraction. The central parts from 34.3–44.2%, the peripheral normal from 50.9–62.6%.

Therefore it must be concluded that calcium was bound to collagen, in calcified as in normal skin, in a similar way as by crosslinks. Calcium extraction changes the collagen in the direction to that of young animals. This effect was present in each case. The single tests vary, but this is not unusual in calcification experiments.

Experiments on aged animals. In the second series of experiments we studied the collagen in the skin of aged animals in its natural slate. The quantity of total collagen (hypro) and the percentage of 'labile' (soluble) collagen (hypro) was measured before and after EDTA treatment.

The animals were 21–29-month-old rats. In 12 animals dorsal and in 2 also ventral skin was analysed.

Each piece of skin was divided into 3 parts: the first was analysed in the natural state; the second after extraction of Ca in EDTA for 40 h; the third part was a control in which we left the skin in Ringer's solution also for 40 h.

The quantity of total collagen did not change in EDTA, nor in Ringer's solution during 40 h (Figure 1). Also the 'labile' collagen did not change in Ringer's solution (Table II).

'Labile' collagen was then measured after Ca-extraction with EDTA. ('Labile' hypro (collagen) of aged animals is much less than in young.) It was found in all cases that 'labile' hypro increases, in mean from 5.2% before to 10.2% after treatment with EDTA (Figure 2). Thus also in old animals calcium takes part in the stabilization of collagen.

Since the denaturation for 'labile' hypro estimation is done in Ringer's solution, it seemed possible that the Ca content counteracted the extraction with EDTA. The estimation was therefore repeated with Ca-free Ringer's solution. This did not change the result as Figure 2 shows. Thus the increase of 'labile' collagen after Ca-extraction with EDTA is not influenced by the Ca content of the Ringer solution.

Table I. Total and 'labile' hypro in calcified (a, b) and normal peripheral skin (c, d, e) before and after EDTA 1%

No. Sex	Place	Total hypro g/100 g		'Labile' hypro %			
				calcified		uncalcified	
		before EDTA	after EDTA	before EDTA %	after EDTA %	before EDTA %	after EDTA %
211	b	2,562	4,896	29.5	44.7		
♀	c	14,628	10,448			38.1	54.3
	d	10,911	10,359			48.6	67.3
	e	9,321	10,565			59.2	70.1
213	a	1,640	1,675	33.8	36.6		
♀	b	2,182	2,239	40.6	63.6		
	c	9,939	7,208			51.5	57.8
	d	9,815	8,559			42.8	60.5
	e	10,027	9,855			47.7	48.8
214	a	1,609	1,756	23.4	22.6		
♀	b	2,782	2,013	29.4	37.5		
	c	8,494	6,329			46.6	57.5
	d	10,227	8,149			51.2	59.3
	e	9,297	4,781			60.9	57.5
215	a	1,779	1,649	44.4	51.7		
♀	b	2,383	3,063	39.2	52.8		
	c	9,124	8,025			55.6	68.2
	d	8,344	11,507			54.1	77.0
	e	9,747	10,987			55.0	72.6
Mean:		7,095	6,530	34.3[a]	44.2[a]	50.9[b]	62.6[b]

Significance: [a] 34.3: 44.2, $t = 3.30$, P 0.05. [b] 50.9: 62.6, $t = 7.25$, P 0.0001.

[7] H. SELYE, *Calciphylaxis* (The University of Chicago Press 1962).
[8] MARIA BOROS-FARKAS, H. SPICHTIN and F. VERZÁR, Gerontologia *13*, 129 (1967).
[9] MARIA BOROS-FARKAS, H. P. v. HAHN and F. VERZÁR, Gerontologia *13*, 136 (1967).
[10] G. S. LAZARUS, R. S. BROWN, J. R. DANIELS and H. M. FULLMER, Science *159*, 1483 (1968).

Table II. Total and 'labile' hypro in aged animals dorsal (d) and ventral (v) skin before and after 1% EDTA

No.	Sex	Age (months)	Region	Total hypro in g/100 g skin			'Labile' hypro %		
				before EDTA	after EDTA	Ringer solution control	before EDTA %	after EDTA %	Ringer solution control
220	♀	24	d	13,160	12,602	11,474	4.8	9.5	4.9
221	♀	24	d	12,090	13,760	13,290	3.2	10.3	5.2
226	♀	25	d	11,320	12,390	18,120	2.6	9.3	4.3
225	♀	25	d	12,800	11,840	11,560	2.9	8.8	3.1
225	♀	25	v	11,530	10,800	12,150	13.6	17.2	11.1
227	♂	24	d	12,050	13,830	12,700	4.3	17.0	6.6
227	♂	24	v	12,780	12,060	14,510	9.3	16.5	11.6
1	♂	22	d	10,631	11,255		7.6	10.5	
6	♂	21	d	12,170	11,379		5.0	9.7	
7	♂	21	d	13,144	13,406		4.1	8.7	
8	♂	26	d	11,951	11,914		3.7	5.9	
10	♂	26	d	12,622	14,342		2.9	7.0	
9	♂	28	d	11,777	13,554		6.0	7.0	
2	♂	29	d	13,610	13,822		2.5	5.1	
Mean:				12,260	12,639	13,330	5.2 ± 0.8 [a]	10.2 ± 1.1 [a]	6.7 ± 1.3

Control after Ringer solution for 40 h.
[a] Significance 5.2 ± 0.8: 10.2 ± 10.2, $t = 6.404$, $P < 0.0001$.

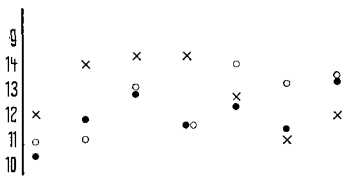

Fig. 1

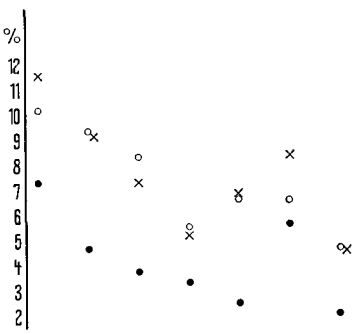

Fig. 2

Fig. 1. Total collagen (hypro) g/100 g of rat skin. ● fresh (untreated) skin, ○ after 40 h in EDTA 1% denatured in Ringer-solution, × after 40 h in EDTA 1% denatured in Ca-free Ringer-solution.

Fig. 2. % 'labile' (soluble) collagen of rat skin. ● fresh (untreated) skin, ○ after 40 h in EDTA 1% denatured in Ringer-solution, × after 40 h in EDTA 1% denatured in Ca-free Ringer-solution.

Discussion. Three completely different procedures showed that Ca extraction with EDTA of skin collagen leads to a decrease of the stability of collagen as measured by an increase of solubility, i.e. an increase of 'labile' hypro (collagen).

This effect is present in collagen of young and also in old animal's skin. Farther it is also present in artificially calcified skin. Calcification of the skin (calciphylaxis) changes the remaining total collagen to a state of increased crosslinks, as in older skin.

Extraction of calcium, by contrast, changes collagen to higher values of 'labile' collagen as in younger animals. This effect is present in normal skin of 2-month-old animals, and in old animals' normal skin and also in calcified skin of young animals.

We conclude that in normal and in calcified skin Ca takes part in the stabilization of the collagen fibre. Decalcification diminished stability; it acts in the direction as of the state of younger animals.

To measure this we used the method of solubility measurement after denaturation. It is not possible to decide whether the role of Ca is one of crosslinking or otherwise; but there is no doubt that Ca is bound to collagen as a template, in a stronger connection than Na or K, which can be extracted by distilled water alone. With the addition of Ca in Ringer's solution it is not possible to counteract the effect of Ca-extraction with EDTA. (In older experiments with THOENEN[11], it was however possible to reverse the Na^+ or K^+ effects on collagen of tendon in experiments with denaturation tension with Na respectively K. Also this suggests a stronger binding of Ca^{++}.)

Zusammenfassung. Verkalkte Haut junger sowie normale Haut junger und alter Tiere wurden mit EDTA behandelt. Das labile Kollagen (löslich in 10 min bei 65 °C) steigt in allen Fällen an. Daraus wird geschlossen, dass das Kalzium an der Stabilisierung des Kollagenmakromoleküls beteiligt ist.

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