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Tom Bauer and Robert Glaeser

DONNER LABORATORY

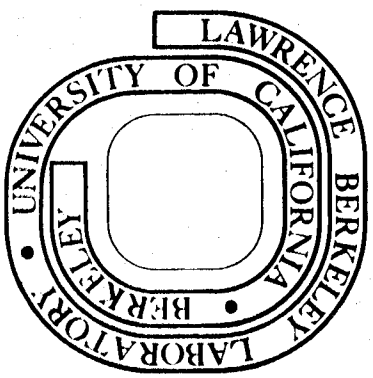
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ELECTRON MICROSCOPY OF HYDROCARBON PRODUCTION IN GREEN PLANTS*

By

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*Prepared for the U.S. Energy Research & Development Administration under Contract W-7405-Eng-48

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I. Introduction

This year the land area of the United States will receive one thousand times as much energy from the sun as our society will use (1). Plants can be cultivated which convert the incident solar flux to chemical bond energy at efficiencies of approximately 1% (2). Three hundred million acres of "energy plantation" would produce our current energy needs forever, or at least until the sun burns out. For comparison, the United States currently has about three hundred fifty million acres under cultivation (3).

Besides energy, our society requires a great number of materials currently derived from declining oil supplies. These materials -- lubricants, plastics, rubber, etc., -- can also be biologically produced; for example, Malaysian rubber trees currently produce two thousand pounds of rubber per acre (4). The potential for growing hydrocarbon materials in the United States is great and largely untapped. This paper describes our current and proposed research into hydrocarbon production in green plants.

II. The Biochemistry of Alkene Production -- The Terpene Metabolism

Figure 1 is a simplified representation of some of the more basic biochemical reactions. The Calvin Cycle operates in the chloroplast to produce phosphoglyceric acid and pyruvic acid. These are the precursors for almost all biological compounds. Also shown is the role of mitochondria.

Our current research is directed towards understanding the production of alkenes -- particularly rubber. However, plants also produce alkanes and acetylenes (5,6), and their generation through the lipid metabolism pathway is shown in Fig. 1.

In alkene production, two molecules of acetyl CoA condense to form acetoacetyl CoA, followed by another acetyl CoA condensation reaction which produces beta hydroxy beta methyl glutaryl CoA. This product is also a

precursor of lipids, as shown in Fig. 1. However, mevalonic acid, produced from beta hydroxy beta methyl glutaryl CoA, is involved only in the terpene (alkene) metabolism.

Referring to Fig. 2, mevalonic acid is twice phosphorylated to produce mevalonic acid pyrophosphate acid (MVAPP). In an important reaction to be dealt with later, a carbon is removed from MVAPP, and isopentyl prophosphate (IPP) -- a 5-carbon compound -- is formed. All chain extensions in the terpenes (including rubber) are by addition of IPP.

Chain initiation is by an isomer of IPP, dimethylallyl pyrophosphate (DMAPP). Condensation of DMAPP with IPP produces the 10-carbon pyrophosphates as shown. From these are formed the monoterpenes, camphor, terpentine, limonene, etc.

IPP can add on to the 10-carbon geranyl pyrophosphate to produce the farnesyl pyrophosphates (Fig. 3). From these are derived 15-carbon sesquiterpenes including wood oils, resins, and some growth regulators. Also, two of the 15-carbon pyrophosphates can dimerize tail-to-tail to produce squalene, the precursor of the steroids.

IPP can add to the end of the 15-carbon pyrophosphate to produce the 20-carbon geranyl pyrophosphate. As shown, these molecules can dimerize tail-to-tail to produce the 40-carbon carotenoid pigments which are involved in photosynthesis. From all the above, the importance of the terpenes should be apparent.

As shown in Fig. 4, chain extension continues by addition of the 5-carbon IPP to produce molecules of greater and greater molecular weight. Rubber and Gutta (stereo isomers) with molecular weights in excess of 100,000 are the largest members of this family. Chain extension of rubber by addition of IPP to already existent rubber molecules has been shown in

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vivo (7). The initiation of rubber synthesis is a mystery so far.

The biochemistry of terpene formation is well-developed. However, there is still a very big step between knowledge of the biochemistry and the ability to manipulate plants to produce more and/or better hydrocarbons. The next section outlines the physical structure of rubber-producing plants, and suggests the benefits to be derived from examining that structure with the electron microscope.

III. The Role of the Electron Microscope -- Structure and Ultrastructure of Rubber-Producing Plants

The view of green plants being taken here is that they are chemical factories which mix freely available carbon dioxide and water with plentiful sunlight to produce valuable materials. The biochemical "machinery" of plants is a network of subcellular components known as "organelles". Probably the best known organelles are chloroplasts (site of photosynthesis), mitochondria (aerobic respiration), and ribosomes (protein synthesis).

Organelles range in size from as large as several microns down to a few hundred Ångströms. Examination of these tiny bodies is best done by means of the electron microscope, an instrument capable of resolving biological structure to a level of less than one nanometer. This amazing resolving power is now being used to unravel the process of photosynthesis, the structure of biomembranes, and diverse other problems.

Unfortunately, the capabilities of the electron microscope cannot yet be fully used to assist in the understanding of rubber production. The problem is that it is not clear yet which cells and organelles are involved in what steps in the biosynthesis of rubber -- or even which are involved at all. If the function of an organelle or organelles could be determined, it would then become reasonable to intensively apply the high resolution electron microscope to determine the structure of that organelle and relate

it to the function. Section V of this paper contains a description of a proposed electron microscope experiment designed to determine the function of at least one organelle involved in alkene production.

Before that discussion, however, we should first consider the structure of rubber-producing plants. Also, it should be noted that the biological function of rubber is unknown, and that we really only know where it is stored, not how or why it is produced.

Rubber is stored in plants in two basically different ways. The vast majority of rubber producers store their rubber (and perhaps produce it) in latex vessels. These vessels are long "tubes", generally found in or near the phloem, which run the length of the stem of the plant and into the leaves and roots.

Latex vessels are formed either by invasive longitudinal growth of a single cell (nonarticulated latex vessels) or by the dissolving of cross walls between longitudinally stacked cells (articulated latex vessels). Additionally, the vessels may form lateral connections with their neighbors, which are called "anastomoses". This process often leads to concentric rings of latex vessels. Hevea brasiliensis, the Malaysian rubber tree, is of the articulated anastomosing type.

It should be noted that the contents of latex vessels are not necessarily high in rubber. Many plants contain no rubber in their vessels, and many more contain only modest to insignificant amounts. In these cases the contents, besides water, are very often high in esters and alcohols of terpenes. Some of these compounds are pharmacologically active (8).

Latex vessels, particularly those of Hevea, have been studied using the electron microscope by an English/Malaysian group obviously interested in improving the production of rubber. It is clear that latex can incorporate

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precursors into rubber, and the organelles of latex have been studied (9). However, no specific function has been attributed to any organelle. Micrographs 1 through 4 of this paper demonstrate the structure and ultrastructure of latex vessels from two different species of rubber producers.

One important plant -- and probably many others -- produces and stores considerable amounts of rubber, and has no latex vessels. This plant is Guayule (Parthenium argentatum), and it stores its rubber primarily in paranchymous tissue associated with its vascular system. Guayule, which grows in the semidesert, was cultivated during World War II in the United States for its rubber. Technology and agricultural techniques were developed to grow the plant commercially, and vast acreages were set aside for its growth when synthetic rubber (made from oil) was developed (10). It seems likely that once again Guayule will be grown in the United States for its rubber, since the price of oil is increasing to the point where synthetic rubber will no longer be competitive with natural rubber in price. Micrographs 5 through 7 are of Guayule. To our knowledge, Guayule has never before been examined with an electron microscope.

IV. The Biological Function of Rubber

As mentioned before, the biological function of rubber is still a mystery. Rubber does not serve as a food reserve, and the highly reduced carbons of rubber are never metabolized once they are formed as rubber (11). Also, the rubber-producing mechanism and latex vessels were probably not evolved to act as sealents for cuts received by the plant. Obviously, almost all plants have mechanisms for sealing cuts without resorting to a specialized mechanism involving rubber. Finally, rubber is almost certainly not used as an insect or micro-organism repellent. The arguments against this idea are basically that there are far easier (less energy consumptive)

ways for a plant to protect itself against attack and, far more damaging, there are several insects and micro-organisms which attack rubber plants anyway. For example, Hevea, a native to the New World jungles, cannot be grown on plantations in the New World. There is a leaf rust which kills rubber trees in Central and South America, and this infection spreads very rapidly under the close conditions of a plantation.

The biochemistry of rubber formation provides a clue as to the function of rubber. Referring back to Fig. 2, it is clear that the conversion of MVAPP to IPP releases carbon dioxide. Also, when a molecule of IPP is added to a growing rubber chain, two phosphate ions are released. But carbon dioxide and phosphates, along with water, are the basic material inputs to chloroplasts for the capture and fixation of sunlight energy. Largely on the basis of the above, it has been suggested that rubber formation is involved with the plant respiration (12). Note that the above is considerably different from the metabolizing of rubber after it is formed.

But why would a plant evolve a system to produce carbon dioxide while sacrificing five reduced carbons? The answer might be that the where and when of the component release may provide an advantage to the plant sufficient to justify the expenditure of energy inherent in rubber production. To understand this idea further, a digression is in order to present other methods used by plants to selectively and advantageously release carbon dioxide.

Under certain conditions, noteably low carbon dioxide concentrations and high light intensities, chloroplasts will reverse the photosynthetic process, consume oxygen, and produce carbon dioxide. This process can be injurious to the chloroplast, and always reduces the net efficiency of carbon fixation. There are at least two known methods by which plants

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minimize this effect.

Some plants, noteably maize and sugar cane, have developed a metabolism known as the "C4 metabolism". These plants have two different types of chloroplasts in their leaves, and carbon dioxide is shuttled between the two. One type of chloroplast is found in the parenchyma cells associated with the vascular tissue running through the leaf, while the other is in the mesophyll tissue of the leaf. Intercellular connections, or plasmodestmata, have been found between these two types of cells in many plants using the electron microscope (13).

Carbon dioxide is first fixed in mesophyll chloroplasts by condensation with the 3-carbon phosphoenol pyruvate (PEP). The 4-carbon product, oxaloacetate (OAA), is then converted to malate or aspartate. These diffuse to the parenchyma chloroplasts where the freshly fixed carbon dioxide is removed and taken into the standard Calvin Cycle by condensation with ribulose diphosphate. The 3-carbon backbone remaining is converted to PEP and then shuttles back to the mesophyll cells (14).

In this way the mesophyll tissue, which receives an adequate supply of carbon dioxide, can transport part of its carbon dioxide to the more interiorly located chloroplasts of the parenchyma. This allows for a higher net rate of photosynthesis and a reduction in damage due to photorespiration.

There is a similar metabolism in certain succulents known as the "crassulacean acid metabolism". Here there is only one type of chloroplast, but carbon dioxide evolved by the plant during respiration at night is condensed with an acceptor to form malate. The malate is stored until daylight when it begins to give up the carbon dioxide, thus increasing the daytime carbon dioxide concentration and reducing photorespiration (15).

The above described metabolisms are cyclic in that the donor/acceptor

molecules are recycled again and again. If the rubber metabolism is involved in plant respiration, it will be unique in that the donor, MVAPP, is not recycled but is converted to IPP and stored as rubber. One of the objects of our proposed experiment is to determine the fate of the carbon released during the conversion of MVAPP to IPP, and to test the hypothesis that rubber production is associated with plant respiration. The other object of the experiment is to determine where in the plant the conversion of MVAPP to IPP takes place.

V. Electron Microscope Autoradiography of Rubber Formation in Guayule -- A Proposed Experiment

Our proposed experiment consists of introducing selectively labelled mevalonic acid to Guayule tissue, and then to examine the exposed tissue. By varying the duration of the exposure to the label and the time between withdrawal of the label and the killing of the tissue, the sequence of the biosynthetic pathway can be followed. Similar experiments with cell homogenates and with Hevea latex led to the determining of the biochemistry of rubber formation.

In the autoradiographic technique, after the tissue is killed, it is fixed, embedded in plastic, and thinly sectioned. A layer of photographic emulsion is applied to the section, and the tissue is then set aside while the disintegrating atoms of the label expose the emulsion. After suitable exposure time, the emulsion is developed, and the section is viewed in the electron microscope. The developed grains are then seen directly over the area which contains the label.

We intend to introduce ^{14}C-labelled mevalonic acid to Guayule tissue, and will attempt to "watch" the biosynthesis of rubber with the electron microscope and the autoradiographic technique. There are two basically different ways to label mevalonic acid, and each of these should produce

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distinctively different autoradiographs. Therein lies the point of the experiment.

Referring to Fig. 2, when MVAPP is converted to IPP, it is the carboxyl carbon of MVAPP which is removed, and the rest of the carbon backbone is processed into rubber. Consequently, if it is one of these backbone carbons which is labelled on the introduced mevalonic acid, the autoradiograph which results will be of the biosynthesis of rubber. However, if the mevalonic acid used as a precursor is labelled in the carboxyl position, the autoradiograph which results will part company with that of the formerly described one at the step wherein MVAPP is converted to IPP. Thus, hopefully, both the site of this reaction and the fate of the severed carbon will become known.

VI. Summary

Recently we began an investigation into hydrocarbon production in green plants. Of the hydrocarbons, the alkenes (terpenoids), particularly rubber, became most interesting. Review of the literature revealed that the biochemistry of terpenoid formation is known, but the biological structures underlying the biochemistry are largely unknown. These conditions are ideal for contributions to knowledge of the process with the electron microscope.

Most rubber-producing plants store their rubber in latex vessels. Hevea brasiliensis is an example of this class of plant, and the latex vessels and latex of it and related plants have been examined, to some extent, in the electron microscope. This paper contains micrographs of the latex vessels of Hevea and a South African bush, Asclepiadeceae fructosa.

One rubber-producing plant of potentially great importance has no latex vessels and appears to be ideal for studies of rubber biosynthesis. This plant is Parthenium argentatum, or Guayule. It is a shrub of the semi-desert which may become the major source of rubber in America in the near

future. This paper contains micrographs of Guayule seedlings, and we believe these to be the first electron micrographs taken of this plant.

Finally, we plan to perform an electron microscope autoradiographic experiment to follow in vivo rubber formation in Guayule. The details of this planned experiment are included in the text.

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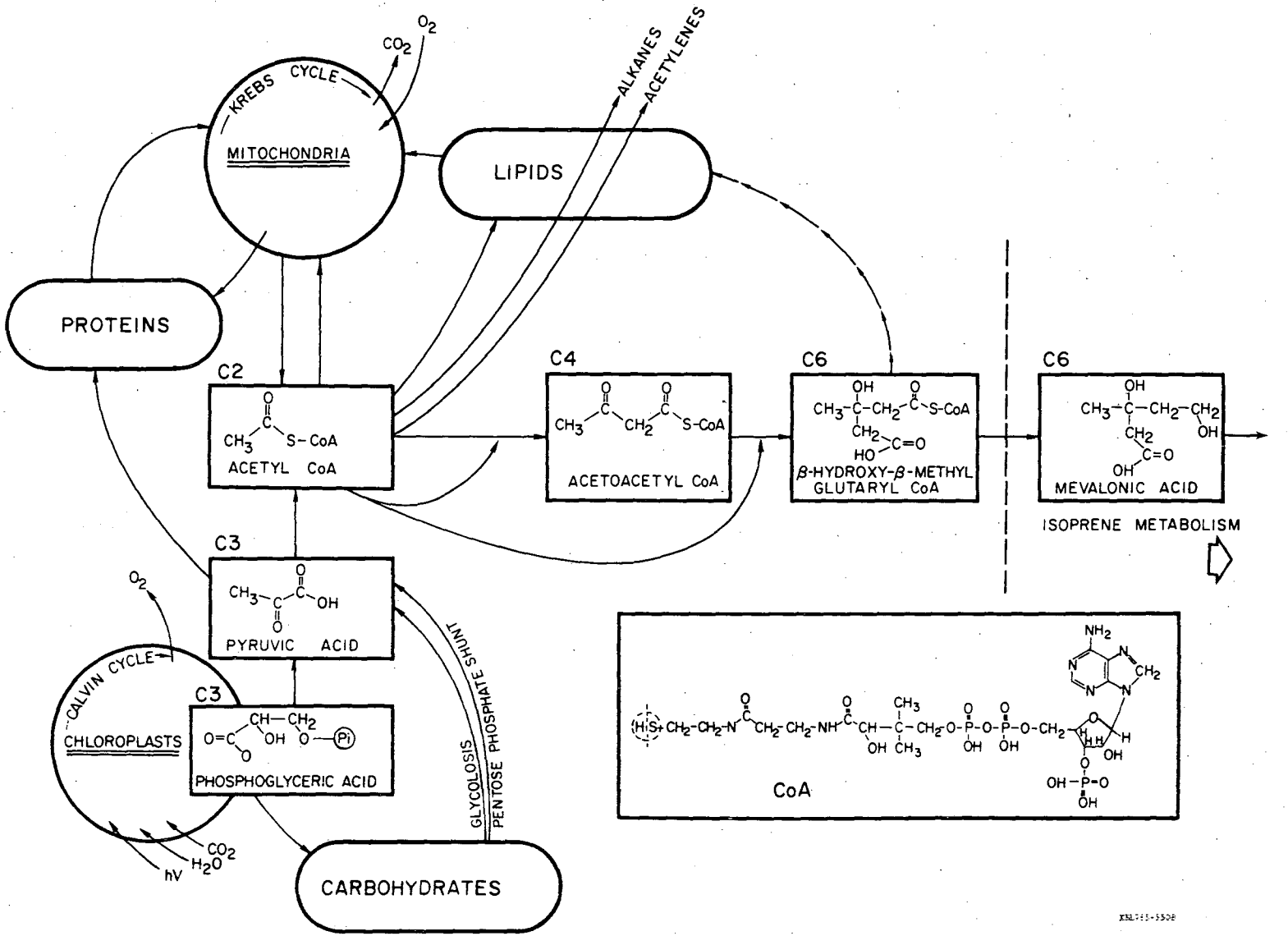


Fig. 1

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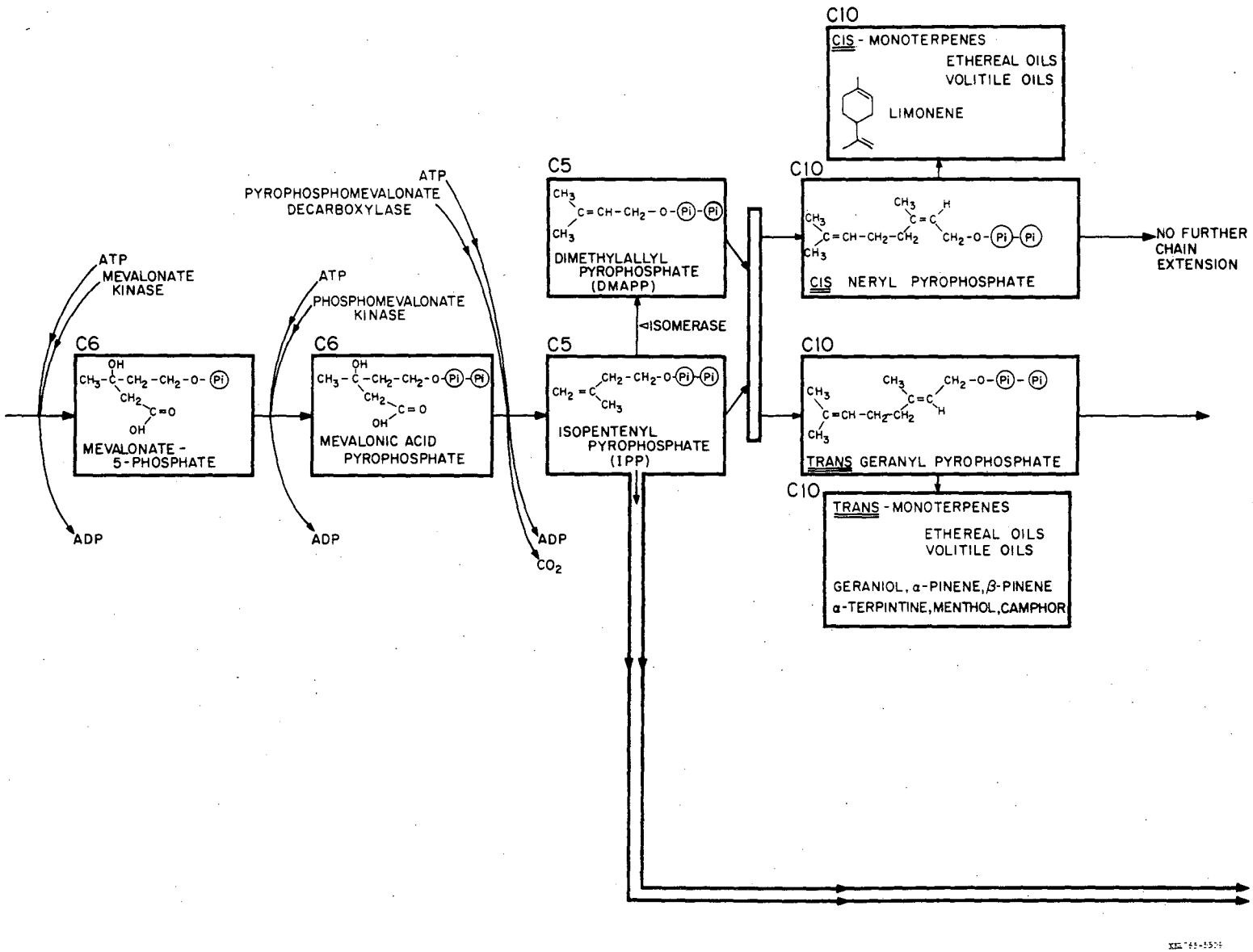

C10
CIS - MONOTERPENES
ETHEREAL OILS
VOLITILE OILS
LIMONENE
C5
DIMETHYLALLYL PYROPHOSPHATE (DMAPP)
C10
CIS NERYL PYROPHOSPHATE
NO FURTHER CHAIN EXTENSION
ATP
PYROPHOSPHOMEVALONATE DECARBOXYLASE
ATP
MEVALONATE KINASE
ATP
PHOSPHOMEVALONATE KINASE
ISOMERASE
C6
MEVALONATE - 5-PHOSPHATE
C6
MEVALONIC ACID PYROPHOSPHATE
C5
ISOPENTENYL PYROPHOSPHATE (IPP)
C10
TRANS GERANYL PYROPHOSPHATE
C10
TRANS - MONOTERPENES
ETHEREAL OILS
VOLITILE OILS
GERANIOL, α-PINENE, β-PINENE
α-TERPINTINE, MENTHOL, CAMPHOR
ADP
ADP
ADP
CO2

Fig. 2

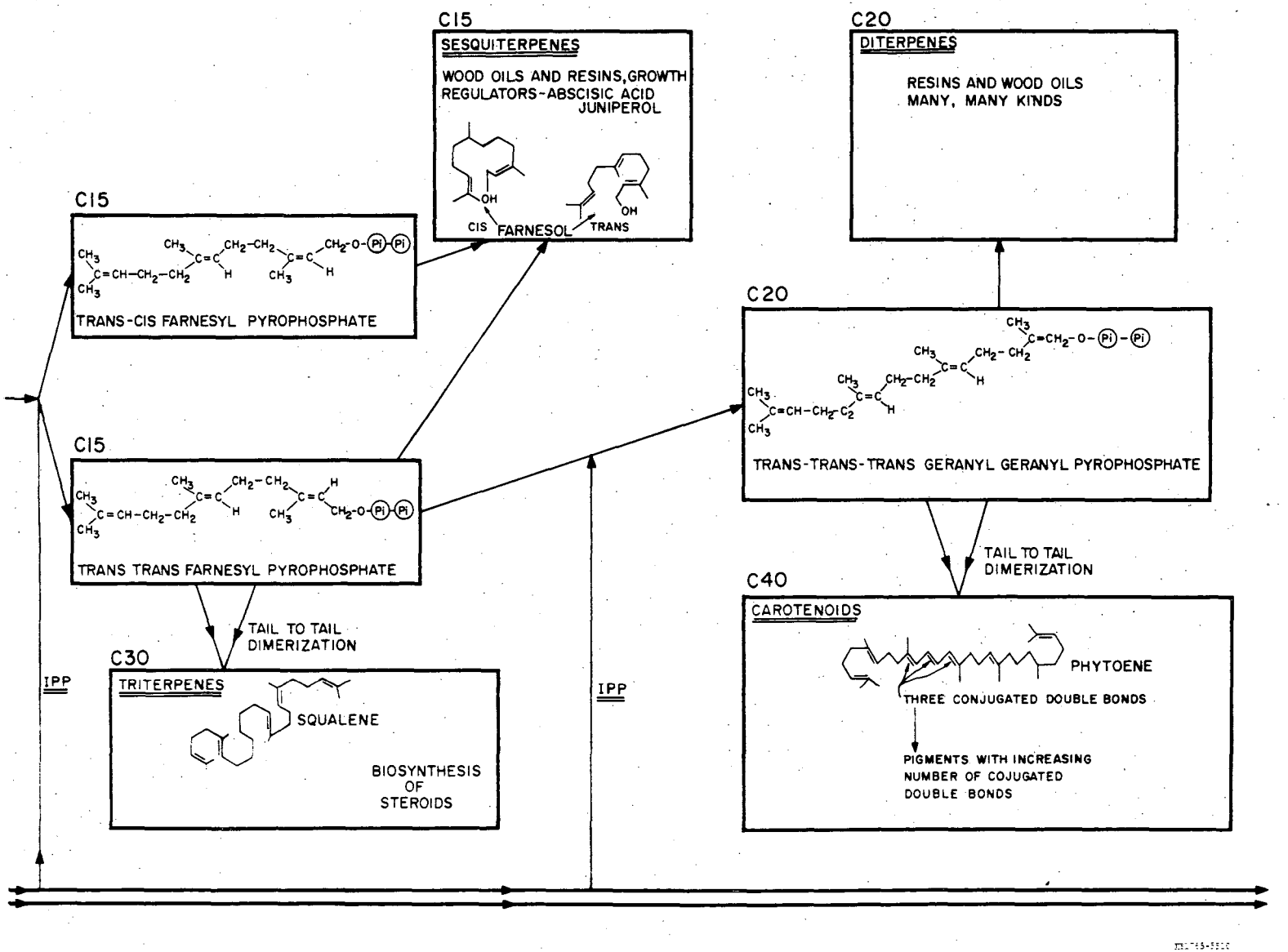


Fig. 3

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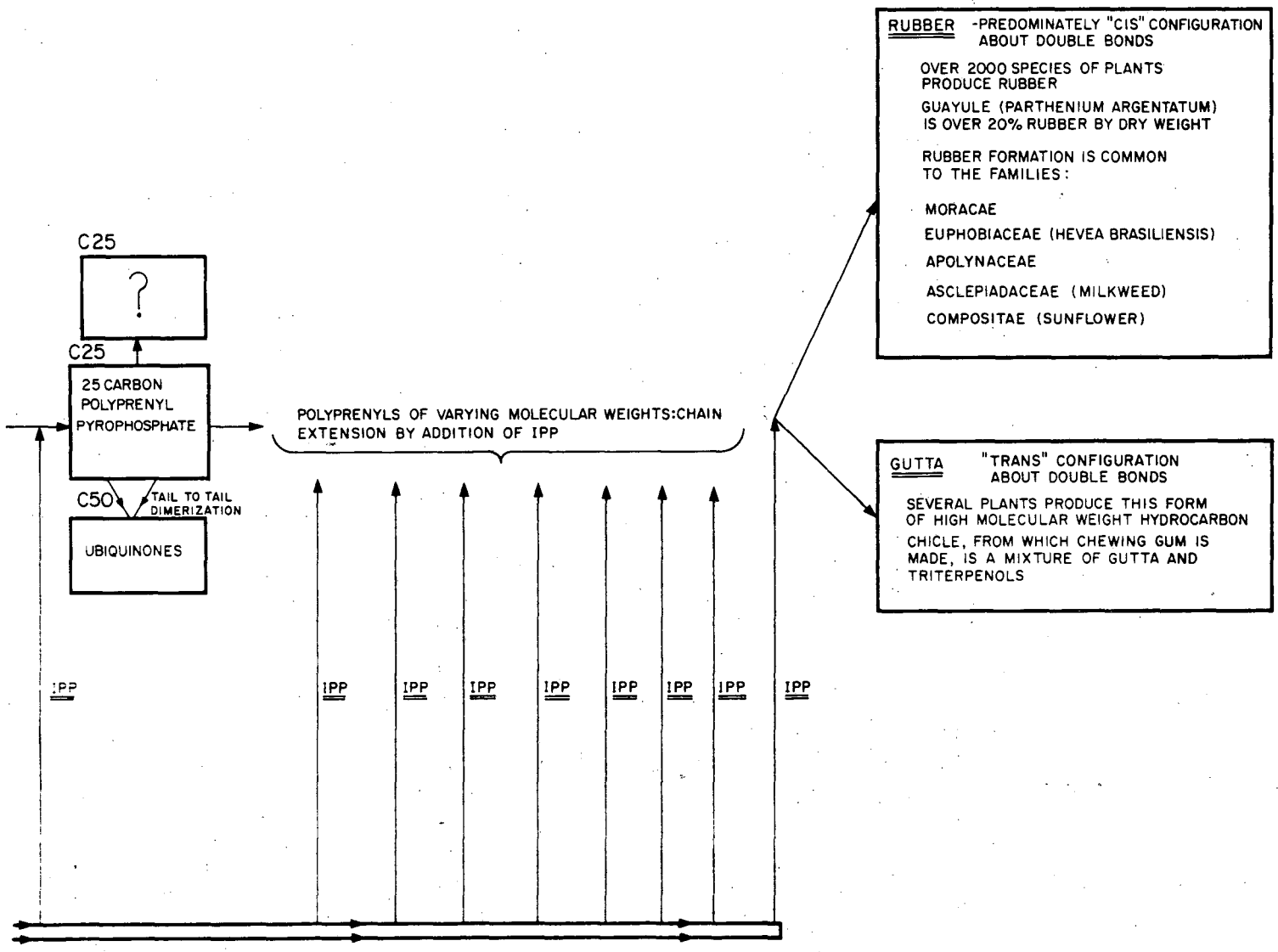
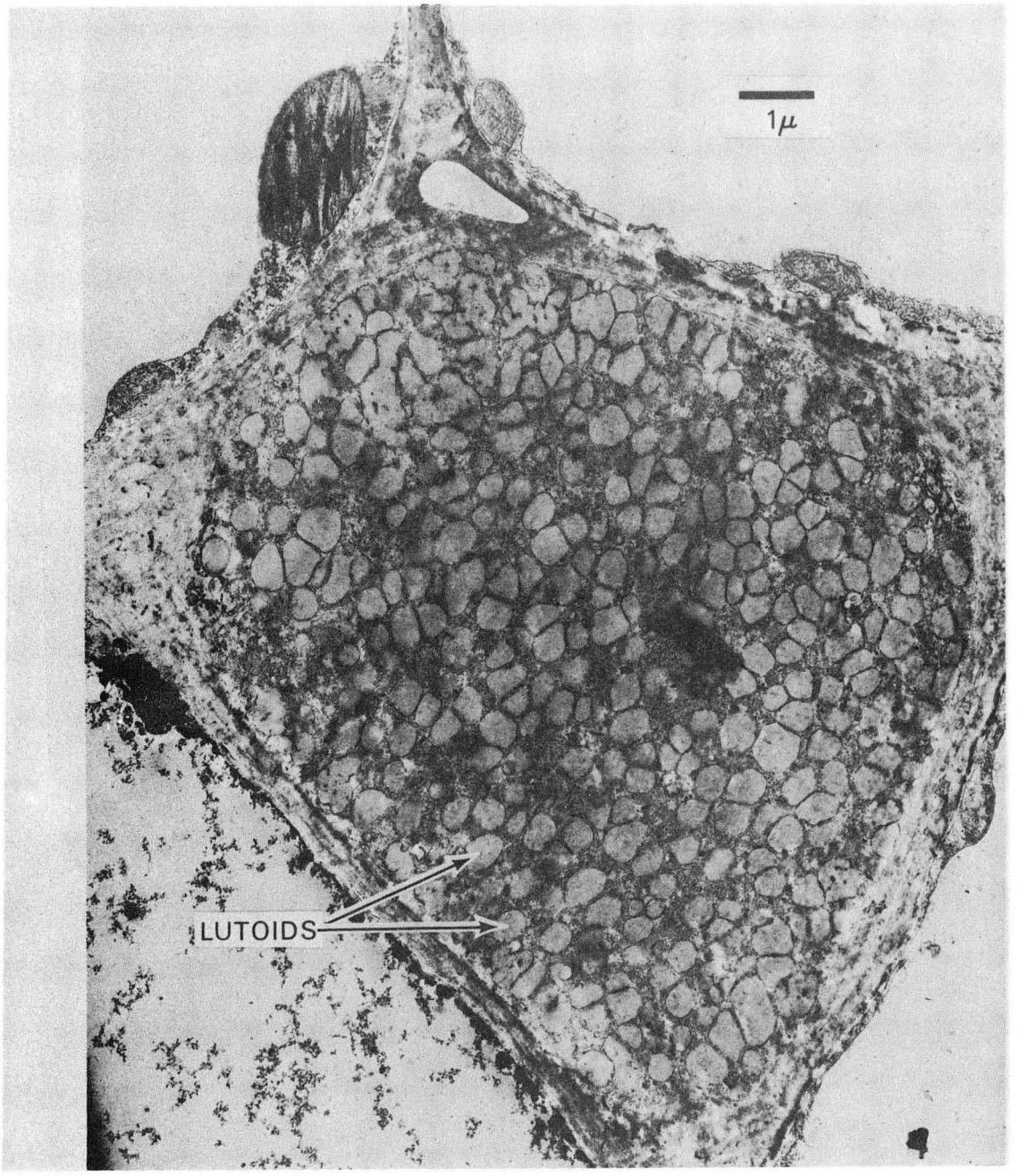

C25
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C25
25 CARBON POLYPRENYL PYROPHOSPHATE
C50
TAIL TO TAIL DIMERIZATION
UBIQUINONES
POLYPRENYLS OF VARYING MOLECULAR WEIGHTS:CHAIN EXTENSION BY ADDITION OF IPP
IPP
IPP
IPP
IPP
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IPP
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IPP
IPP
RUBBER -PREDOMINATELY "CIS" CONFIGURATION ABOUT DOUBLE BONDS
OVER 2000 SPECIES OF PLANTS PRODUCE RUBBER
GUAYULE (PARTHENIUM ARGENTATUM) IS OVER 20% RUBBER BY DRY WEIGHT
RUBBER FORMATION IS COMMON TO THE FAMILIES:
MORACAE
EUPHOBIACEAE (HEVEA BRASILIENSIS)
APOLYNACEAE
ASCLEPIADACEAE (MILKWEED)
COMPOSITAE (SUNFLOWER)
GUTTA "TRANS" CONFIGURATION ABOUT DOUBLE BONDS
SEVERAL PLANTS PRODUCE THIS FORM OF HIGH MOLECULAR WEIGHT HYDROCARBON
CHICLE, FROM WHICH CHEWING GUM IS MADE, IS A MIXTURE OF GUTTA AND TRITERPENOLS

Fig. 4

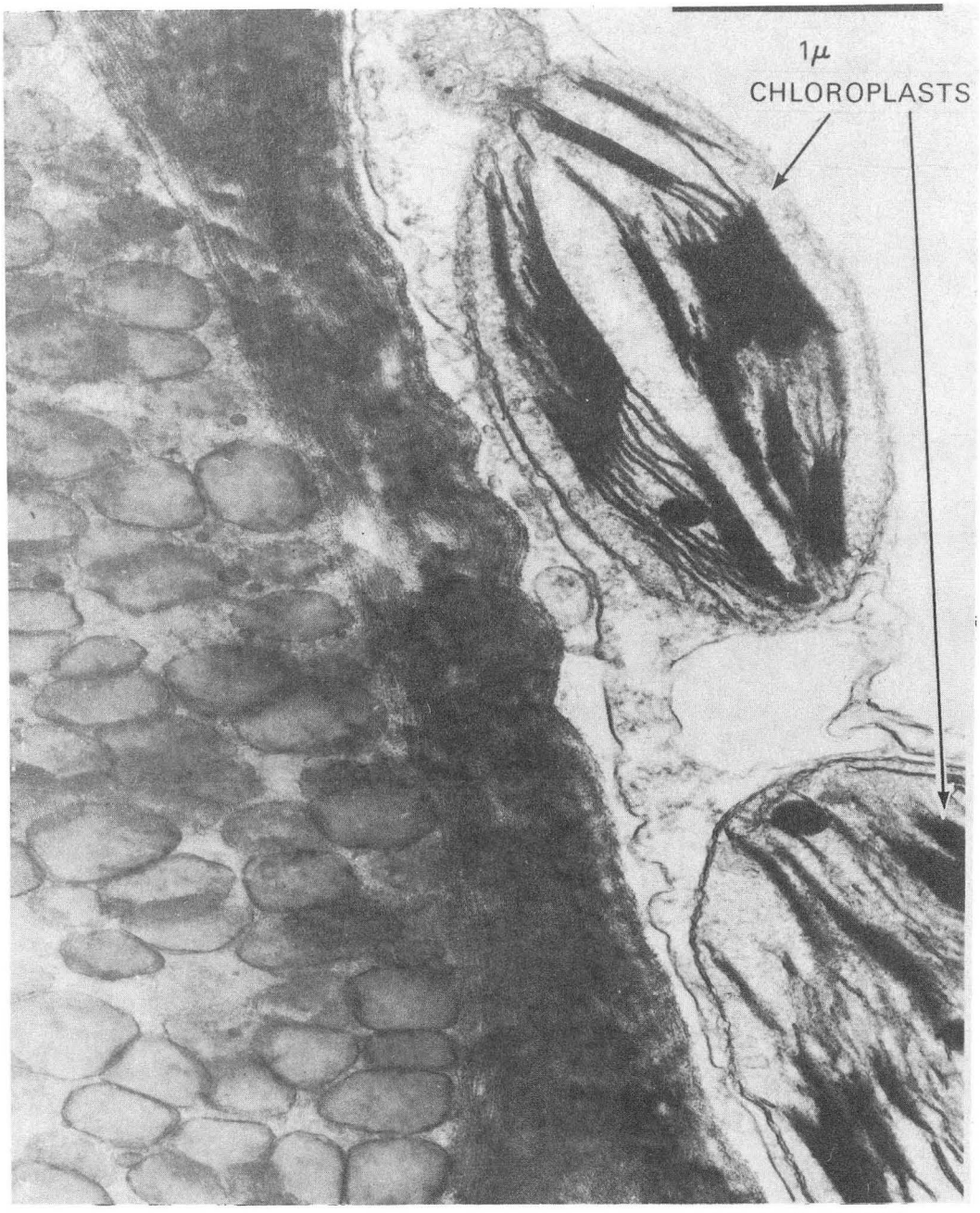
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Micrograph 1. Hevea brasiliensis.

Cross section of a latex vessel of a very young stem. The contents are primarily lutoid bodies. There is very little rubber in evidence.

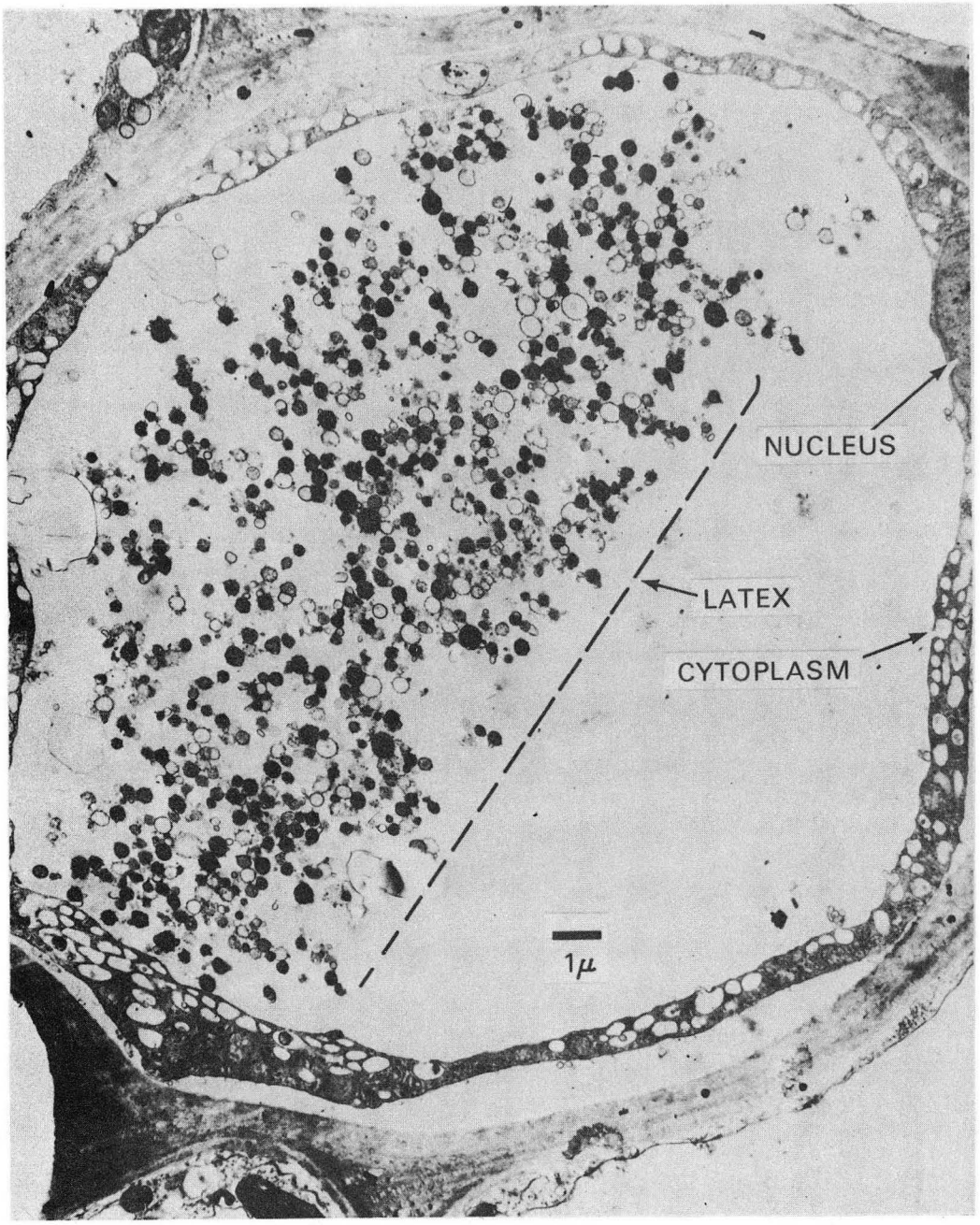


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Micrograph 2. Hevea brasiliensis.

Higher magnification view of the edge of a latex vessel, the highly structured cell wall, and the closely lying chloroplasts of an adjacent cell.

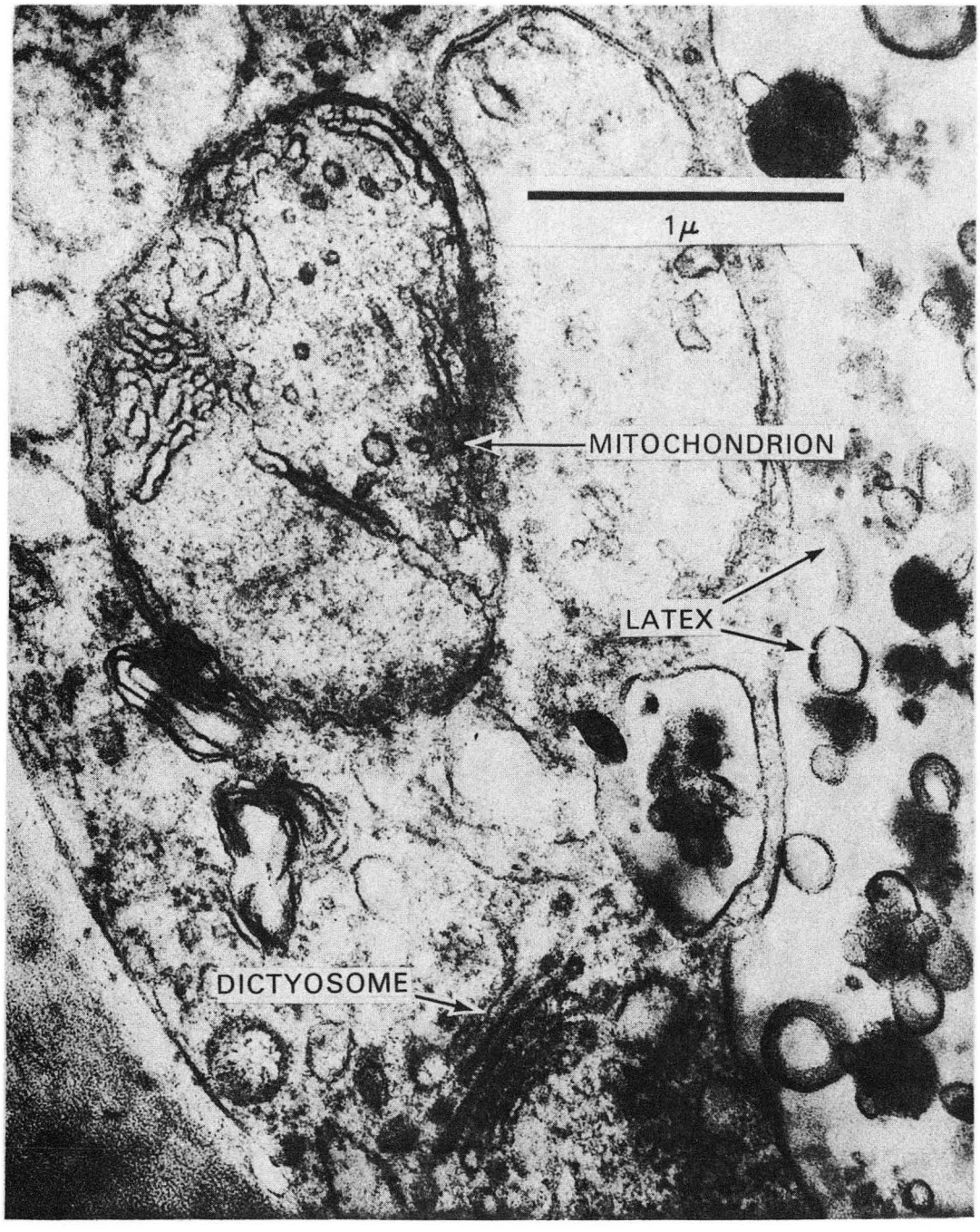
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Micrograph 3. Asclepiadeceae fructosa.

Cross section of a latex vessel from a young stem. Different from Hevea in that the latex appears to be expelled into a large central vacuole surrounded by a cytoplasm.

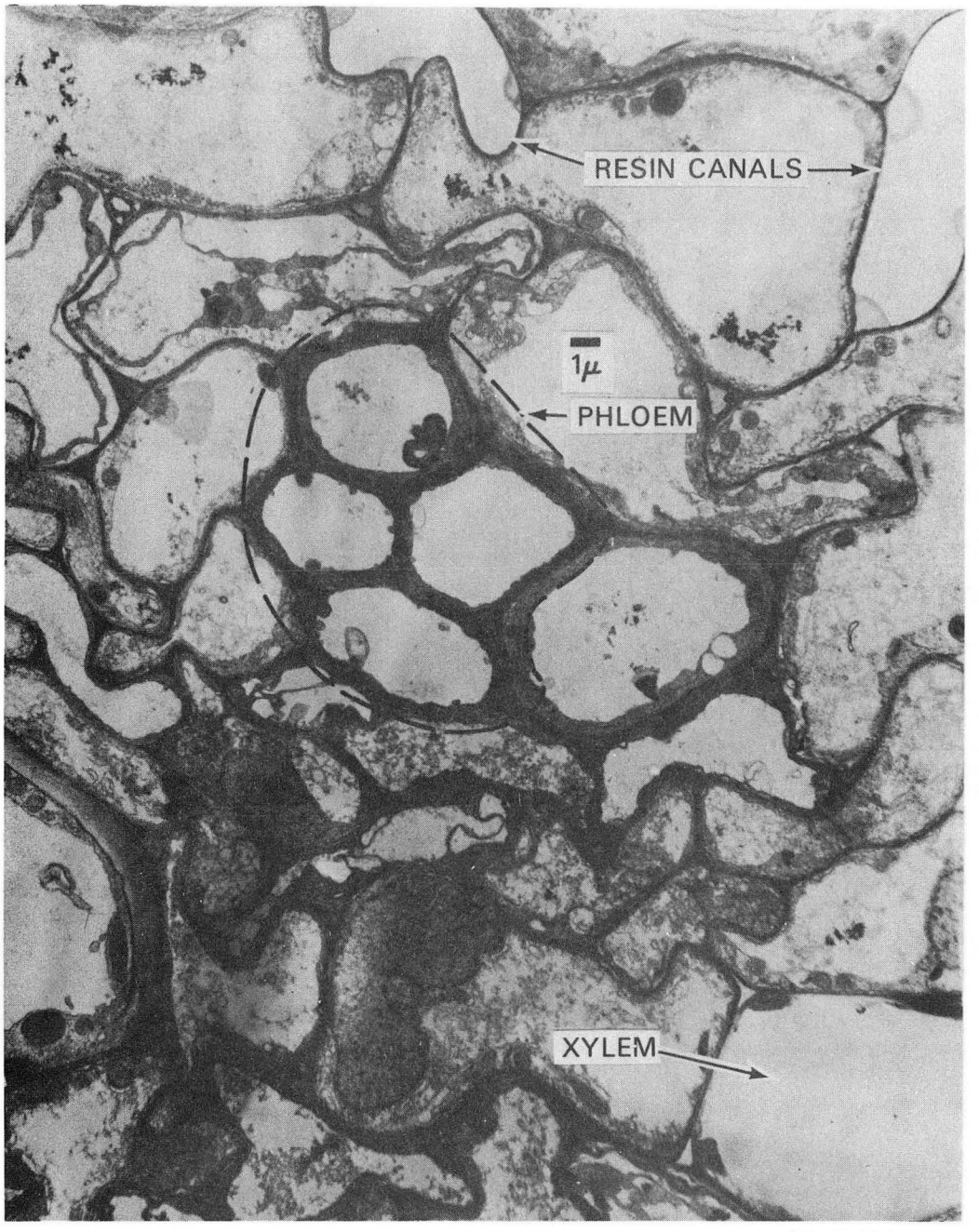


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Micrograph 4. Asclepiadeceae fructosa.

Higher magnification view of a latex vessel showing some organelles of the cytoplasm and the edge of the latex containing vacuole.

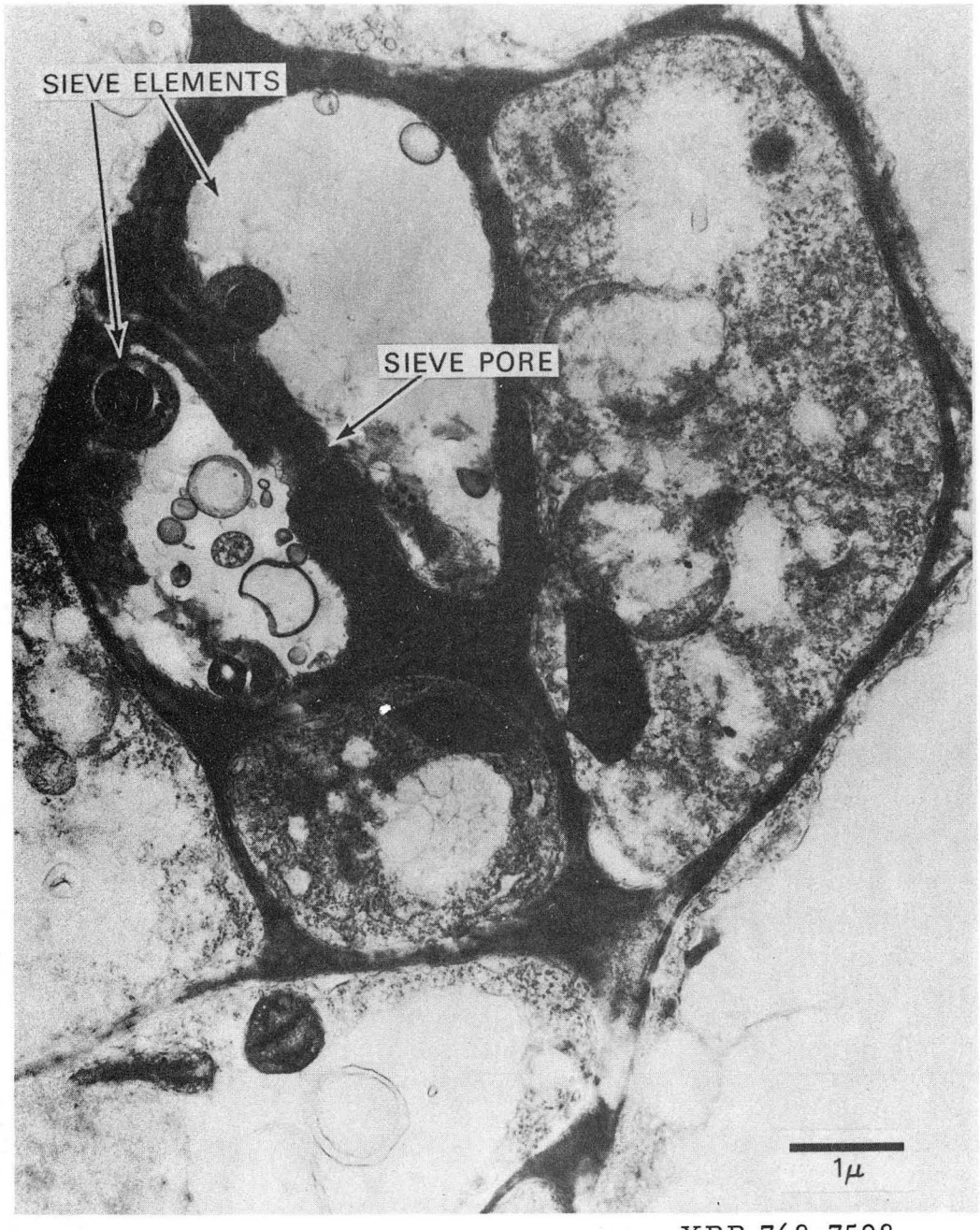
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Micrograph 5. Parthenium argentatum.

Cross section of the root of a week old seedling. The xylem is approximately at the center of the root and the resin canals are about half way to the epidermis.

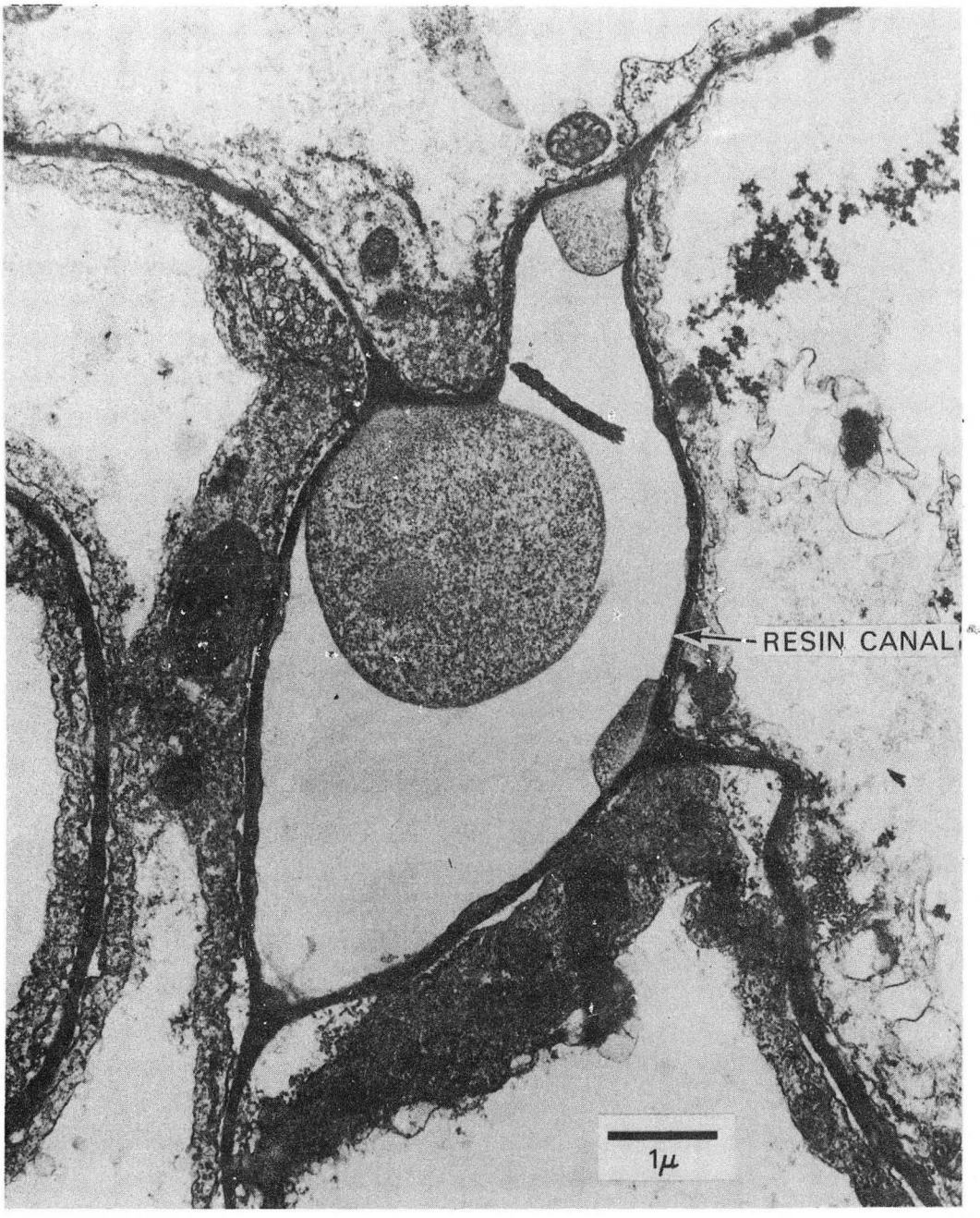


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Micrograph 6. Parthenium argentatum.

Higher magnification view of the primary phloem of a week old stem. The paranchyma cells surrounding the phloem become the primary rubber storage areas.

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Micrograph 7. Parthenium argentatum.

Resin canal of the root of a week old seedling surrounded by endodermal cells. It is probable that resin is transported to the canal by means of channels in the cell walls.

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