nous blood is saturated with O_2 there can be no chemical removal of heat from tissue by blood. Heat can then accumulate in a tissue, such as the brain, which is not readily able to increase its perfusion rate. Hence it is interesting that neurological oxygen toxicity becomes manifest at about the point of venous saturation of hemoglobin and that it is particularly sensitive to temperature—a man with fever readily convulses in hyperbaric oxygen (*13*).

Finally, this discussion has merely touched upon a few of the most obvious implications of this novel concept of chemical facilitation of heat transfer in physiological systems.

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Collagen Synthesis: Localization of Prolyl Hydroxylase in Tendon Cells Detected with Ferritin-Labeled Antibodies

Abstract. *An improved procedure was employed for linking ferritin to antibodies against prolyl hydroxylase, the enzyme that synthesizes the hydroxyproline in collagen. By electron microscopy, the enzyme was then found to be localized in cisternae of the rough endoplasmic reticulum of embryonic tendon cells; this indicates that hydroxylation of proline occurs while newly synthesized polypeptides are fed into the cisternae.*

The collagen molecule is synthesized by a series of steps that include (i) assembly of amino acids into each of the three polypeptide chains of the molecule, (ii) hydroxylation of some of the prolyl and lysyl residues in the polypeptide chains, and (iii) glycosylation of some of the hydroxylated lysyl residues before the molecule is secreted [for review, see (*1*)]. Some of the hydroxylations of proline and lysine can occur while the nascent chains are still being assembled on ribosomes, but these residues can also be hydroxylated after chain completion. If the prolyl and lysyl hydroxylases are inhibited, freshly isolated connective tissues or cells synthesize and accumulate an unhydroxylated form of collagen known as protocollagen (*1, 2*). If inhibition of the hydroxylases is reversed under appropriate conditions, the accumulated protocollagen is hydroxylated and secreted. Although these and related observations demonstrate that the molecule must be extensively modified before it is ready for secretion, relatively little is known about which cellular organelles are involved. For example, although collagen-like aggregates have been observed in Golgi vacuoles (*3*), it has not been established that collagen is synthesized and secreted via the rough endoplasmic reticulum and Golgi complex (*4*), as shown for several other proteins synthesized for "export" (*5*). In the experiments reported here, improved procedures with ferritin-labeled antibodies were used to locate prolyl hydroxylase, the enzyme that synthesizes hydroxyproline in collagen.

Prolyl hydroxylase was purified from chick embryos by means of an affinity column procedure (*6*), and antibodies were prepared in rabbits (*7*). The antibodies were purified by immunoadsorption on a column containing pure prolyl hydroxylase covalently bound to 1 percent agarose (Bio-Rad, A-150m) (*8*). The column was eluted with 3*M* sodium thiocyanate in 0.01*M* sodium phosphate, *p*H 6.0 (*9*), and the purified antibody was linked to ferritin with glutaraldehyde by an improved procedure (*10*). Ferritin (Polysciences; six times recrystallized) was activated by incubation (3.3 mg/ml) in a solution of 1.7*M* glutaraldehyde (Baker) in 0.1*M* sodium phosphate, *p*H 7.3, at room temperature for 30 minutes. The activated ferritin was separated from excess glutaraldehyde by gel filtration on a Sephadex G-25 (Pharmacia) column equilibrated and eluted with 0.1*M* sodium phosphate buffer, *p*H 7.3. Purified antibody (1 mg) was then added to 2 mg of activated ferritin in 11 ml of the phosphate buffer. After 26 hours at 4°C, the product was isolated by gel filtration on a 6 percent agarose column (Bio-Rad, A-5m) equilibrated and eluted with 0.1*M* tris(hydroxymethyl)-aminomethane (tris) hydrochloride buffer, *p*H 7.5, at 4°C. The method was an improvement over earlier procedures (*11*) in that the yields were more than tenfold greater, the product was free of unconjugated antibody, the antibody retained most of its immuno-

Treatment	Enzyme protein per 10^8 cells	
	Micrograms	Percent of total
Homogenate (nonfixed)	2.7	100
Supernatant 1	0.25	9
Supernatant 2	0.57	21
Cell fraction	1.36	50

Table 1. Determination of prolyl hydroxylase in fixed cell preparations by passive hemagglutination inhibition. Tendon cells (5×10^8) were incubated in 66 ml of modified Krebs medium at 37°C for 3 hours (*2*). The sample was divided in half, and the cells were separated from the medium by centrifugation. The cells from half of the sample were suspended in 2.5 ml of 0.1*M* sodium phosphate buffer, *p*H 7.3, and homogenized with 60 strokes in a Teflon and glass homogenizer at 1740 rev/min with a constant-torque motor (Schwaben Präzision, Nördlingen, Germany). The homogenate was used to measure total enzyme antigen. The cells from the other half of the sample were fixed by incubation for 1 hour at 4°C in 2.5 ml of a solution containing 1 percent formaldehyde (Baker), 0.06*M* sodium phosphate buffer, *p*H 7.3, and 0.14*M* sucrose. The cells were removed by centrifugation and washed with 2.5 ml of 0.1*M* sodium phosphate buffer, *p*H 7.3. The wash was combined with the fixing solution; dialyzed against 500 ml of buffer containing 0.2*M* NaCl, 0.1*M* glycine, and 0.01*M* tris-HCl, *p*H 7.8, at 4°C; and concentrated to 0.5 ml in an Amicon ultrafiltration cell with a PM-30 membrane. This sample was supernatant 1. The cell pellet was suspended in 2.5 ml of 0.1*M* sodium phosphate buffer, homogenized with 30 strokes as described above, and centrifuged at 20,000*g* for 20 minutes. The supernatant and pellet were supernatant 2 and the cell fraction, respectively. Enzyme protein was measured by a passive hemagglutination inhibition technique as described by Eskeland *et al.* (*15*), except that 0.1 percent bovine serum albumin was used instead of 0.1 percent gelatin. Formalin-treated sheep blood cells were coated with prolyl hydroxylase (*7*) and a standard curve with purified prolyl hydroxylase was prepared (*6*).

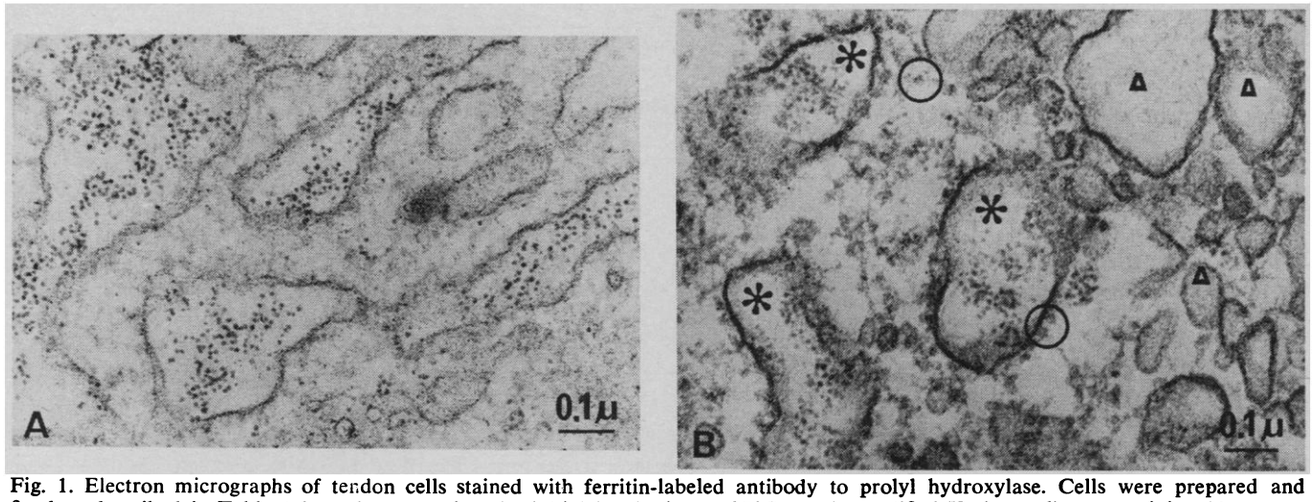

Fig. 1. Electron micrographs of tendon cells stained with ferritin-labeled antibody to prolyl hydroxylase. Cells were prepared and fixed as described in Table 1 legend, except that the initial incubation at 37°C was in modified Krebs medium containing 10 percent fetal calf serum. The cell fraction (Table 1) from 5×10^7 cells was incubated with 150 μl of ferritin-labeled antibody to prolyl hydroxylase (1.3 mg of ferritin per milliliter) at 4°C for 24 hours. The cell fragments were washed three times with 5 ml of 0.1*M* sodium phosphate buffer, *p*H 7.3, and fixed for 1 hour at 4°C with a solution of 3 percent glutaraldehyde, 0.06*M* sodium phosphate buffer, *p*H 7.3, and 0.14*M* sucrose. The cell fragments were washed with 0.1*M* sodium phosphate buffer and postfixed for 1 hour at 4°C with a solution of 1 percent OsO_4, 0.06*M* sodium phosphate buffer, *p*H 7.3, and 0.16*M* sucrose. After washing with 0.9 percent NaCl, the cell fragments were stained with 0.5 percent magnesium uranyl acetate (Polysciences) for 30 minutes at room temperature. The sample was dehydrated with ethanol and embedded in Araldite (Taab). Ultrathin sections were stained with bismuth subnitrate (*14*) and examined with the JEM-100B electron microscope (JEOL). (A) Electron micrograph of a section lightly stained with bismuth subnitrate to emphasize the distribution of the ferritin within membrane-bound compartments. Because of the light staining, the ribosomes on the outer surface of the rough endoplasmic reticulum are not sharply defined. (B) Electron micrograph of a section more heavily stained with bismuth subnitrate to demonstrate that the membrane-bound compartments containing the ferritin consist of rough endoplasmic reticulum. The asterisks indicate rough endoplasmic reticulum containing ferritin, circles indicate ribosomes, and triangles indicate smooth-surfaced elements of the Golgi complex. The Golgi complex was consistently free of ferritin.

logical activity, and the conjugate was free of ferritin-ferritin aggregates (*10*).

Experiments for locating prolyl hydroxylase were done with matrix-free cells from chick embryo tendons. The cells had been characterized in terms of their capacity to synthesize and secrete the precursor form of collagen known as procollagen (*2*), and studies with peroxidase-labeled antibodies and light microscopy demonstrated that prolyl hydroxylase was distributed in localized regions of the cytoplasm (*7*). Electron microscopy (*10*) showed that the cells were rich in rough endoplasmic reticulum and had a prominent Golgi complex. In addition, the cytoplasm contained relatively large amounts of microtubules and microfilaments.

To overcome problems inherent in the use of labeled antibodies for determining intracellular localization of antigens (*11*), the tendon cells were fixed and homogenized before labeling with the antibody. This new procedure allowed adequate penetration of the ferritin-antibody conjugate, preserved the morphology of intracellular organelles, and permitted quantitation of the antigenicity of the protein visualized by electron microscopy. Incubating either purified prolyl hydroxylase or tendon cells with 1 percent glutaraldehyde for 1 hour at 4°C appeared to destroy most of the antigenicity of the enzyme. Therefore, glutaraldehyde was not used as fixative when enzyme localization was studied. In contrast, treatment with formaldehyde had little effect on the antigenicity of pure enzyme, and after fixation with 1 percent formaldehyde for 1 hour at 4°C, 60 to 80 percent of the initial antigenicity was still present in the cell preparation (Table 1). To assure adequate penetration of the ferritin-antibody conjugate, the cells were homogenized after fixation with formaldehyde. Most of the antigen remained in the cell fragments after homogenization (Table 1).

When the fixed and homogenized cells were treated with ferritin-labeled antibody, most of the ferritin was seen within the rough endoplasmic reticulum (Fig. 1A). The ferritin appeared to be distributed throughout the lumen of the cisternae. Some of the cisternae were highly labeled, but ferritin distribution was variable, and a few of the cisternae were unlabeled. As discussed elsewhere (*10*), the variation in the labeling of the cisternae might reflect variations in enzyme content of various regions of the endoplasmic reticulum, incomplete penetration by the labeled antibody, or some loss of cisternal contents during the preparative procedures. There was a small amount of labeling of nuclei which appeared to be nonspecific (as indicated in a control experiment described below), but there was no significant labeling of any other cellular compartment or structure, including the smooth-membraned elements in the Golgi region of the cells. In further experiments, the cells were incubated for 3 hours with 0.3 m*M* α,α'-dipyridyl and 10 percent fetal calf serum so that they synthesized and accumulated protocollagen (*2*). The distribution of ferritin-labeled antibody to prolyl hydroxylase was the same as in cells not incubated with α,α'-dipyridyl, a result indicating that the enzyme was not displaced in cells that had accumulated protocollagen.

Two types of control experiments were done with fixed and homogenized cells. In one, it was shown that free ferritin could enter all the subcellular organelles, including cisternae of endoplasmic reticulum, mitochondria, nuclei, and Golgi vacuoles (*10*). In a second, the cells were treated with nonimmune rabbit immunoglobulin G labeled with ferritin by the same proce-

dure used to prepare the antibody-ferritin conjugate. There was no significant labeling of any cellular organelle except the nucleus, which was slightly labeled.

The observations with ferritin-labeled antibody demonstrate that prolyl hydroxylase is found within the cisternae of the endoplasmic reticulum. Since separate assays of antigenicity and of enzyme activity in cell homogenates established that most of the enzyme in the matrix-free tendon cells was in an active form (*10*), the distribution of ferritin-labeled antibody reflects the location of active enzyme. The ferritin label was distributed throughout the cisternae, but the results do not exclude the possibility that the enzyme is loosely bound to the inner surface of the cisternal membrane and is displaced into the lumen by the preparative procedures. However, the results do appear to exclude the possibility that the active enzyme is on the ribosomal side of the endoplasmic reticulum, in the cytoplasm, or in the Golgi vacuoles.

The localization of prolyl hydroxylase in the cisternae has several consequences. Some hydroxylation of proline can occur in nascent, incomplete peptides (*1*), and prolyl hydroxylase cannot hydroxylate protocollagen polypeptides in a triple-helical conformation (*12*). Therefore, the newly synthesized polypeptides are fed into the cisternae during synthesis, and when the polypeptides enter the cisternae, they are in a random-coil form. Since under appropriate conditions protocollagen accumulates in connective tissue cells and can be hydroxylated 1 to 3 hours after synthesis (*13*), accumulated protocollagen must be retained in the cisternae until the hydroxylation occurs.

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Plant Mycoplasmas: Serological Relation between Agents Associated with Citrus Stubborn and Corn Stunt Diseases

Abstract. *Growth-inhibition and precipitin tests established that antigens of the helical mycoplasma-like organism* (Spiroplasma citri) *associated with citrus stubborn disease are serologically related to antigens in corn infected with stunt disease but not in healthy corn.*

There has previously been no reason to suspect a relationship between citrus stubborn and corn stunt diseases. Recently, however, an agent suspected to cause the citrus stubborn disease was cultured from diseased citrus (*1*), and was shown to have many of the properties of the class Mollicutes (mycoplasmas). More detailed biochemical and serological studies of the organism indicated that it was a new distinct species, but also confirmed its general similarity to other mycoplasmas (*2*). Phase-contrast microscopy of broth cultures revealed that helical filaments were the predominant form of the organism. Ultrastructural studies reaffirmed the morphological structure of these microbes and also revealed the presence of a tailed bacteriophage (*3*). It was proposed that the organism recovered from citrus stubborn be named *Spiroplasma citri* (*2*); the organism has not been assigned to higher taxons.

Table 1. Growth-inhibition tests (disc method) with *Spiroplasma citri* and antiserums to corn stunt disease and healthy corn tissues. Fractionation of corn and corn stunt antigens is described in the text. Tissue was adsorbed with freeze-dried antigen from centrifuged cultures or fractions of plant extracts.

Antiserum	Growth-inhibition zones (mm) to *Spiroplasma citri* cultures	
	Moroccan isolate	California isolate
Unadsorbed serum		
Corn stunt fraction	5–6	4–5
Corn fraction	0	0
S. citri		
Moroccan isolate	10	7–9
California isolate	7–9	6–8
A. laidlawii (PG-8)	0	0
Adsorbed serum		
Corn stunt tissue		
Unadsorbed	3–4	4–5
Absorbed (corn stunt)	3	1
Adsorbed (healthy corn)	3	4
Adsorbed (*S. citri* Morocco)	0	0
Adsorbed (*A. laidlawii* PG-8)	4	5

The helical organisms associated with citrus stubborn disease are similar to bodies found associated with, and suspected to cause, corn stunt disease (*4*), a disease that affects both plants and the insect vectors that transmit it (*5*). The helical bodies associated with corn stunt, whose appearance in ultrathin sections is mycoplasma-like, can be seen as helices in the phloem of infected corn by techniques such as freeze-etch electron microscopy (*4*). Unfortunately, although the corn stunt agent can be maintained in an infectious state for up to 48 days in primary cultures (*6*), it has not been grown in continuous cell-free culture.

Both *S. citri* and the corn stunt agent have helical structures and since *S. citri* was characterized as an unusual mycoplasma we believed that techniques employed in the serological analyses of mycoplasmas might yield useful information on the relationship between the two microorganisms.

Antiserums to the corn stunt agent were prepared from diseased plant