Chemical Modifications of Cartilage Matrix During Endochondral Calcification

The pre-osseous cartilage is a provisional tissue, which is gradually modified before calcification, resorbed with a complicated and partially known mechanism and, finally, replaced by bone tissue. As far as we know, in spite of the fact that analysis of cartilage has been carried out by many workers[1,2], the chemical composition of the cell-free matrix has never been established.

The present investigation has been undertaken in order to determine the chemical variations of matrix of calf scapula and costo-chondral cartilage, occurring in endochondral calcification. The analytical study has been performed using the matrix obtained from different functional zones of cartilage.

Materials and methods. Slices of the tissue, practically devoid of cells, were obtained by cutting the cartilage with a freezing-microtome, adjusted to a thickness of 2.5 µm. By this procedure the cutting itself produces disruption of cells. Staining of preliminary preparations with toluidine-blue, hematoxyline-eosine and histochemical test for succinic dehydrogenase, in order to reveal mitochondria, have confirmed the absence of cellular material in sections prepared by this way. The cartilage slices of scapula were derived from the following zones: resting, that part of cartilage distal to the front of calcification; transforming, the region preceding the zone of the columnar cells; transforming-ossifying, the zone of columnar cells; ossifying, the layer, often as wide as 1 or 2 diameters of a degenerating cell, just in front of the newly laid down bone. Rib cartilage slices were derived only from the former (resting) and the latter (ossifying) zone.

Matrix slices from the same zone were very rapidly passed through distilled water and 50% ethanol. They were then microscopically controlled (phase contrast), in order to check the zone and to exclude cellular contamination. Finally, they were pooled, lyophylized and weighed with an electrobalance Cahn, Mod. G.

The matrix was hydrolized with 6 *N* HCl at 105 °C for 6 h before determination of hexosamines, with a micro-modification of the Elson-Morgan reaction, as reported by EXLEY[3]. Hydrolysis was extended to 40 h prior to determination of hydroxyproline, according to SERAFINI-CESSI and CESSI[4], of total phosphorus, as reported by LINDBERG and ERNSTER[5] and of total nitrogen, according to MINARI and ZILVERSMIT[6].

The material was digested with 0.1 *N* H_2SO_4 for 1 h at 80 °C to determine sialic acid according to WARREN[7]. Uronic acid has been also determined according to BITTER and MUIR[8].

Results and discussion. Table I reports the amount of some organic and inorganic constituents of the different types of cartilage matrix, the values being expressed as percent of dry weight. It appears that, in scapula cartilage, hydroxyproline gradually decreases in the transition from resting to ossifying cartilage: 74% of hydroxyproline disappears during the maturation process of the tissue. As a consequence, total nitrogen also decreases, but to a less dramatic extent than hydroxyproline (46%). Actually, in the transition from resting to the transforming zone, total nitrogen and hydroxyproline decrease virtually by the same percentage (20%). As a matter of fact, in these 2 regions of cartilage, total nitrogen is practically that of collagen, since, as indicated by data collected in the third column of Table I, non-collagenous nitrogen is almost negligible. At the level of the transforming-ossifying region, on the contrary, a significant amount of the latter appears. This counteracts the decrease of nitrogen-collagen. Parallel to the formation of non-collagenous nitrogen, mucopolysaccharide components increase significantly; in fact, uronic acid and hexosamine, on one hand, and sialic acid, on the other, increase by 50% and 145%, respectively, in the transition from the resting to the ossifying cartilage. As expected, phosphorus is present only in the ossifying region.

[1] A. J. BARRETT, *Comprehensive Biochemistry* (Eds. M. FLORKIN and E. H. STOTZ; Elsevier, Amsterdam 1968), vol. 26B, p. 425.
[2] G. M. HERRING, Clin. Orthop. Rel. Res. *60*, 261 (1968).
[3] D. EXLEY, Biochem. J. *67*, 52 (1957).
[4] F. SERAFINI-CESSI and C. CESSI, *Structure and Function of Connective and Skeletal Tissue* (Eds. S. FITTON-JACKSON, R. D. HARKNESS, S. M. PARTRIDGE and G. R. TRISTRAM; Butterworths, London 1965), p. 222.
[5] O. LINDBERG and L. ERNSTER, *Methods of Biochemical Analysis* (Ed. D. GLICK; Interscience Publishers Inc., New York 1956), vol. 3, p. 1.
[6] O. MINARI and D. B. ZILVERSMIT, Analyt. Biochem. *6*, 320 (1963).
[7] L. WARREN, J. biol. Chem. *234*, 1971 (1959).
[8] T. BITTER and H. M. MUIR, Analyt. Biochem. *4*, 330 (1962).

Table I. Chemical analysis of calf scapula and costal cartilage matrix

	Nitrogen	Hydroxy-proline	Non collagenous nitrogen[a]	Uronic Ac.	Hexosamines	Sialic Ac.	Phosphorus
Scapula							
Resting	12.16 ± 0.36 (8)[b]	9.04 ± 0.29 (9)	0.38	5.88 ± 0.19 (10)	5.88 ± 0.19 (10)	0.24 ± 0.01 (9)	< 0.04
Transforming	9.57 ± 0.34 (8)	7.02 ± 0.37 (9)	0.28	6.45 ± 0.42 (8)	7.02 ± 0.54 (12)	0.29 ± 0.01 (9)	< 0.04
Transforming-ossifying	10.27 ± 0.35 (5)	5.86 ± 0.21 (7)	2.26	8.58 ± 0.23 (8)	8.68 ± 0.12 (7)	0.59 ± 0.02 (9)[c]	< 0.04
Ossifying	6.56 ± 0.18 (9)	2.37 ± 0.12 (14)	2.89	9.08 ± 0.40 (11)	8.96 ± 0.32 (13)		2.25 ± 0.22 (7)
Rib							
Resting	12.09 ± 0.27 (7)	8.48 ± 0.18 (7)	0.98	6.29 ± 0.23 (7)	6.40 ± 0.32 (7)	0.46 ± 0.01 (5)	< 0.04
Ossifying	8.69 ± 0.52 (7)	2.22 ± 0.09 (7)	4.95	12.21 ± 0.17 (8)	12.41 ± 0.59 (12)	0.49 ± 0.05 (4)	0.34 ± 0.04 (8)

Lyophilized material: % of weight. [a] Calculated as follows: N tot — (N coll. + N hex.). N coll. = Hypro × 7.14/5.7 and N hex. = Hex. × 0.078. [b] No. of experiments. [c] Value obtained from the combined zones: transforming-ossifying and ossifying.

Table II. Zonal composition of calcifying cartilage matrix

	Collagen[a]	Other protein[b]	Muco-polysaccharides[c]	Sialic acid	Mineral[d]	Water
Scapula						
Resting	64.54	2.37	15.81	0.24	<0.22	4.91
Transforming	50.12	1.75	18.11	0.29	<0.22	5.45
Transforming-ossifying	41.84	14.12	23.21		<0.22	6.00
Ossifying	16.92	18.05	24.26	0.59	12.15	6.19
Rib						
Resting	60.55	6.12	17.08	0.46	<0.22	6.13
Ossifying	15.85	30.94	33.11	0.49	1.62	6.17

Lyophilized material: % of weight. [a] Calculated from Hypro × 7.14. [b] Calculated from non-collagenous Nitrogen × 6.25. [c] Calculated from [(Uronic Ac. + Hex.)/2] × 2.69. [d] Calculated from Phosphorus × 5.4.

Virtually the same observations can be made for the data obtained from the 2 zones of rib cartilage.

From the analytical data reported in Table I, we have calculated the amount of collagen, non-collagenous protein, mucopolysaccharides and hydroxyapatite present in the matrix of cartilage at the different zones. The new data are collected in Table II. Since in the transition from resting to ossifying cartilage the increase of non-collagenous protein is accompanied by an increase of mucopolysaccharides and sialic acid, one is tempted to correlate the 2 facts. In this case chondromucoproteins and sialoproteins would be the increasing components, as indicated also by the data reported by LINDENBAUM and KUETTNER[9].

While this manuscript was in preparation[10], a paper appeared by WUTHIER[11] who reports a similar loss of collagen in the calcification front of cartilage from foetal calves. In this material he described also a zonal distribution pattern of mucopolysaccharides, which, however, is different from ours. This discrepancy may be a consequence of the drastic washing procedures employed by WUTHIER in his experiments. A significant release of mucopolysaccharides is known to occur in tissues treated with distilled water or saline solutions.

To conclude, the most important events which occur during endochondral calcification of cartilage are a dramatic decrease of collagen and an increase of non-collagenous proteins and mucopolysaccharides. This changes are obviously the expression of changes in turnover of the compounds at the level of the different functional zones of cartilage. Relevant in this connection may be the recent observation that lysosomal enzymes are more active in cartilage before calcification[12,13].

In spite of the fact that it is not known to what extent cartilage and bone calcification processes are comparable, it is at least suggestive to recall the fact that a non-collagenous fraction is changing during calcification of osteons[14,15].

Résumé. On a étudié la composition chimique de différentes zones fonctionelles de la matrice du cartilage ossifiable scapulaire et costal. On a constaté qu'en s'approchant du front de calcification, le collagène diminue fortement tandis que les mucopolysaccarides et les protéines non collagènes augmentent.

F. VITTUR, M. C. PUGLIARELLO and B. DE BERNARD[16]

Istituto di Chimica Biologica dell'Università, Via Fabio Severo 150, I-34127 Trieste (Italy), 20 July 1970.

[9] A. LINDENBAUM and K. E. KUETTNER, Calc. Tiss. Res. *1*, 153 (1967).
[10] F. VITTUR, M. C. PUGLIARELLO and B. DE BERNARD, VI FEBS Meeting, Madrid, 1969, Abstract 821.
[11] R. E. WUTHIER, Calc. Tiss. Res. *4*, 20 (1969).
[12] A. O. JIBRIL, Biochim. biophys. Acta *141*, 605 (1967).
[13] J. L. GRANDA and A. S. POSNER, J. Bone Jt Surg. *50A*, 1073 (1968).
[14] E. BONUCCI, A. ASCENZI, F. VITTUR, M. C. PUGLIARELLO and B. DE BERNARD, Calc. Tiss. Res. *5*, 100 (1970).
[15] M. C. PUGLIARELLO, F. VITTUR, B. DE BERNARD, E. BONUCCI and A. ASCENZI, Calc. Tiss. Res. *5*, 108 (1970).
[16] This investigation was supported by grants from the Italian National Research Council.

Steigerung der $^{14}CO_2$-Bildung aus ^{14}C-Glukose bei der Ratte durch das orale Antidiabetikum Butylbiguanid-hydrochlorid[1]

Über den Mechanismus der blutzuckersenkenden Wirkung der Biguanide gibt es mehrere, z.T. widersprüchliche Vorstellungen (vgl. hierzu die Übersichten[2–6]). Manche Autoren[7–10] beobachteten eine Stimulierung der peripheren Glukoseutilisation durch Biguanide, andere[11–15] eine Hemmung der Glukoneogenese in der Leber. Kürzlich berichteten SEARLE et al.[16,17] über eine Zunahme der Glukoseoxidation bei stoffwechselgesunden und diabetischen Menschen nach Biguanid-Applikation. Wir untersuchten, ob sich eine gleichartige Steigerung der Glukoseutilisation auch im Tierexperiment nachweisen lässt.

Methodik. 7 männliche Wistar-Ratten mit einem Gewicht von 120–160 g (Züchter: Hagemann, Bösingfeld) erhielten nach 24 h Nahrungsentzug 0,1 µC uniform markierte ^{14}C-D-Glukose (spez. Aktivität: 1,1 mC/mg, The Radiochemical Center, Amersham), gelöst in 1 ml 0,9% Kochsalzlösung, i.p. appliziert. Unmittelbar vor der