Collagen Polypeptides: Normal Release from Polysomes in the Absence of Proline Hydroxylation

Abstract. *It is not necessary that proline be hydroxylated for the completion and release of nascent collagen chains from polysomes. Hydroxylation of collagen proline in vivo normally takes place predominantly on nascent polypeptides; however, in the presence of an inhibitor of hydroxylation, unhydroxylated chains are released. These chains may subsequently be hydroxylated when the inhibition is removed. The results clarify a controversy over the site of proline hydroxylation.*

Whether the hydroxylation of collagen proline occurs on nascent collagen chains or on completed and released polypeptides has been a subject of controversy. Recently, however, we have obtained clear evidence that hydroxyproline is present in nascent chains (*1*). After a 10-minute incubation with [^{3}H]-proline, the polysomes isolated from cultured mouse fibroblasts, line 3T6, were shown to contain nascent collagen polypeptides. These polypeptides were identified by their unique content of proline residues which would undergo hydroxylation and which were, for the most part, already hydroxylated by the end of the short incubation period. Evidence that hydroxylation of collagen proline occurs on nascent chains has also been obtained by Miller and Udenfriend (*2*) who observed that hydroxylation was present in the peptidyl-puromycin complexes released from a homogenate of pig granuloma.

The conclusion that collagen proline residues are normally hydroxylated while present as nascent polypeptides raises the following questions. Is this hydroxylation necessary for the release of nascent collagen peptides from the polysomes? If not, can released but unhydroxylated chains be subsequently hydroxylated in vivo, or does hydroxylation occur only on nascent chains? We now report answers to these questions and show (i) that hydroxylation is not needed for the release of collagen chains from polysomes, and (ii) that such released chains can also be hydroxylated in vivo. These findings provide an explanation for much of the controversy over whether nascent or completed and released polypeptides represent the site of proline hydroxylation since this site is different with respect to hydroxylation-inhibited and uninhibited cells.

To examine the effect of inhibition of hydroxylation on the release of nascent collagen chains, 3T6 cells in early stationary growth phase were incubated for 12 minutes with [3,4-^{3}H]proline in the presence or absence of α,α'-dipyridyl, a compound that completely inactivates collagen proline hydroxylase by chelating the Fe^{2+} ions required for activity of this enzyme. The cells were gently lysed, and a cytoplasmic fraction was prepared as previously described after removal of debris, nuclei, and mitochondria (*1*). The cytoplasmic fractions were treated with ribonuclease and then centrifuged through linear sucrose gradients under conditions that separate released peptides, at the top of the gradient, from the monosomes that are carrying the nascent peptides (*1*).

The appropriate fractions from the gradients were combined as indicated by regions A and B of Fig. 1 to give, respectively, the soluble proteins containing the released peptides and the ribonuclease-derived monosomes containing the nascent chains. The amount of collagen peptides in each fraction was measured by determining the amount of radioactivity in hydroxyproline residues or in proline residues susceptible to hydroxylation by collagen proline hydroxylase.

The results (Table 1) show that the release of nascent collagenous peptides as well as the release of the nascent peptides of the total protein fraction proceeds at the same rate, whether or not the hydroxylation of collagen peptides is allowed. Thus, the ratio of nascent to released chains stays constant within experimental error when hydroxylation is inhibited and does not show the increase that would be expected in the case of the unhydroxylated collagen peptides (protocollagen) if the absence of hydroxylation was preventing the release of nascent chains. Direct evidence that the concentration of dipyridyl used was effectively inhibiting the hydroxylation of proline in the protocollagen samples was obtained by measuring the radioactivity in hydroxyproline and in proline residues susceptible to hydroxylation. This latter measurement was made by incubating samples in vitro with collagen proline hydroxylase under conditions in which the reaction goes to completion. Since the hydroxylation of [3,4-^{3}H]-proline produces equal amounts of tritiated water and tritiated hydroxyproline (*3*), the radioactivity incorporated

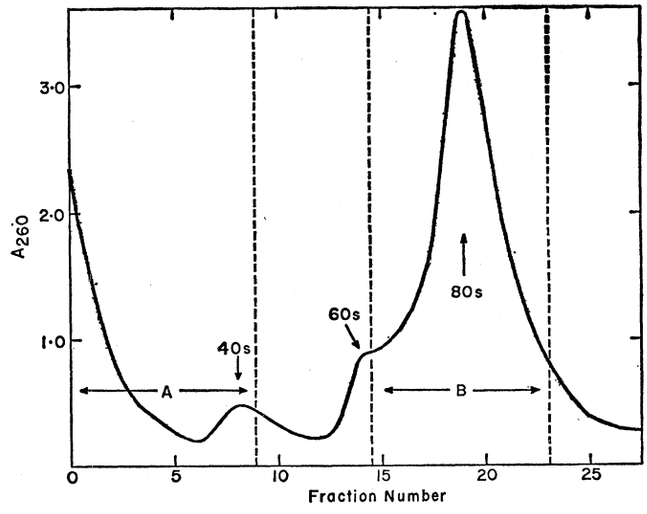

Fig. 1. Separation of released peptides from monosomes on sucrose gradients. After incubation (see Table 1) the cells were collected and lysed (*1*). The debris and mitochondria were removed; an equal amount of supernatant (2.5 ml) was prepared from both the control and dipyridyl-treated cultures and each was treated with ribonuclease (20 μg/ml) for 2 minutes at 37°C. The samples were layered on top of three sucrose gradients (11.3 ml; 15 to 30 percent (weight to volume). The gradients were centrifuged in the SW 41 rotor of the Beckman L2-65B ultracentrifuge at 2°C at 41,000 rev/min for 255 minutes. The gradients were displaced by addition of 60 percent sucrose to the bottom of the tube, and the absorbance at 260 nm was monitored by a recording spectrophotometer. Fractions (20 drops) were collected, and the indicated fractions were combined for further analysis (fractions 9 to 14 and 23 to 27 were discarded). The monosome area (region B) was sedimented by centrifugation in the SW 50.1 rotor at 50,000 rev/min at 2°C for 150 minutes in polyallomer tubes, and the part of the gradient containing soluble released proteins (region A) was concentrated and dialyzed as described in Table 1.

into either of these products provides a measure of the radioactive susceptible proline residues; in the experiments reported here, the radioactivity in both products was measured and showed excellent agreement. The percentage of the susceptible residues which were hydroxylated in vivo could then be calculated as 100 times the number of counts per minute in hydroxyproline before incubation with hydroxylase divided by the number of counts per minute in hydroxyproline after incubation with hydroxylase; alternatively, the latter number may be replaced by the sum of the number of counts per minute in hydroxyproline before incubation with hydroxylase plus those released to tritiated water after incubation with hydroxylase. These methods of calculation showed that a maximum of 1 to 2 percent of the susceptible proline residues were hydroxylated in the dipyridyl-inhibited cultures, while in the uninhibited cultures, hydroxylation reached 82.5 percent in the nascent peptide fraction and 93 percent in the released peptides. This latter finding illustrates the conclusion discussed above that, in the absence of an inhibitor of hydroxylation, the hydroxylation of collagen proline occurs for the most part on nascent chains. The smaller amount of radioactivity incorporated into protocollagen, as compared to collagen (see Table 1), does not represent a specific effect of dipyridyl on collagen synthesis, since this level of dipyridyl causes a similar inhibition (35 to 40 percent) of total protein synthesis.

The fact that hydroxylation of collagen proline residues is not required for the completion and release of nascent collagen polypeptides leads us to the second question posed above, whether completed and released protocollagen peptides can be hydroxylated in vivo. To answer this question, the following experiment was performed.

Six stationary phase plates were incubated for 1 hour with 3.5 m*M* dipyridyl in fresh medium, after which the medium was removed, and each plate was incubated with 133 μc of [3,4-^{3}H]proline for 12 minutes in 10 ml of phosphate-buffered saline, containing also 3.5 m*M* dipyridyl. At the end of this incubation, the medium was discarded, and the cell layers were rinsed with fresh dipyridyl-containing saline to which 1 m*M* unlabeled L-proline had been added. The plates were then incubated for 15 minutes in complete medium which contained also 3.5 m*M* dipyridyl and 1 m*M* unlabeled proline, in order to deplete the free [^{3}H]proline pool and to insure that the labeled nascent chains would be completed and released from the polysomes. Half of the plates were then chilled, and the nascent and released peptide fractions were prepared as described in Fig. 1 and Table 1. To see whether these released polypeptides could be hydroxylated by the cells when the inhibition of hydroxylation was removed, the remaining three plates were washed with fresh medium to which was added 1 m*M* $FeSO_4$ to reverse the inhibition of hydroxylase and which also contained 1 m*M* unlabeled L-proline but no dipyridyl, and these plates were incubated further for 30 minutes in fresh medium of the same composition. At the end of the incubation, the nascent and released peptide fractions from these plates were also prepared. These fractions were analyzed to determine the extent of hydroxylation of the susceptible proline residues, as were the corresponding fractions from the duplicate plates in which the inhibition was not reversed.

Table 1. Normal release of nascent collagen chains in the absence of proline hydroxylation. One hour before incubation with [^{3}H]-proline, three stationary phase plates (15 cm in diameter) were incubated with fresh medium (*7*), while three identical plates were incubated with fresh medium containing 3.5 m*M* α,α'-dipyridyl. At the end of the preliminary incubation the medium was removed, and the plates were incubated with 200 μc of [3,4-^{3}H]proline (5 c/mmole, New England Nuclear) for 12 minutes in 10 ml of phosphate-buffered saline, containing 3.5 m*M* dipyridyl in the case of the three plates which were first incubated with dipyridyl. The subsequent separation of the released and nascent peptides is described in Fig. 1. The ribosomal pellet containing the nascent peptides was resuspended in 2.9 ml of H_2O, while the completed and released peptides were concentrated to about half their original volume in dialysis bags coated with dry Sephadex G-200. These peptides were then dialyzed against H_2O to remove excess [^{3}H]proline and sucrose. Total protein radioactivity in each fraction was measured by counting 50 μl plus 0.95 ml of 1.2 percent sodium dodecyl sulfate in dioxane-based liquid scintillation counting fluid. Protocollagen (unhydroxylated collagen) was measured from its content of proline residues susceptible to hydroxylation by collagen proline hydroxylase. Samples (1.3 ml) were incubated in a final volume of 1.5 ml with hydroxylase and the required cofactors (*4*). The ^{3}HOH released as a result of the in vitro hydroxylation was collected by lyophilization of the reaction mixture and was counted by liquid scintillation; the residue was hydrolyzed in the presence of 50 μmole of carrier hydroxyproline, which was then purified to constant specific activity (*6*). Duplicate control samples (1.3 ml) were identically treated except that heat-denatured hydroxylase was added and the mixtures were lyophilized directly without incubation. The radioactivity released as H_2O from the protocollagen samples equaled within experimental error the number of counts per minute in hydroxyproline after incubation with hydroxylase; the latter values are reported for protocollagen in the table. The uninhibited samples were treated identically and the collagen radioactivity shown in the table again represents that in hydroxyproline after incubation with hydroxylase. The calculations of the percentage of hydroxylation of the susceptible proline residues in the dipyridyl-containing cultures and in the uninhibited cultures are given in the text.

	Count/min in peptides		Ratio of nascent to released (%)
	Nascent	Released	
Hydroxylation normal			
Collagen	1,654	21,600	7.7
Total protein	290,000	3,549,000	8.2
Hydroxylation inhibited by α,α'-dipyridyl			
Protocollagen	1,015	15,053	6.7
Total protein	174,580	2,249,000	7.8

The released polypeptide fraction, prior to the reversal of hydroxylation, had 251 count/min in hydroxyproline and 9400 count/min in proline residues susceptible to hydroxylation (measured as ^{3}HOH, see above); these figures show that only 2.6 percent hydroxylation had occurred in the presence of dipyridyl. When the inhibition was reversed by incubating the cultures for 30 minutes with Fe^{2+}, the released polypeptide fraction had 10,310 count/min in hydroxyproline and 188 count/min in susceptible proline residues (measured again as ^{3}HOH); these figures show that 98 percent of the susceptible proline residues in these released peptides were now hydroxylated. This result shows that completed and released unhydroxylated collagen chains can be hydroxylated in vivo when the inhibition of hydroxylation is removed. The nascent peptide fractions from either set of cultures contained negligible radioactivity in either hydroxyproline or in susceptible proline residues, as would be expected after the 15-minute incubation with unlabeled proline (159 and 52 count/min, total, respectively, before and after the incubation with Fe^{2+}). Furthermore, only modest increases occurred during the 30-minute incubation with Fe^{2+} in the radioactivity in released collagenous chains (9 percent, measured as the sum of the counts per minute in hydroxyproline and in susceptible proline residues—10,498 compared to 9,400 count/min). The total trichloroacetic acid–insoluble radioactivity showed a similar modest increase (1,033,000 count/min after, compared to 919,000 count/min before, the incubation with Fe^{2+}). The effectiveness of these "chase" conditions in preventing further incorporation of radioactive proline into protein, insures

that the hydroxylation observed on the released peptides was not obscured by the hydroxylation that was taking place normally on the polysomes.

While ours is, we believe, the first direct demonstration that hydroxylation is not needed for release of nascent collagen chains, and that released chains can be hydroxylated, these conclusions were suggested by, and are consistent with, earlier work with chick embryos. Bhatnagar and Prockop had reported that, when hydroxylation was inhibited by dipyridyl, protocollagen continued to be synthesized for at least 4 hours at about two-thirds the normal rate of collagen synthesis (*4*). Furthermore, after reversal of the inhibition, the accumulated protocollagen was hydroxylated by the tissues at the same rate as newly synthesized collagen chains (*5*). It had also been shown (*4*) that apparently complete, unhydroxylated alpha chains could be isolated from chick embryo minces incubated with dipyridyl, a finding consistent with release as well as completion of nascent chains, in the absence of hydroxylation. Our work confirms these conclusions and leads to the realization that, while hydroxylation of proline normally occurs on nascent collagen chains, it can also occur on released chains when hydroxylation has been temporarily inhibited. It appears therefore that it is not crucial whether the collagen chain is nascent or released in order to have hydroxylation of proline take place.

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References and Notes

1. E. Lazarides, L. N. Lukens, A. A. Infante, *J. Mol. Biol.* **58**, 831 (1971).
2. R. L. Miller and S. Udenfriend, *Arch. Biochem. Biophys.* **139**, 104 (1970).
3. J. L. Hutton, Jr., A. L. Tappel, S. Udenfriend, *Anal. Biochem.* **16**, 384 (1966).
4. R. S. Bhatnagar and D. J. Prockop, *Biochim. Biophys. Acta* **130**, 383 (1966).
5. ———, J. Rosenbloom, *Science* **158**, 492 (1967).
6. L. N. Lukens, *J. Biol. Chem.* **245**, 453 (1970).
7. B. Goldberg and H. Green, *J. Mol. Biol.* **26**, 1 (1967).
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Isolation of Mycoplasmatales Viruses and Characterization of MVL1, MVL52, and MVG51

Abstract. *Eleven Mycoplasmatales viruses are now known. Burst size, burst time, sensitivity to ultraviolet and the host range of three viruses that were studied are different. However, all three are naked, rod-shaped particles of similar size. Plaque morphology and the isolation of immune cells suggest that both virulent and nonvirulent infections are possible.*

Gourlay (*1*) described the first isolation of a Mycoplasmatales virus, which infects *Mycoplasma laidlawii* strain BN1 [renamed *Acholeplasma laidlawii* BN1 (*2*)], and named this agent MVL1 (*2*). Using BN1 as an indicator strain, we have isolated two more viruses, designated MVL52 and MVG51, which have properties different from those of MVL1. During an examination of other possible host strains, eight additional virus isolations have been made; these eight have not yet been characterized.

Cells of BN1 were grown either in a liquid tryptose medium or on a solid tryptose medium containing 1 percent agar (*3*). The transfer to this medium from the richer GS medium of Gourlay (*1*) affected neither cell growth (the generation time is about 2 hours) nor MVL1 viral growth.

For plaque assays, viruses are added to plates containing 2- to 6-hour-old indicator lawns; plaques are counted after 24 hours' growth at 37°C (Fig. 1). Because a naladixic acid–resistant clone of BN1, designated BN1 Nal^r, gives more distrinct plaques than BN1, it is used for the indicator lawn. To prepare virus stocks, plates with confluent plaques are washed for 6 to 16 hours at 37°C with phosphate buffer (*p*H 7.4) and filtered through a Millipore filter (pore size, 0.22 μm); this yields virus titers of about 10^{12} per milliliter.

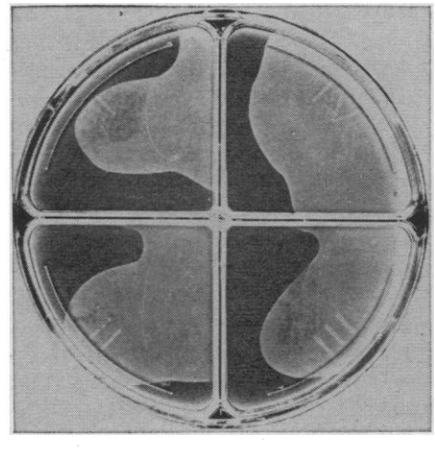

Fig. 1. Growth of BN1 Nal^r lawn (light areas) on 100-mm tryptose agar plates, showing Mycoplasmatales virus plaques. Sector I, lawn infected with MVL1; sector II, with MVL52; sector III, with MVG51; and sector IV, uninfected control lawn. Many plaques have a halo appearance; this can be seen particularly in sector III.

In an attempt to isolate more viruses, 24-hour lawns of three *Mycoplasma* were washed and plated on BN1. Plaques were observed from the washings from *A. laidlawii* B and *Mycoplasma* sp. strain 14 (goat) [these strains are described by Tourtellotte and Jacobs (*4*)]. The resulting plaque-forming units were designated MVL52 and MVG51, respectively. As an amendment to the nomenclature scheme of Gourlay, Bruce, and Garwes (*2*), MV, the Mycoplasmatales virus symbol, is followed by a letter designating the species origin of the virus (in our case, either G or L for goat or *laidlawii*) and then an isolation number (in our case, starting in the fifties).

In order to determine the degree of similarity of the three Mycoplasmatales viruses, a number of their properties were measured. Ultraviolet irradiation studies showed that MVL1, MVL52, and MVG51 inactivation follows one-hit kinetics (Fig. 2). The three viruses have different inactivation cross sections: 0.0020 mm^2/erg for MVL1, 0.0030 mm^2/erg for MVL52, and 0.0044 mm^2/erg for MVG51. Since, as discussed below, the three viruses are about the same size, the different cross sections may reflect differences in the virus nucleic acid structures.

To study virus growth, about 10^6 viruses and 10^6 cells were mixed in liquid media. After allowing 10 minutes for adsorption, a portion was filtered through a Millipore filter (pore size, 0.22 μm) to remove cells, and the filtrate was assayed for virus. Only about 0.1 percent of the virus was recovered in the filtrate. Corrections for the free virus titers were made in the studies below.

When BN1 Nal^r cells were mixed with MVL1, at a multiplicity of infection of 0.5, 32 percent of the cells were assayed as infective centers. When one considers a Poisson distribution of