constitute acceptable proof that they are in fact viruses. However, these results do provide evidence that human milks contain particles that are similar in several respects to the known RNA tumor viruses of animals: a density of 1.16 to 1.19 g/ml and the presence of both the reverse transcriptase and the 60*S* to 70*S* RNA.

Further experiments relating particles to human neoplasia now become possible. Human breast tumors contain RNA that is homologous to the RNA of the MMTV (*11*). Of particular interest will be the outcome of molecular hybridization experiments that examine the extent of homology between the RNA of the particle in human milk and the RNA of human neoplastic tissues as well as the RNA of known animal tumor viruses.

J. SCHLOM, S. SPIEGELMAN
Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York 10032
D. H. MOORE
Institute for Medical Research, Camden, New Jersey 08103

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15 December 1971

Procollagen: Conversion of the Precursor to Collagen by a Neutral Protease

Abstract. *An enzymatic activity (procollagen peptidase), capable of converting the biosynthetic precursor procollagen to collagen at neutral* p*H, has been identified in rat and chick calvarial bone. Limited proteolysis of procollagen with chymotrypsin resulted in a similar transformation. The activity in bone can be demonstrated in vitro despite inhibition of new collagen synthesis by cycloheximide. Preservation of the collagen precursor in preparations extracted with acetic acid results from inhibition of the enzymatic activity at low* p*H.*

Recent studies have provided evidence for the existence in rat calvaria of a biosynthetic precursor of collagen, procollagen (*1*). Procollagen was shown to contain the pro-α1 chain (*2*) which differed from α1 of extracellular fibrous collagen in its chromatographic properties, molecular weight, and relative proline and hydroxyproline contents. We have evidence that the procollagen molecule also contains an analogous pro-α2 chain.

A time-dependent conversion of molecules containing pro-α1 to those containing α1 was shown by short-term labeling experiments (*1*). Such conversion could be simulated in vitro by limited proteolysis with pepsin. A collagen fraction with some of the properties of procollagen was also identified in the medium of cultured human fibroblasts (*3*) and chromatographic evidence for the pro-α1 chain was obtained in studies of collagen synthesis in which a cell-free system was used (*4*). In chick calvaria, inhibition of proline hydroxylation with the chelating agent α,α'-dipyridyl resulted in the synthesis of underhydroxylated precursors to the pro-α1 chain (*5*).

It was proposed that the additional sequences in procollagen serve to initiate helix formation and to inhibit intracellular fibrogenesis (*1*). The conversion of procollagen to collagen would therefore be required before a collagen molecule could be incorporated into a functional extracellular fiber. The limited proteolysis necessary for this conversion might occur intracellularly during the secretion of collagen, on the cell membrane, or extracellularly. We now report that a soluble proteolytic activity obtained from chick and rat calvaria, for which we propose the trivial name procollagen peptidase, is capable of converting procollagen to collagen. Some characteristics of this activity and a similar limited proteolysis of procollagen by chymotrypsin are also described.

Calvaria (frontal and parietal bones) from newborn rats or 17-day-old chick embryos were incubated in Dulbecco's modification of Eagle's medium supplemented with β-aminopropionitrile fumarate (64 μg/ml), sodium ascorbate (100 μg/ml), penicillin (8 unit/ml), and streptomycin (8 μg/ml). After an initial 60-minute incubation at 37°C, bones were transferred to medium containing L-[2,3-^{3}H]proline (20 μc/ml) and incubated for 18 minutes. A labeled procollagen fraction was extracted from homogenized bones with cold 0.5*M* acetic acid and dialyzed extensively against this solution. In other experiments labeled bones were extracted with 1*M* NaCl containing 0.05*M* tris-HCl, *p*H 7.5, or with the NaCl-tris buffer with 0.1 percent Triton X-100.

In order to prepare procollagen peptidase, chick or rat calvaria were homogenized and extracted at 4°C for 18 hours with a buffer containing 0.15*M* NaCl, 0.05*M* tris-HCl, *p*H 7.5, and $5 \times 10^{-3}M$ $CaCl_2$. The extract was centrifuged at 100,000*g* for 60 minutes, and the supernatant was concentrated by pressure filtration. Incubation of the enzymatic activity with labeled procollagen substrate was performed at 20°C for 24 hours, and the reaction was terminated by dialysis against *p*H 4.8 acetate buffer at 4°C.

The ability of a neutral salt extract of chick calvaria to convert chick calvarial procollagen to collagen is demonstrated in Fig. 1. In these and subsequent chromatograms the procedure for the fractionation of collagen chains on carboxymethylcellulose (*6*) was modified by addition of 4*M* urea to all buffers (*1*). The content of procollagen and collagen in the starting material, as judged by the relative sizes of the pro-α1 and α1 peaks in Fig. 1A, was very similar to that in preparations from rat calvaria (*1*). Since the recovery of counts was the same in the two chromatograms, selective degradation or precipitation of procollagen by the tissue extract is excluded. Incuba-

tion of procollagen under identical conditions with as much as 1 ml of fresh, adult chicken serum resulted in no conversion to collagen. Enzymatic activity was lost by heating the tissue extract to 65°C for 20 minutes, but no inhibition was seen with soybean trypsin inhibitor (0.5 mg/ml) or $10^{-4}M$ diisopropylfluorophosphate (DFP).

Although chick calvarial extracts readily converted procollagen to collagen, no effect was noted on the carrier rat collagen, as judged by the similar contents of α and β components in Fig. 1, A and B. This was not due to a species difference in enzyme and substrate since the use of rat procollagen, calvarial extract, and carrier collagen yielded the same results.

In contrast to the action of calvarial extracts, limited proteolysis of native collagen with chymotrypsin effects conversion of molecules with intramolecularly cross-linked β components to molecules containing largely α-like chains (*7*). To determine whether chymotrypsin is also able to achieve conversion of procollagen to collagen, [^{3}H]procollagen was supplemented with carrier acid–extracted rat skin collagen and treated with chymotrypsin (10 percent by weight of the carrier protein) at *p*H 7.6 and 20°C for 24 hours. The reaction was terminated by addition of DFP ($10^{-3}M$), and the material was chromatographed on carboxymethylcellulose. The data (not shown) indicated that limited proteolysis of the carrier acid–extracted rat skin collagen occurred, as was demonstrated by a marked increase in the ratio of α to β components. Furthermore, chymotrypsin effectively converted procollagen to a collagen-like molecule, since, as in the case of preparations of procollagen treated with calvarial extract, the activity in the α1 region of the chymotrypsin-treated preparation equaled the sum of the activities in pro-α1 plus α1 in the control. Selective complete degradation of procollagen by chymotrypsin is therefore excluded.

If procollagen is converted to collagen enzymatically in vivo, this conversion might be expected to proceed in calvaria in the absence of new protein synthesis. After an initial preliminary incubation, rat calvaria were labeled for 18 minutes with [^{3}H]proline. A portion of the bones was homogenized directly and extracted with acetic acid. A second portion was transferred to medium containing 250 μg of cycloheximide and 3 μc of L-[^{14}C]proline per milliliter and incubated for an additional 60 minutes. A third portion was incubated for the same time in medium containing [^{14}C]proline but lacking cycloheximide. The carboxymethylcellulose elution pattern of procollagen incubated in the presence of [^{3}H]proline for 18 minutes is shown in Fig. 2A. When such calvaria were transferred to medium containing cycloheximide and [^{14}C]proline, essentially complete conversion of procollagen to collagen occurred in the absence of new collagen synthesis. Thus, the pro-α1 fraction is replaced by chains eluting in the position of α1 despite the lack of incorporation of [^{14}C]proline into collagen (Fig. 2B). However, the calvaria were capable of new protein synthesis in the absence of cycloheximide (Fig. 2C). In this last experiment the labeled pro-α1 fraction was small in comparison with the α1 fraction because a relatively long biosynthetic period (60 minutes) was used.

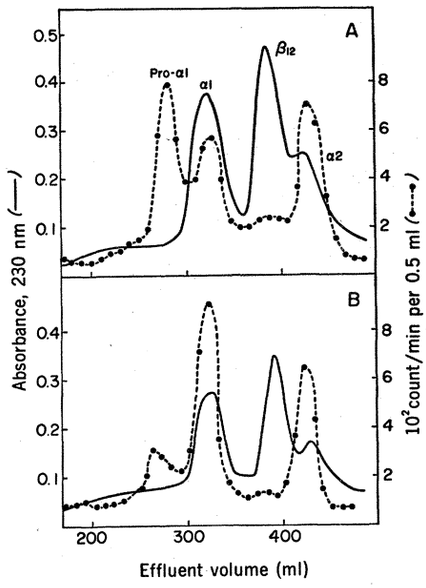

Fig. 1. Carboxymethylcellulose chromatography of preparations of [^{3}H]procollagen from chick calvaria. Samples were heat-denatured and chromatographed at *p*H 4.8 in 4*M* urea. Elution was achieved with a linear gradient of NaCl from 0 to 0.10*M* over a total volume of 620 ml. A constant amount of carrier acid–extracted rat skin collagen was added to each sample. (A) Buffer control; (B) treated with calvarial extract.

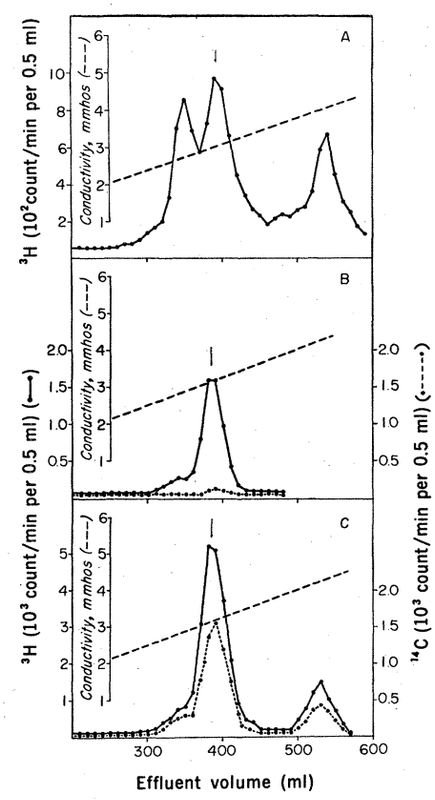

Fig. 2. Carboxymethylcellulose chromatography of acetic acid extracts of rat calvaria incubated with [^{3}H]proline for 18 minutes. (A) Control. (B) Extract of bones incubated for an additional 60 minutes with cycloheximide and [^{14}C]proline. (C) Extract of bones incubated for an additional 60 minutes with [^{14}C]proline. The arrow indicates the position of elution of the α1 chain. Solid line [^{3}H]incorporation; dotted line, [^{14}C]incorporation.

When calvaria were extracted at 4°C with 1*M* NaCl containing Triton X-100 at neutral *p*H over a period of 48 hours, little or no pro-α1 chain was found. However, homogenization of calvaria in this buffer and immediate rapid dialysis against *p*H 4.8 buffer yielded large amounts of pro-α1 (data not shown). Salt solutions without detergent extracted considerably smaller amounts of procollagen, which was also converted to collagen unless the extract was acidified. These findings indicate that both acidic and neutral salt solutions extract procollagen and suggest that procollagen peptidase, extracted concomitantly at neutral *p*H, converts procollagen to collagen even at 4°C. This enzymatic activity is apparently irreversibly inhibited at low *p*H since the procollagen extracted at low *p*H is not converted to collagen when such solutions are subsequently neutralized. These observations may account for the absence of procollagen

in 1*M* NaCl extracts of calvaria in the experiments of Vuust and Piez (*8*).

Our studies demonstrate that a proteolytic activity, active at neutral *p*H, is capable of conversion of procollagen to collagen in bone. The existence of a preformed enzymatic activity is consistent with the observation that conversion of procollagen to collagen proceeds despite inhibition of collagen synthesis by cycloheximide. The enzymatic activity is not trypsin-like and is not a serine protease as indicated by lack of inhibition with soybean trypsin inhibitor or DFP. However, since relatively unpurified extracts were used as a source of enzymatic activity, it is possible that the physiologically important enzyme is susceptible to inhibition by these compounds and that, under these circumstances, conversion of procollagen is achieved in a less specific fashion by extraneous resistant enzymes in the extract.

Recently, cattle afflicted with a heritable disorder, dermatosparaxis, were found to contain a dermal collagen fraction which was defective in its fibrogenic properties and contained chains higher in molecular weight than α chains (*9*). This collagen fraction may represent procollagen, or a derivative thereof, which accumulates as a result of a defect in procollagen peptidase. Relatively large amounts of a collagen fraction having some of the characteristics of procollagen were also identified in the culture medium of normal human fibroblasts (*3*). Unlike procollagen, however, this medium fraction does not dissociate to pro-α chains under denaturing conditions. Procollagen might escape limited proteolysis in cell culture if procollagen peptidase were inhibited by a factor in the fetal calf serum used as a component of the culture medium or if the enzymatic activity were bound to the cell membrane and thence unable to efficiently cleave a substrate capable of diffusion into the medium.

PAUL BORNSTEIN
H. PAUL EHRLICH
ANNE W. WYKE
Departments of Biochemistry and *Medicine, University of Washington, Seattle 98195*

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10 September 1971

Epidemic Strain of Venezuelan Equine Encephalomyelitis Virus from a Vampire Bat Captured in Oaxaca, Mexico, 1970

Abstract. *A vampire bat,* Desmodus rotundus, *captured in Oaxaca, Mexico, in August 1970, was found to be infected with the epidemic strain of Venezuelan equine encephalomyelitis virus at the same time that an equine epizootic was occurring there.*

In 1968 an explosive and lethal outbreak of encephalitis in horses occurred in Guatemala (*1*). The strain of Venezuelan equine encephalomyelitis (VEE) virus that was isolated from horses there is antigenically related to the epidemic strain of VEE virus that has been isolated in Venezuela (*2*) and other Latin American countries (*3*). By 1969 this virus had spread to Costa Rica (*4*), and by 1970 into the southeastern Mexican states of Chiapas and Oaxaca (*5*). In early 1971 the virus appeared to have "jumped" into the Tampico, Tamaulipas (Mexico) area, with the occurrence of equine deaths there, also (*6*).

The existence of VEE virus in Mexico was first established in 1962 (*7*), but its geographic range appeared limited to the southeastern states of the republic (*8*). In 1966, an equine encephalitis epizootic in the Tampico area (*9*) killed approximately 300 of 1000 horses. Of 231 surviving horses 52 (22.5 percent) had hemagglutination-inhibiting antibodies to VEE virus 3 months after the epizootic (*10*).

During the 1970 outbreak in Mexico various specimens were collected from the affected area of eastern Oaxaca and tested. We now report on one isolation from that study.

Eighteen bats were examined for virus: these included six *Balantiopteryx plicata,* eight *Mollosus* sp., and four unsexed vampire bats (*Desmodus rotundus*). The vampires were collected at an abandoned well in San Francisco Ixhuatan, Oaxaca, on 10 August 1970. At the time of capture they were exsanguinated by cardiac puncture; the blood was allowed to clot, and serum was removed, frozen, and stored. When the animals were killed, the submaxillary salivary glands, brown fat, brain, and viscera (lung, heart, kidney, liver, and spleen) were removed; separate, sterile instruments were used for each organ or set of organs; the specimens were frozen at −70°C. The specimens were taken to the Instituto Nacional de Investigaciones Pecuarias, Palo Alto, D.F., Mexico, and later sent to the Center for Disease Control in Atlanta, Georgia, where they were processed for virus isolation.

Four of six suckling mice inoculated intracerebrally with a 10 percent clarified suspension of the viscera of one of the four vampire bats (No. 111) died 2 days after the inoculation. The virus was established in suckling mice by two subsequent intracerebral passages. The titer of the material of the isolate after the third passage was $10^{10.8}$ suckling mouse intracerebral LD_{50}/ml. The isolate was identified as VEE virus by neutralization tests in weanling mice with the use of hyperimmune mouse ascitic fluid prepared

Table 1. Identification of bat 111 isolate as VEE virus by neutralization test performed in suckling mice. The results are expressed as the log of the neutralization index; VEE, Venezuelan equine encephalomyelitis; EEE, Eastern equine encephalomyelitis; WEE, Western equine encephalomyelitis. Strains are shown in parentheses.

Virus	Neutralization by hyperimmune ascitic fluid to:		
	VEE (FE3-7C)	EEE (NJO)	WEE (Fleming)
Bat 111	2.7	0	0
VEE (GJ9/1BJ)	4.7	0	0
EEE (NJO)	< 1.0	2.9	< 1.0
WEE (Fleming)	< 1.0	< 1.0	3.0