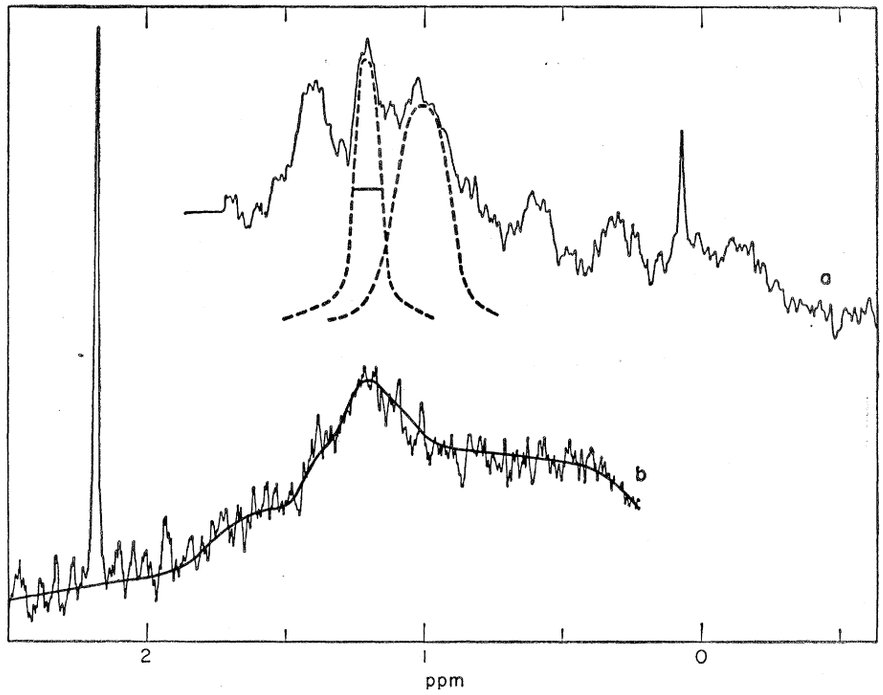

Fig. 2. *Spectrum a*: 2 percent solution of bacterial cytochrome c in D_2O-phosphate buffer; accumulated scans, 296; sweep rate, 2 cycle/sec^2. Other conditions as in Fig. 1. *Spectrum b*: 3.4 percent solution of leucine-substituted deuterated phycocyanin in D_2O-phosphate buffer; accumulated scans, 510; sweep rate, 2.5 cycle/sec^2. The sharp line at 2.23 ppm is internal acetate.

indicate that in phycoerythrin the (observable) leucine side chains occur in two different environments. From integration of the spectra, we estimate that at least 75 percent of the incorporated ^{1}H-leucine is observed in the protein. This calculation represents a very conservative estimate, in that the placement of the base line is difficult, and essentially all of the ^{1}H-leucine may in fact be detectable.

Proton magnetic resonance analysis of ^{1}H-leucine-substituted C-phycocyanin (under our conditions mainly a trimer with molecular weight of 1.4×10^5) gives a result similar to that obtained with phycoerythrin (Fig. 2,*b*). Here the main resonance peak of leucine centers at 1.23 ± 0.02 ppm. With deuterated-cytochrome c containing ^{1}H-leucine extracted from *Rhodospirillum* (molecular weight 1.2×10^4) we observe much sharper lines (Fig. 2,*a*). There are major resonance peaks at 1.24 ± 0.02 ppm and 1.06 ± 0.02 ppm; the line widths at half height are 11 and 22 hz, respectively. Smaller peaks are observed at 0.63, 0.32, and -0.15 ppm. These data indicate that, in these proteins, a sizable portion of the leucine side chains (those at about 1.24 ppm) are in a very mobile, aqueous environment. That portion of the leucine immediately upfield from the line at 1.24 ppm could represent leucine in a less mobile hydrophobic environment, for this line is broader than the one at 1.24 ppm and an upfield shift is to be expected if the surrounding environment is more hydrocarbon-like. In cytochrome c, the lines observed at very high field are most likely from leucine residues adjacent to aromatic residues of the protein.

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Partial Hydroxylation of Certain Lysines in Collagen

Abstract. *Peptides derived from the $\alpha 1$ chain of collagen have been isolated in small amounts and have been shown to differ from ones found in expected amounts only by substitution of hydroxylysine for lysine. This observation indicates that hydroxylation of these lysines by protocollagen hydroxylase has been effected to a very minor extent.*

The biological formation of hydroxyproline and hydroxylysine in collagen involves hydroxylation of proline and lysine after incorporation of the latter amino acids into polypeptides (*1*) about the size of α-chains (*2*). The hydroxylation of both proline and lysine is probably catalyzed by one enzyme (*3*), namely protocollagen hydroxylase (*4*). Only proline located in position 3 of the collagen triplet (the repeating amino acid sequence—gly X.Y.—with glycine occupying position 1) appears to be hydroxylated (*5*).

The hydroxylysine contents of collagens derived from different tissues of the same animal are quite variable, although the total amounts of lysine and hydroxylysine, as well as the overall amino acid composition of these collagens, are constant (*6*). In the collagen of chick bone, the hydroxylysine content progressively decreases during maturation of the chicks (*7*). Since certain prolyl residues in rat skin and tendon collagens are incompletely hydroxylated (*8*), it was suggested that the variations observed in the amounts of hydroxylysine of chick bone collagen reflect an alteration in the ability of lysine (protocollagen) hydroxylase to act upon lysine residues that are candidates for hydroxylation.

I now present evidence that in rat skin collagen certain lysyl residues are hydroxylated to a very minor extent. These observations were made during investigations on the primary structure of collagen. Initial experiments involved splitting α-chains at methionyl residues with cyanogen bromide (*9*). Eight resultant peptides have been isolated and characterized from the $\alpha 1$ chain of rat skin collagen. One of these peptides, $\alpha 1$-CB5, contained equimolar quantities of glucose and galactose presumably attached glycosidically to the hydroxyl group of hydroxylysine (*10*).

Initial degradation experiments aimed at determining the amino acid sequence of $\alpha 1$-CB5 involved digestion of the

peptide with chymotrypsin. The products were separated by gel filtration on Sephadex G-25 by elution with 0.2*M* acetic acid. Two well-resolved peptide peaks were observed, the larger of which contained 31 of the original 37 amino acids. The presence of homoserine in the absence of phenylalanine (see "original," Table 1), is evidence that this peptide is derived from the COOH-terminal portion of α1-CB5. The remaining six amino acids and two hexose residues were found in a smaller peptide peak (α1-CB5-C2) eluting just prior to the salt.

The next step in the sequence studies involved cleavage of the larger peptide, α1-CB5-C1, with trypsin at the two lysyl residues. After incubation with 5 percent by weight of trypsin for 18 hours at *p*H 8, the products were separated by chromatography on a column of Dowex 50 X-2 with a pyridine acetate buffer gradient. Three principal peptide peaks were observed (T1, T2, and T3, Fig. 1), a result consistent with the lysine content. In addition, three smaller peptide peaks were observed, one eluting prior to T2 (T2a) and two just before T3 (T3a and T3b). The small peak seen just before T3a on the chromatogram is believed to be ammonia since the peak was variable in size and did not contain a significant quantity of amino acids.

Amino acid analyses were performed as described (*9*), except hydrolyses were only for 18 hours, and corrections for losses of serine and threonine were not made. The composition of T2a was identical to that of T2 (Table 1), except that a hydroxylysine replaced the lysine moiety, an indication that the residue of lysine in this sequence has been partially hydroxylated. The amount of hydroxylated peptide was 13 to 15 percent of that in T2. A substantial amount of hydroxylysine was also found in T3b (Table 1), although its overall amino acid composition was the same as that of T3, giving evidence for partial hydroxylation of the lysine in this sequence. The amount of hydroxylated peptide in T3b was estimated to be only about 3 percent of the amount in T3. No difference was found in the compositions of T3a and T3.

These observations suggest that throughout the polypeptide chains of collagen there may be a number of lysyl residues which are substrates of protocollagen hydroxylase but which are acted upon by the enzyme only to a minor extent. Thus the differences in the amounts of hydroxylysine seen in various tissues may be due to a variation of the amount or the activity of protocollagen hydroxylase and not to the production of collagens with differing amino acid sequences. Another possibility is that certain tissues elaborate hydroxylases with different affinities for the lysine side chains. Extreme examples are dentine collagen (*6*) and glomerular basement membrane (*11*) whose hydroxylysine contents are two to three times that of soluble collagens.

These investigations suggest that the extent of hydroxylation of, and thus the affinity of protocollagen hydroxylase for, the lysine side chains is dictated by amino acid sequences adjacent to the lysines rather than by the tertiary structure in this particular area of the molecule. The pertinent data are as follows. In spite of the scant hydroxylation of the two lysyl residues referred to above, the amino acid composition of α1-CB5 indicates that a third lysine is hydroxylated to the extent of 90 to 100 percent. Subsequent studies (*12*) have located the resultant hydroxylysine in the NH_2-terminal portion of the peptide; it is recovered in the hexapeptide (α1-CB5-C2) previously alluded to after chymotryptic cleavage of α1-CB5. Furthermore three hydroxyprolines, but no proline, are found in T1 (Table 1), which indicates that complete hydroxylation of the prolines in this sequence has taken place. One of the hydroxyprolines is located only three residues from the lysine of T2 which is hydroxylated to a minor extent (*12*). A similar conclusion concerning the importance of the primary structure in the hydroxylation of lysine and proline has been reached by Nordwig and Pfab (*13*).

Finally the three lysines (or hydroxylysines) that were encountered in these studies were located in position 3 of the collagen triplet. Only prolines located in position 3 seem to be hydroxylated (*5*). Whether the same requirement is generally true for lysines will have to be verified by further sequence studies or studies on the specificity of protocollagen hydroxylase.

Table 1. Amino acid compositions of peptides isolated after hydrolysis of α1-CB5-C1 with trypsin. Results are given as residues per peptide. A dash indicates the entire absence of an amino acid or that its content was less than 0.1 residue per peptide.

Amino acid	Residues (No.)						
	Original	T1	T2	T2a	T3	T3a	T3b
Hydroxyproline	2.92	2.81	—	—	—	—	—
Aspartic acid	2.98	1.04	1.03	0.95	0.99	0.92	0.95
Threonine	1.02	—	0.89	0.90	—	—	—
Serine	1.99	0.94	—	—	1.08	1.14	1.06
Glutamic acid	3.01	2.80	—	0.15	0.10	0.26	0.35
Proline	2.10	—	2.15	2.20	—	—	—
Glycine	10.26	5.04	3.37	3.21	2.09	2.18	2.76
Alanine	3.09	1.07	1.11	1.05	1.05	1.36	1.13
Leucine	0.96	—	—	—	0.96	0.98	0.96
Hydroxylysine	0.16	—	—	0.95	—	—	0.64
Lysine	1.64	—	0.96	—	0.94	0.96	0.21
Homoserine*	1.02	1.02	—	—	—	—	—

* Includes homoserine lactone.

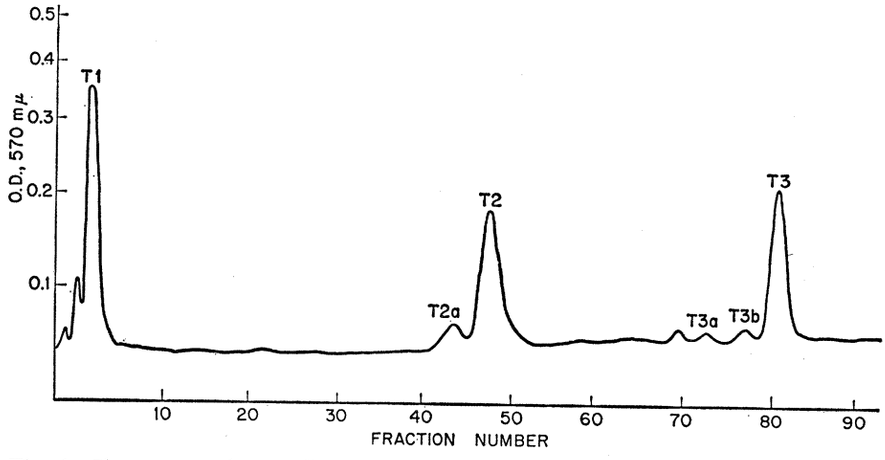

Fig. 1. Chromatography on Dowex 50 X-2 of the peptides produced by hydrolysis of α1-CB5-C1 with trypsin. The effluent was continuously monitored for ninhydrin-positive material with a Technicon peptide analyzer.

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Cell Proliferation: Enhancement by Extracts from Cell Surfaces of Polyoma-Virus-Transformed Cells

Abstract. *Dispersion of confluent monolayers of BHK21 cells with ethylenediaminetetraacetate yields a material that inhibits cell proliferation, whereas identical extraction of polyoma-virus-transformed cells provides material which enhances cellular proliferation. The material was partially characterized.*

The loss of contact inhibition and subsequent uncontrolled growth demonstrated by tumor-virus-transformed cells has been correlated with the neoplastic process (*1*). Defendi and Gasic (*2*) have reported that polyoma-virus-transformed cells histochemically exhibit an abnormally heavy layer of acid mucopolysaccharide at the cell surface, and they suggested that this increase of material could be correlated with the loss of contact inhibition demonstrated by those transformed cells. Stoker (*3*) has reported that incorporation of thymidine into polyoma-virus-transformed cells is inhibited when they are in contact with stationary, nondividing BHK21 cells, and he has suggested that this effect results from the passage of molecules between the contiguous cells. Addition of conditioned medium obtained from high concentrations of chick embryo cells to small numbers of these cells enhances their growth (*4*). Furthermore, conditioned medium obtained from either very crowded cultures of these cells or from chick cells transformed with Rous sarcoma virus contains a substance which inhibits growth (*4, 5*). These active factors are suggested to be derived from the cell surface as an exudate, and to reflect conditions at the cell periphery.

I now describe the isolation, some chemical characteristics, and the growth-enhancing effect of extracts derived from the surfaces of polyoma-virus-transformed cell by treatment with ethylenediaminetetraacetate (EDTA).

Cultures of hamster kidney cells (BHK21) demonstrating contact inhibition were used as controls. Two polyoma-virus-transformed derivatives of this line, TC-1 and H-6, demonstrating loss of contact inhibition and continuous mitosis, were also used as sources of cell surface material (*6*). Ethylenediaminetetraacetate was used to disperse confluent cell cultures and to obtain the extracellular material because it removes from the coating of the BHK21 cells and its transformed derivatives that material which is detected with Hale's stain, and does so as completely as the various enzymatic treatments reported by Defendi and Gasic (*2*). Viability of these cells, as determined by the trypan-blue dye-exclusion method usually exceeded 95 percent; any preparations revealing less than 95 percent viability were discarded. These preparations were centrifuged, and the clarified supernatants were divided into high-molecular-weight (HMW) and low-molecular-weight (LMW) moieties by ultrafiltration (*7*). The HMW and LMW fractions from transformed and nontransformed cells were tested for biological activity by addition of constant volumes of each isolate to either the transformed or the nontransformed cell cultures. Stock EDTA solutions, subjected to the ultrafiltration and concentration procedures, were used as controls.

Addition of LMW fractions derived from either transformed or nontransformed cells, as well as the HMW fraction obtained from BHK21 cells, markedly inhibited cell proliferation. However, addition of HMW fractions obtained from the polyoma-transformed cell line TC-1 enhanced cell growth (Fig. 1 and Table 1). The extent of inhibition or enhancement was dependent upon the number of cells, as well as upon the volume of material added to this test system. The effects were greatest when larger volumes of test materials (0.3 ml) were added to lesser numbers of cells ($< 150{,}000$).

Inhibition of growth was transitory, and eventually all cell cultures attained confluency. The morphology and contact orientation of BHK21 cells stimulated by addition of transformed cell HMW material were normal when such cells reached confluency. It is unclear whether any loss of contact inhibition occurred during the stages of rapid proliferation. No cytomorphological effects were noted when test samples were added to confluent monolayered cultures. Addition of control solutions neither enhanced nor inhibited cell proliferation, even when volumes as large as 0.4 ml were added to the test system. The dry weights of the test samples were not significantly different; each sample contained 30 to 40 μg of material per 0.1 ml.

Table 1. Effect of EDTA extracts obtained from dispersed, viable cells, on rates of cell growth. To each Leighton tube containing 350,000 cells in 1.5 ml of MEM was added 0.1 ml of the fraction to be tested. All tests were performed in duplicate, and the average elapsed time necessary for a confluent monolayer to be attained was determined microscopically. Cell numbers were determined in a hemocytometer at 24 hours and at the time confluency was attained.

Source of added material	Cell counts ($\times 10^5$)						Hour confluency reached		
	BHK21		H-6		TC-1				
	24 hours	Confluency	24 hours	Confluency	24 hours	Confluency	BHK-21	H-6	TC-1
None added	6.25	10.1	7.00	16.8	7.30	16.2	75	48	52
Control EDTA HMW	7.35	12.3	8.20	17.5	7.10	15.1	75	55	55
Control EDTA LMW	6.90	12.5	8.50	17.3	8.10	17.1	70	50	60
BHK21 HMW	4.90	10.3	5.70	15.5	7.10	14.7	95	60	75
TC-1 HMW	8.95	13.5	9.70	17.2	8.80	16.2	62	40	45
BHK21 LMW	5.10	11.2	6.20	16.9	5.50	15.1	85	65	60
TC-1 LMW	5.90	10.7	6.50	14.9	6.50	16.2	82	55	65