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THE ELECTRON MICROSCOPY OF HYDROCARBON PRODUCTION IN PARTHENIUM ARGENTATUM (GUAYULE)

Thomas E. Bauer
(Ph. D. thesis)

DONNER LABORATORY

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THE ELECTRON MICROSCOPY OF HYDROCARBON PRODUCTION IN PARTHENIUM ARGENTATUM (GUAYULE)

Thomas E. Bauer

Abstract

Current rates of consumption of fossilized hydrocarbons will soon exhaust the world's supply of these materials. The biological conversion of solar energy to produce hydrocarbons is suggested as a renewable source of these valuable materials. The work reported in this thesis is aimed at assisting in the development of the biological production of hydrocarbons.

Mankind's use of biologically converted solar energy is not new. Wood has been burned for energy, and crops have been cultivated for fiber and other materials since the beginning of history. Even the biological production of hydrocarbons has been exploited; for example, rubber, turpentine, and essential oils. What is a new idea is the expansion of biological production of hydrocarbons to meet a substantial part of our nation's total needs. Section I of this thesis expands on these ideas.

A serious hindrance to achieving the above-stated goal is the incomplete knowledge which exists regarding the biological processes involved in hydrocarbon production. The work described in this thesis revolves around the use of the

electron microscope to assist in the understanding of the biological production mechanism. The work has four primary divisions.

First, a plant was selected for study. There are thousands of species of plants which produce hydrocarbons in some amount. Of these thousands, only a few produce significant amounts of hydrocarbons. The pragmatic necessity of finding a plant which grows on marginal lands reduces the number still further. The advantages inherent in studying a plant about which much is already known finally directed our attention to the little desert shrub Guayule (*Parthenium argentatum*). This shrub can produce hydrocarbons (rubber) in concentrations up to 1/4 of its dry weight. It grows on semi-arid land and has been extensively studied, particularly during World War II in this country. Section II of this thesis describes the potential of Guayule in further detail.

The second phase of our work was an investigation into the morphology of Guayule at the electron microscope level. Section III of this thesis reports the results of this investigation. Also presented in Section III are light microscope morphology studies of Guayule that were performed during World War II.

Our third task was to develop experiments which would allow us to follow the biosynthesis of hydrocarbon in Guayule. In order to do this, the knowledge of the biochemistry of rubber formation was used to select a tracer, mevalonic acid.

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Mevalonic acid is the precursor of all the terpenoids, a large class of hydrocarbons which includes rubber. It was found that when high enough concentrations of mevalonic acid are administered to seedling Guayule plants, build-ups of metabolized products are found within the chloroplasts of the seedlings. Also, tritium labeled mevalonic acid was used as a precursor, and its metabolic progress was followed by using the technique of electron microscope autoradiography. The results of these experiments also implicated chloroplasts of the Guayule plant in hydrocarbon production. Section IV of this thesis presents the details of these experiments along with their results.

Our final task was the development of a system to produce three-dimensional stereo reconstructions of organelles suspected of involvement in hydrocarbon biosynthesis in Guayule. The techniques are designed to reconstruct an object from serial sections of that object. The techniques use stereo imaging both to abstract information for computer processing, and also in the computer produced reconstruction. Section V of this thesis details the development of these techniques.

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I. INTRODUCTION

A. Background

The United States currently has an energy budget of around 80 quads (1 quad = 10^{15} BTU) per year (Calvin, 1977:3). Of this budget, currently around half is supplied by petroleum, another quarter by natural gas, and slightly less than a quarter by coal. The remainder is supplied by a few percentage points each from hydropower, geothermal, nuclear, and solar (Calvin, 1977:11).

The current situation described as the "energy crisis" evolves from the fact that our supplies of oil and gas are rapidly being depleted. It is estimated that there is only about a 20-year supply of natural gas left, and oil reserves are variously estimated at between 50 to 100 years supply (Hubert, 1971:62-63). The longer estimates for oil reserves depend upon the obtaining of oil from places, such as shale oil, where retrieval is technologically difficult and possibly ecologically harmful.

These considerations require us to investigate alternative sources of energy. The next few sections of this introduction briefly list these alternatives and their limitations. The last alternative described, the biological conversion of solar energy (biomass), appears to be a nearly ideal solution.

The work reported in this thesis is almost exclusively confined to electron microscope studies of one plant (Guayule) which has excellent potential as a biological hydrocarbon producer. The listing of other biomass alternatives and other solar and non-solar alternatives is provided simply as background material.

The motivation for the work described in this thesis is a wish to contribute to the development of biological conversion of solar energy. This general motivation is sharply constrained by the author's realm of competence, the field of electron microscopy. This constraint provides several implications for the design of experiments.

First, the use of the electron microscope suggests that only one species of plant be intensively studied. The electron microscope is designed to allow very high resolution examination of small areas. Thus a superficial survey of several species would not really make use of the microscope's power, while an exhaustive study of several species would be a task considerably greater than ordinarily undertaken by one investigator for a thesis project.

Clearly, the decision to study just one species demands great care in the selection of a plant for study. Section F of this Introduction provides the reasoning behind the choice of Guayule. Also, Section II of this thesis is devoted to a description of the potential of Guayule for hydrocarbon production.

A second implication which derives from the use of the electron microscope is that some means be found to identify precisely the components of the chosen plant which are directly assiciated with biological process (hydrocarbon production) under study. Again, this requirement stems from the high resolving power of the electron microscope. A green plant is a large, complex structure, and an exhaustive study of every component would be prohibitively time consuming. Consequently, the general morphology study of the plant presented in Section III of this thesis concentrates on those structures of Guayule which previous light microscope studies had found to be associated with hydrocarbon (rubber and resin) storage. Additionally, biochemical tracer experiments were undertaken to further isolate and identify those structures of the plant directly associated with hydrocarbon production. These biochemical-based experiments and their results are described in Section IV of this thesis.

Finally, the use of the electron microscope implies that any structure studied will be examined by means of relatively thin sections (.1μ-.3μ thickness). Since the structures of interest in the plant are three-dimensional and generally considerably larger than .3μ in diameter, a thorough characterization of such a structure requires that some means be developed to reconstruct the three-dimensional shape of the structure from information contained in the sections. Section V of the thesis describes the techniques developed to

accomplish such reconstructions.

To summarize, the first tasks undertaken for the research reported in this thesis were the selection of a mechanism (hydrocarbon production) and a plant (Guayule) for study with the electron microscope. Secondly, biochemically based experiments were performed to identify the structures within the plant directly associated with hydrocarbon production. Finally, techniques were developed to reconstruct the three-dimensional shape of identified structures from the relatively thin sections examined in the electron microscope.

Appendices to the thesis present a brief study of other hydrocarbon-producing plants, a description of the computer programs used in the reconstruction effort, and a description of distortions which may arise with the use of stereo imaging techniques which were part of the reconstruction effort. Also, Sections B through E of this Introduction present general background material to give a perspective to the research described in the rest of this thesis.

B. Non-Solar Energy Alternatives

A substitute for oil and gas often mentioned and currently being developed is coal. The United States possesses coal reserves sufficient for at least 100 years if all our energy needs were supplied at current usage levels by coal (Hubert, 1971:62-63). However, the ecological problems associated with extensive strip mining of coal, the problems of

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air and water pollution, and the clearly finite nature of our coal supply suggest that since we will eventually have to turn to renewable resources for energy it might be better to develop these before we destroy the land digging up the last supplies of fossil fuel.

Nuclear fission has long been touted as the fuel of the future. However the list of ecological consequences arising from the production of sufficient energy by means of nuclear reactors need not be detailed here. Additionally, unless breeder reactors are developed--and that does not appear likely at the present time--we are again faced with a very finite supply of fissile materials. It is estimated that the total amount of uranium available in this country would only supply our energy needs for 100 years (Hubert, 1971: 62-63). And this would be gained at a cost of a storage problem of nuclear waste which would extend for hundreds of thousands of years (deMarisly et al., 1977). Clearly, fission energy has several severe drawbacks.

Nuclear fusion is often suggested as a nearly infinite supply of energy for the future. However, the problems with nuclear fusion only begin with the fact that there has never yet even been a laboratory demonstration of a sustained fusion reaction. Even if this hurdle were overcome, there are still other drawbacks.

First, the fusion reactors currently under development use lithium as fuel. Unfortunately lithium is no more abundant than the uranium in terms of its energy production

capacity (Metz, 1976a). Fusion schemes which involve the far more plentiful fuels of deuterium or hydrogen with boron are technically considerably more difficult to bring about (Metz, 1976b).

A little-known problem with fusion is that it possesses a waste storage problem of its own. The neutron flux from the fusion reactor makes the structural material of the reactor intensely radioactive as well as weakening the structure itself. It is estimated that the waste disposal problem of these radiated structural materials would amount to 250 tons of material every year from each 100 megawatt reactor (Metz, 1976a).

Recognition of the problems of nuclear energy result in interest in other "exotic" energy sources. A resource often mentioned in geothermal energy. However, pilot projects in the area have already uncovered a major pollution problem. In addition to a heat pollution problem there is a very serious problem with hydrogen sulfide released by the geothermal wells (Hammond et al., 1973). Hammond et al. estimate that the amount of sulfur pollution generated by geothermal plants is equivalent to that produced by a fossil fuel plant burning high sulfur fuel.

Tidal energy is often presented as a pollution-free energy resource. Unfortunately, tidal energy is very limited. If the total available tidal energy could be harnessed for human use at 100% efficiency, it would supply only 1/3 of

the world's needs (Hubert, 1971). Consequently, tidal energy is viable only for local needs at very special parts of the world.

C. Solar-Related Alternatives

The preceding paragraphs have outlined the potential problems of non-solar energy alternatives. In this section we begin an investigation of solar-related alternative energy sources. These alternatives are hydropower, and energy derived from wind and waves.

The solar flux striking the earth causes evaporation of water which later returns to the earth as snow and rain. Some of this "secondary solar energy" can be captured by hydropower facilities. Unfortunately this alternative is very limited. The United States now currently obtains less than 2% of its energy needs from hydropower plants, and the maximum potential of this resource in the United States is less than three times that (Hubert, 1971).

There is substantial energy available in winds and waves created by the incident solar radiation. It is estimated that over 40 times mankind's current energy needs are contained in wind and waves (Hubert, 1971).

Windmills to extract energy from the wind have been in use on the small scale for hundreds of years. It is estimated that there have been over 6 million windmills built in the United States since the middle of the nineteenth

century (Metz, 1977).

However, recent developmental efforts for large-scale production of energy from wind has run into several technical problems (Metz, 1977). In addition to developmental problems, large-scale windmills create problems with television reception, are unsightly, and may produce environmental problems (Metz, 1977). These considerations require us to investigate other alternatives for supplying the United States' large energy needs.

D. Non-Biological Direct Solar Energy Utilization

Each day the earth receives 20 thousand times as much energy from the sun as mankind currently uses (Calvin, 1977: 11). If we could develop means to convert this energy into usable forms even at a very low efficiency, we could easily supply the United States' energy needs. These considerations have spurred research in areas of direct conversion of solar energy.

One way in which the solar energy might be used is by focusing the sunlight on boilers to produce steam which will in turn drive generators. Such projects are under development, but unfortunately because of the distributed nature of solar energy they require large areas. For instance, a thousand megawatts power station would require approximately 30 square kilometers of area to collect the necessary solar energy (Hammond et al., 1973). Since the United States' current

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U.S. Energy Requirements = 8×10^{16} BTU/Year*

= 2.7×10^{6} Megawatts, average

U.S. Annual Average Surface Insolation = 6×10^{6} BTU/M^2 Year*

= 2.4×10^{10} BTU/Acre Year

Area Required to Produce 8×10^{16} BTU/Year from U.S. Average Insolation at Conversion Efficiency of:

1% = 3.3×10^{8} Acres = 5.2×10^{5} Sq. Miles

10% = 3.3×10^{7} Acres = 5.2×10^{4} Sq. Miles

20% = 1.6×10^{7} Acres = 2.6×10^{4} Sq. Miles

100% = 3.3×10^{6} Acres = 5.2×10^{3} Sq. Miles

Total U.S. Land Area	=	23×10^{8} Acres
Presently Cultivated Area	=	3.8×10^{8} Acres**
Pasture and Range Land	=	7.4×10^{8} Acres**
Forest Land	=	4.7×10^{8} Acres**
Urban Land	=	$.4 \times 10^{8}$ Acres**
Potentially Arable Land	=	$.75 \times 10^{8}$ Acres**

---

* (Calvin, 1975)

** (Pimentel, et al., 1976)

FIGURE I-1

energy needs are about 2.7×10^6 megawatts (see Figure I-1), a simple calculation shows that to supply our total energy budget would require 80,000 square kilometers of area for solar collection.

To get around the requirements of large areas of collection on the surface, it has been suggested to collect solar energy in space by use of a satellite and then to beam the collected energy to the earth by means of microwaves (Glaser, 1977). However even if there were no other objections, the expense of such a system is prohibitive. Even very optimistic estimates suggest a cost of over 40 billion dollars to produce approximately 10% of the United States' current energy budget in this fashion (Glaser, 1977). Other problems associated with this futuristic scheme include potential damage to the earth's atmosphere from the microwave radiation, hazards to human health from the microwave radiation, and hazards from the extra heat loading of the earth.

There is one area with bright prospects for producing a significant fraction of our energy needs by converting solar energy into useful forms. This area consists of solar collectors mounted on individual homes and businesses designed to extract home heating and even some electricity from the incident solar flux. Approximately 40 million acres of land in the United States has been converted already to urban use (Pimental et al., 1976). If we assume that one tenth of this area could reasonably be outfitted with solar convertors, we

have available 4 million acres of surface to collect and convert the incident solar flux. If the available area were provided with solar collectors operating at a 20% efficiency, the energy so collected would be over 1/4 of our total energy budget (see Figure I-1).

E. Biological Conversion of Solar Energy: I--Non-Hydrocarbon Alternatives

All of the above-mentioned energy alternatives have been technological in nature. This section is devoted to exploring the potential for biological conversion of solar energy. This solution not only has the potential to supply most of our energy needs, but also has an associated potential to produce many valuable materials.

As previously stated, the earth receives around 20,000 times as much energy from the sun each day as mankind uses (Calvin, 1977:11). Green plants operating at an earth average collection and conversion efficieny of only .025% (Hubert, 1971) are already collecting and fixing approximately 5 times as much energy each year as the world uses. Under cultivated conditions we can do considerably better than .025% fixation.

Some crops such as eucalyptus or alfalfa can be cultivated in conditions to produce over 1% fixation of the incident solar energy (Livingston & McNeill, 1975). If we could convert the fixed chemical energy in plants to usable fuel at a 50% conversion rate, 300 million acres of energy

plantations could produce half of our current energy needs forever (see Figure I-1). For comparison, the United States has about 380 million acres under cultivation, with about 90 million acres of usable cropland still be to developed (Pimental et al., 1976). Additionally there are about 740 million acres in pasture and range land, 470 million acres in forest, and an estimated 75 million acres of potentially arable land (Pimental et al., 1976).

Consequently it can be seen that the United States does possess enough area to seriously consider energy plantations. An additional point is that the agricultural crops already grown for food and fiber can have a secondary use as energy sources after serving their primary functions.

A recent editorial in Science magazine (Hammond, 1977) indicates that the wood-products industry is now deriving approximately 40% of their total energy needs (about 1 quad) from waste products of their operation. It is estimated Hammond et al., 1973) that the United States currently produces about 880 million tons of dry, ash-free organic waste each year. This waste has an energy content of between 14 and 18 million BTUs per ton (Hammond et al., 1973). Thus conversion of waste to fuel could produce over 12 quads of energy per year, or approximately 1/7 of the nation's needs.

An interesting sidelight to this discussion was provided by a story on the evening news program produced by the San Jose outlet of the American Broadcasting Company

(KNTV Channel 11) on August 23, 1977. At the site of a new waste-to-methane conversion facility being constructed in Mountain View, an engineer on the project stated that methane production from waste land fill operations has always been a problem. According to the engineer, the basements of homes near land fills are sometimes invaded by methane generated by the decomposing waste. The engineer stated that occasionally explosions and property damage have occurred as a result of this "undesired" methane production. This story underlines the technical feasibility of the conversion of waste to fuel.

However, even if we do obtain 1/7 of our energy from converting waste to fuel and obtain another quarter of our energy from individual home and business solar collectors, we still must develop energy resources of about 60% of our current needs. In order to develop the remainder of that energy, biomass-energy plantations are strongly suggested. The rest of this section is devoted to a survey of potential energy and material crops.

Technically the simplest biomass solution is to grow a high sugar containing crop such as sugar cane and then to ferment sugar to alcohol. This solution is already being pursued in Brazil where they are producing enough alcohol to extend their gasoline supply by about 2% (Calvin, 1975). According to Calvin, the Brazilian aim is to raise the average alcohol content in their gasoline to around 10% in 5

years (Calvin, 1975). The problem with this simple solution is that only land suitable for growing sugar can can be used, and sugar cane only has an efficiency of converting sunlight to chemical energy in sugar of around .25% (Calvin, 1975).

A more complex but much more widely adaptable method of converting biologically fixed solar energy is to enzymatically convert cellulose (wood) to sugar and then to convert sugar to alcohol by fermentation. This process has been developed at the University of California, Berkeley, by Professor Charles Wilke of the Chemical Engineering Department. Laboratory process plants for this technology have been developed, and currently a small pilot plant is being developed (Wilke & Yang, 1975). Although somewhat more expensive and technologically difficult, this process would throw open the doors for the conversion of all the world's waste cellulose materials into alcohol. It also allows the utilization of high efficiency energy crops such as eucalyptus which store their energy in the form of cellulose.

In a recent paper (Bassham, 1974), James Bassham of the University of California at Berkeley has reviewed the energy fixing efficiencies of several high-yield plants. What follows is a brief review of the materials presented by Bassham.

In discussions of energy plantations, alfalfa is a crop commonly mentioned. Although not having the highest efficiency of conversion of sunlight into energy, alfalfa is

a widely grown crop with potential for future exploitation. Bassham reports that the alfalfa production has been measured at 29 metric tons per hectare per year. This corresponds to a fixation efficiency of around .7% of the local insolation (Bassham, 1974).

Eucalyptus can be considered as an energy crop. Although the hard woody nature of the plant would make processing somewhat more difficult, it has the advantage of growing quite well on marginal land (Livingston & McNeill, 1975). Bassham reports that eucalyptus yields of 54 metric tons per hectare per year are currently possible. This corresponds to a fixation efficiency of 1.3% of the total incident local insolation (Bassham, 1974).

The most widely discussed potential energy crop is sugar cane. This seems appropriate since Bassham has found that sugar cane growing in Texas has produced 112 metric tons per hectare per year. This corresponds to a conversion efficiency of sunlight to energy locked in cellulose of 2.8% of the local insolation (Bassham, 1974). This is by far the most efficient plant studied. Unfortunately, sugar cane requires a great deal of moisture to grow and consequently would compete with land needed for valuable food crops.

Finally, whenever discussions of energy plantations take place it is suggested that there is considerably more surface of the earth under water than dry. Thus a likely candidate for future energy plantations would be an aquatic

or marine plant, with some form of algae leading the list. Bassham reports yields of algae equaling 87 tons per hectare per year of surface. This corresponds to a conversion efficiency of 2.2% of the local insolation, and this makes algae a prime candidate for an energy crop (Bassham, 1974). Additionally, since algae are far more easily processed than a woody crop and since they grow in a marine environment, they would require no irrigation and would not compete with food crops, algae are by far the star of the group.

F. Biological Conversion of Solar Energy: II--Hydrocarbons

A third alternative for biological conversion of solar energy is the production of hydrocarbons by green plants. Although the fixation energy efficiencies are somewhat lower, the advantage of growing hydrocarbons is that the so produced materials could be more easily inputted into our current industrial petroleum based energy system. Consequently we are very interested in the potential of biological hydrocarbon production.

Before discussing the various plants which produce hydrocarbons in large quantity, it is advisable at this point to be more precise in the definition of the word hydrocarbon. As implied by the name, hydrocarbons are chemicals made up of combination of carbon and hydrogen only. Because of the nature of the carbon atom this allows for four distinct types of hydrocarbons. The first major group of hydrocarbons are

the alkanes. These are hydrocarbons which contain only single atomic bonds between the carbons. Octane, an eight-carbon hydrocarbon, is the primary ingredient in gasoline. Alkanes are produced to some extent by almost all plants, and sometimes in fairly large concentrations (Smith, 1971).

A second class of hydrocarbons are the alkenes, which by definition have double bonds between at least some of the carbon atoms. Thousands of species of plants produce alkenes to some extent (Smith, 1971), and these will be discussed somewhat more fully after a brief mention of the other two classes of hydrocarbons.

The third class of hydrocarbons are the acetylenes, which contain triple atomic bonds between at least some of the carbon atoms. No further mention will be made of this class of hydrocarbons and they are mentioned here only for completeness.

Similarly the fourth class of hydrocarbons is mentioned only for completeness. These are the aromatics, and they are like the alkenes in that there are double bonds between some of the carbon atoms. They differ however in that while the alkenes are open-chain structures sometimes achieving high molecular weight, the aromatics have cyclic structures. The aromatics get their name because of their low boiling points and often odorous qualities.

As stated above, many species of plants produce alkenes. One particular alkene, rubber, already is a major world cash crop. Only a very few plants produce rubber in any substantial

quantity, but many produce lower molecular weight alkenes which have potential as an easily processed replacement for our petroleum resources.

Professor Melvin Calvin at the University of California, Berkeley, has recently been exploring the variaties of alkene producers for a potential "gasoline tree." In a recent work (Calvin, 1977), he reviews a half dozen different alkene producers as candidates for energy suppliers. The candidate for which he has the most fondness is a member of the Euphorbia family, Euphorbia trigona.

For completeness I should point out that the search for plants which produce hydrocarbons of the right kind and in large enough amounts is not new. For example, between the years 1928 and 1932 Thomas Edison surveyed over 2,000 plants looking for a variety capable of becoming an American rubber tree (Calvin, 1977). Searches for a good hydrocarbon producer predated and followed Edison, and a review of almost any book in the field of economic botany will list many (Smith, 1971, for example).

The best known rubber producer is the Hevea rubber tree, a native of South America which is grown in plantations in Southeast Asia. Unfortunately Hevea cannot be grown in the United States because it requires high temperatures and high humidity, conditions generally not found outside of tropical areas. Unfortunately, areas of the world in which Hevea is cultivated are politically unstable and unfriendly

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to the United States. Thus we have a great interest in locating a plant suitable for cultivation in the United States which produces large amounts of rubber.

A candidate for a domestic production of rubber is the Russian dandelion (Taraxacum kok-saghyz). The plant was discovered in Russia to be producing about 25 pounds of rubber per acre (Whaley, 1948). During World War II, the plant was grown in several areas of the United States and breeding experiments resulted in productions of up to 70 pounds per acre (Whaley, 1948). These yields are rather deficient, and fortunately for the United States another plant is available with substantially greater potential.

The third species of plant known to produce large amounts of rubber is Guayule (Parthenium argentatum). This little plant possesses an exciting potential for the production of rubber and other hydrocarbons, and the bulk of this thesis will be dedicated to exploring aspects of Guayule.

II. GUAYULE: HISTORY, ECOLOGY, PRODUCTIVITY, AND GENERAL MORPHOLOGY

A. General Background

Figure II-1, due to Artschwager (1943), is a photograph of a mature Guayule shrub (*Parthenium argentatum*). It is a member of the tribe Heliantheae of the family Compositae. The genus *Parthenium* has 12 members, all native to the Western Hemisphere, distributed from Massachusetts (*P. hysterophorus*) to Texas and Mexico (Guayule) (Taylor, 1951). The smallest member of the genus is *P. alpinum*, a few-inch tall plant of the Colorado mountains (Taylor, 1951). The largest member of the genus is *P. stramonium*, a 25-foot giant native to northwestern Mexico (Taylor, 1951). No member of the genus except Guayule produces any rubber.

Guayule is a native of the 130,000 square miles of the Chihuahuan desert of Texas and Mexico (Taylor, 1951). It is estimated to actually grow on 10% of this range which has conditions especially favorable to it. It requires an elevation of 2,000 to 6,000 feet and rainfall of 10 to 16 inches annually. Its range is further confined to limestone ridges with scant topsoil and a maximum daytime temperature of 95° Fahrenheit to a minimum nighttime temperature of 0°F (Taylor, 1951). Figure II-2 graphically presents this information. Guayule can be grown under cultivated conditions over a far larger area. Figure II-3 presents the potential range of

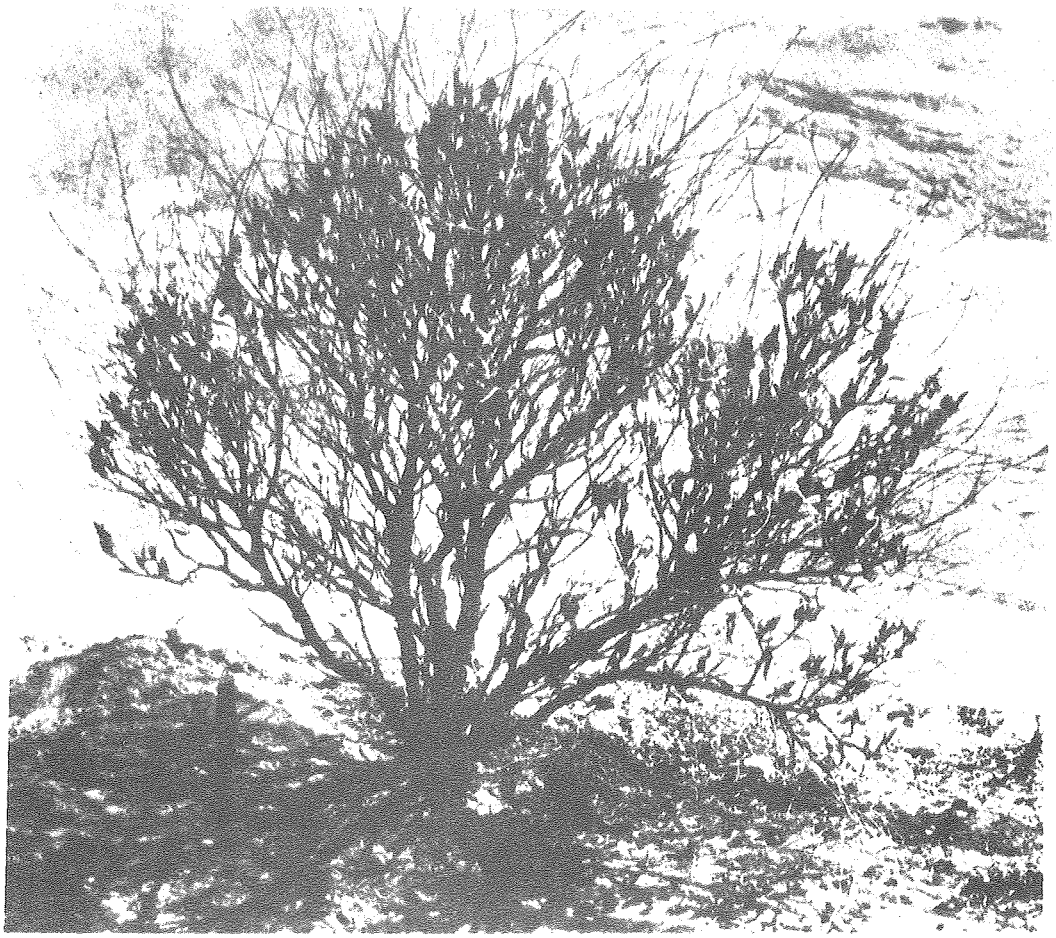

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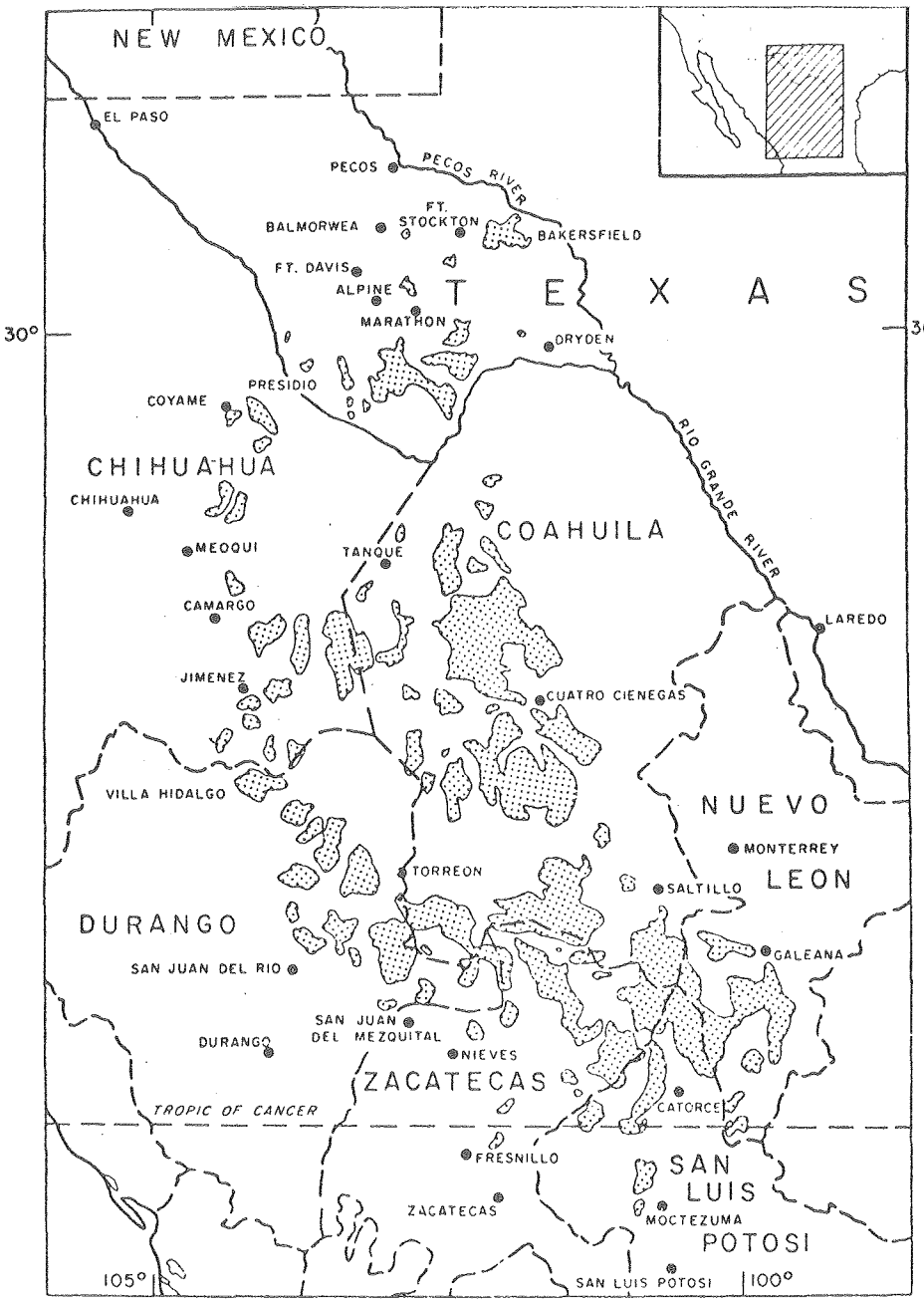
FIGURE II-1



Distribution of native guayule in Mexico and Texas.

FIGURE II-2

FROM (Vietmeyer,1977)

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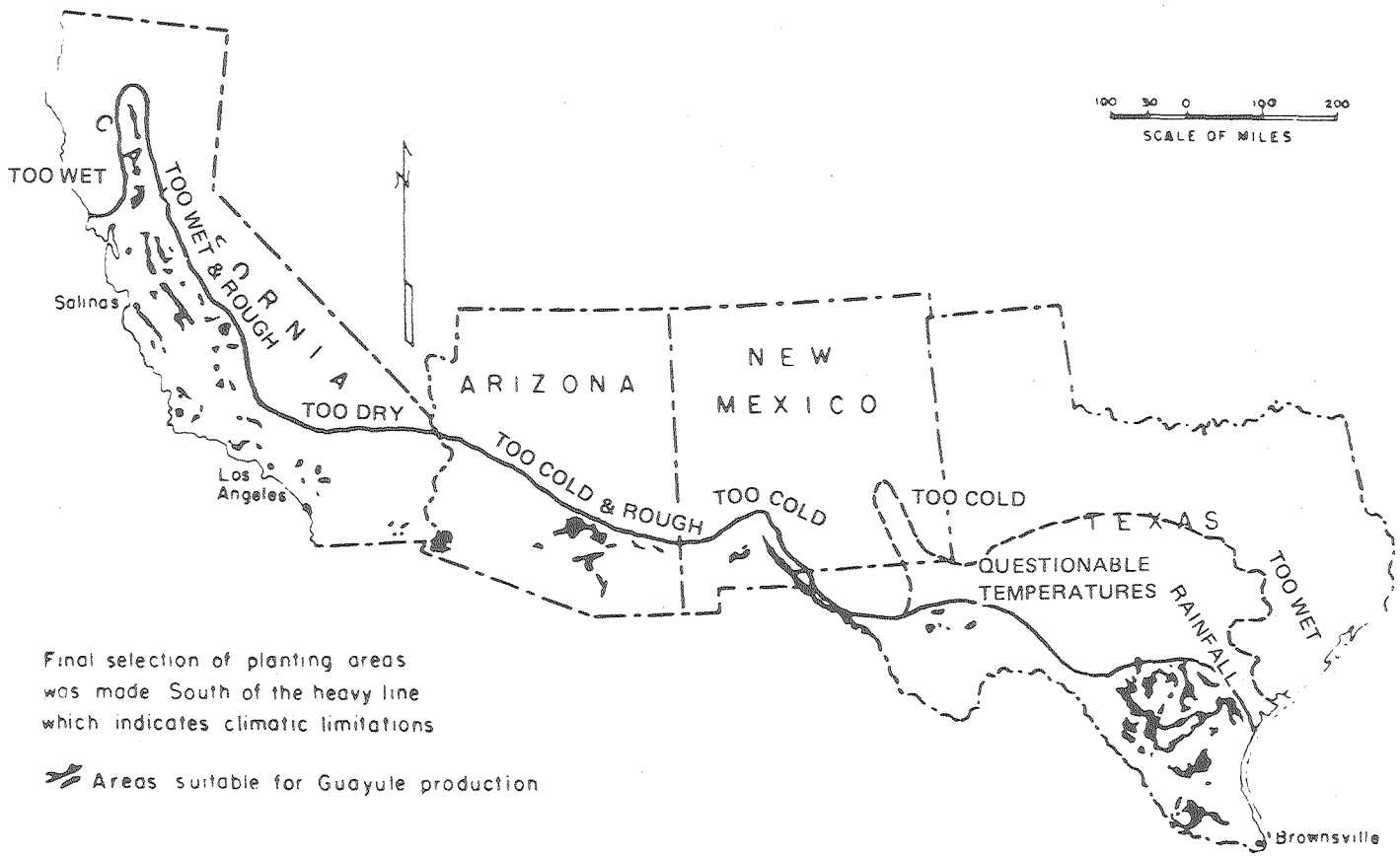

Areas in the United States with climate considered suitable for the cultivation of guayule (as reported by the Emergency Rubber Project, 1944).

FIGURE II-3

FROM (Vietmeyer,1977)

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cultivation of Guayule. However, only under the limited conditions specified above can it successfully compete in the natural state.

Historically, Guayule's capacity as a rubber producer was first exploited by the native Indians of Mexico who produced rubber balls for their games by communal chewing and spitting of the rubber-containing bark (Hammond & Polhamus, 1965). United States attention was first drawn to Guayule rubber potential in 1876 when the Mexican government sent a sample as an exhibit to the Centennial Exhibition in Philadelphia (Hammond & Polhamus, 1965). The first commercial extraction of rubber in the United States was performed in 1888 by the New York Belting and Packing Co. They imported 100,000 lbs. of baled shrub from Mexico, ground the plant, and extracted the rubber by solution in hot water (Hammond & Polhamus, 1965).

A brief but concise description of Guayule development from the turn of the century to the outbreak of World War II is found in Technical Bulletin No. 1327 published by the United States Department of Agriculture (Hammond & Polhamus, 1965):

> In 1902, American capitalists financed a series of experiments by William A. Lawrence, which led to the successful mechanical extraction of crude rubber from Guayule shrub in 1904 by the pebble mill extraction method. Extraction was accomplished by a rotating shell containing flint pebbles, which comminuted the shrub.
>
> The first lot of rubber prepared was shipped to the United States and used by the Manhattan Rubber Co. As a result of the success of the mechanical method of extraction, the Continental-Mexican Rubber Company completed a large factory at Torreon in 1906. Then followed the establishment of factories of various sizes in San Luis

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Potosi, Saltillo, Monterrey, Gomez Palacio, and Jimulco. In 1909, a mill built by the Texas Rubber Co. at Marathon, Tex. began operation. Production was stepped up to a point where, in 1909, Mexico exported 9,542 long tons of Guayule rubber to the United States. Much of the rubber was used in the manufacture of automobile tires.

The limited natural sources of Guayule shrub were rapidly depleted to a point where most of the factories were forced to close down. The four owned by the Continental-Mexican Rubber Co. remained in operation, but their supply of shrub was getting low. They foresaw the need for carrying out cultural experiments and, in 1910, initiated such a program under the leadership of the late W. B. McCallum.

Threatened in 1912 by the Mexican Revolution, cultural operations were transferred to California under the name of the American Rubber Producers, Inc., of the Intercontinental Rubber Co. Headquarters were first established by Dr. McCallum near San Diego. Indicator plots were established throughout California and the southwest to determine areas best suited to Guayule culture. Owing to the lack of sufficient acreage to support full-scale factory operations, his headquarters were moved in 1916 to southern Arizona where a large tract of land was purchased by the company and the township of Continental was established about midway between Tucson and Nogales. Although large shrub could be grown under irrigation at Continental, the very low rubber content was disappointing.

In 1925, headquarters were moved from Arizona to the Salinas Valley in California where conditions for rubber accumulation were more favorable. An extensive commercial development was initiated and, during four campaigns between 1931 and 1941, 3,068,630 pounds of rubber were milled from Guayule planted largely in the Salinas Valley.

In 1930, the War Department was very aware of the strategic value of rubber and consequently sent then Major Dwight D. Eisenhower into the Salinas Valley to study Guayule. Eisenhower filed a report which was very favorable to the encouragement of the production of Guayule and especially to its potential as a domestic supply of natural rubber upon which we might rely in "grave emergency" (Taylor, 1951).

However, the federal government did not act on the recommendations of the report until the outbreak of World War II.

On March 5, 1942, Congress passed public law no. 473, billed as the "Emergency Rubber Project" (Taylor, 1951). The emergency was of course that the Japanese conquests in Southeast Asia had entirely cut off our primary, almost exclusive supply of rubber. When one recalls that at the time there was no synthetic rubber, the very real nature of the emergency becomes clear.

Although the primary mission of the Emergency Rubber Project was to produce as much rubber as possible as quickly as possible, much research was performed on Guayule and a great amount about its growing habits and rubber producing potential was learned. The information developed with regard to agricultural techniques for maximum rubber production, deresination of the rubber, rapid rubber assays, and the results of the breeding programs is beyond the scope of this work, but is reviewed in a very comprehensive fashion in the above-mentioned United States Agriculture Department Technical Bulletin No. 1327 (Hammond & Polhamus, 1965).

However, after all this effort, the Emergency Rubber Project was scrapped at the end of World War II. Synthetic rubber had just been invented and was rapidly being improved and reduced in price. Also the Southeast Asia rubber plantations were again producing natural rubber and supplying it to the West. Consequently, interest in Guayule languished until

0 0 0 0 4 6 0 7 3 0 1

quite recently. The present interest is spurred by the rapidly escalating price of oil from which synthetic rubber is derived, in conjunction with political instability and even unfriendliness in the producing nations of Southeast Asia. These considerations have produced renewed efforts in both Mexico and the United States.

In Mexico, the government has embarked on a rubber production effort designed to use the estimated 10 million acres of Guayule growing wild in the states of Coahuila, Zacatecas, Chihuahua, Nuevo Leon, and San Luis Potosi. They plan to harvest 300,000 tons of shrub annually and from this to produce 30,000 tons of deresinated rubber. A pilot plant designed to produce one ton of rubber a day was completed in March of 1976, and that plant is now producing tires which are undergoing testing (Vietmeyer, 1977).

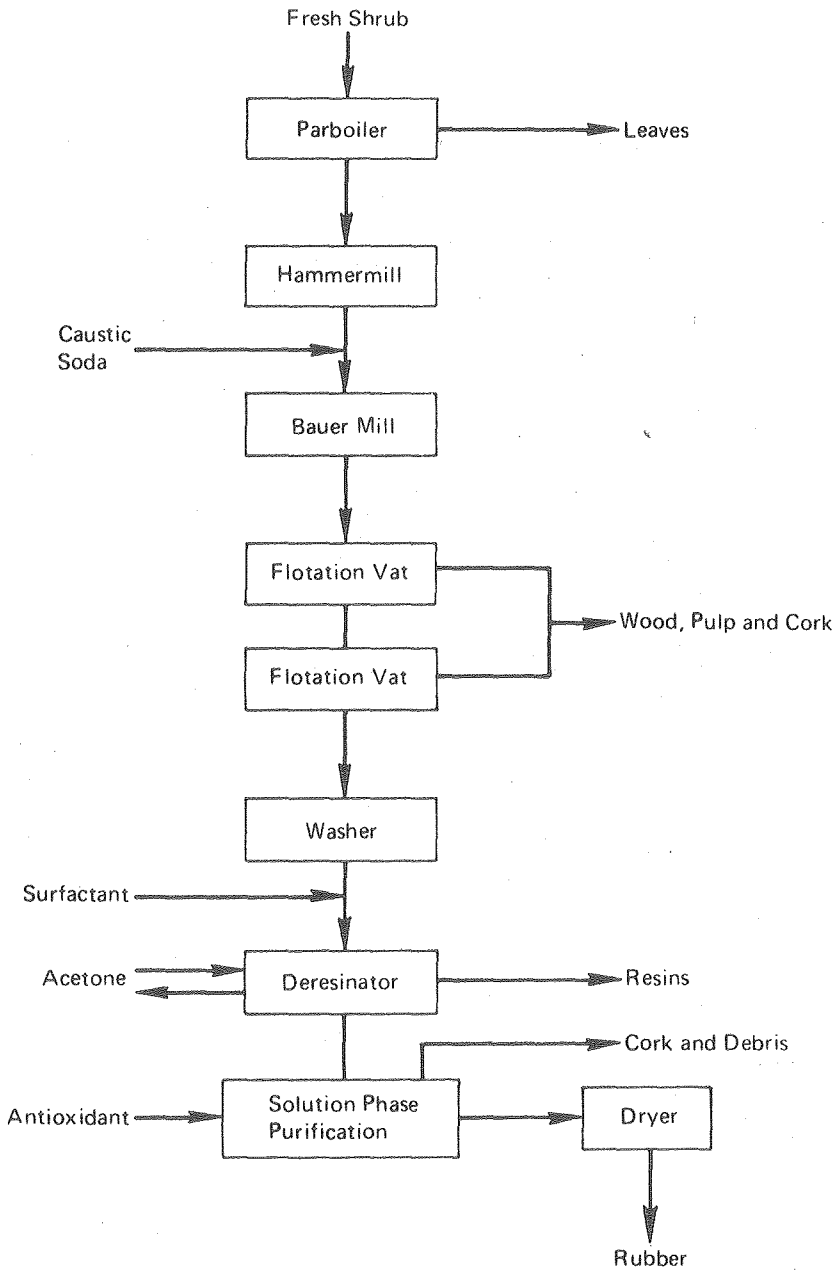
In the United States there is considerably renewed scientific interest. The National Academy of Science has recently produced a report very favorable to the development of Guayule as a national resource (Vietmeyer, 1977). Investigators at the Los Angeles Arboretum the the University of Arizona's arid land study group in Tuscon are performing breeding experiments in an attempt to develop superior strains of Guayule. At Lawrence Berkeley Laboratory, research into the rubber producing mechanism of Guayule is underway and is of course the subject of this report.

B. General Economics

As previously mentioned, over two thousand different species of plants produce hydrocarbons in some amounts. The circumstances that make Guayule interesting as a crop are its high rubber production--up to 26% of its weight can be rubber (Vietmeyer, 1977)--and its low water needs which make it viable on marginal soil. The primary drawback to rubber production with Guayule is the cost of extraction of the rubber. As will be described in greater detail later on, Guayule does not contain its rubber in latex vessels as in the Hevea tree, but rather in individual cells. Thus to release the rubber, the plant must be crushed and ground to remove the rubber from the cell walls. Crushing and grinding however produce aglomerates of the rubber, wood, and cork. Consequently a large number of physical and chemical steps are required to process rubber from Guayule.

Figure II-4 outlines the steps in the preparation of Guayule rubber. The fresh shrubs are dipped in hot water to coagulate the rubber in the cells, remove soil from the roots, and remove the leaves, which reduces by 20% the amount of material inputted to the mill.

The next two operations are grinding and hammering operations in mills. The mills are essentially the same as those used to pulp wood fiber for paper production. Caustic soda is added to the process because it helps to break open the cells and in some way promotes the separation of rubber



The method for commercially extracting rubber from guayule shrubs recently developed in Mexico.

FIGURE II-4

FROM(Vietmeyer,1977)

from vegetable material (Vietmeyer, 1977). The disposal of the spent caustic soda has environmental problems, and hopefully further research will develop a more acceptable separation technique.

The next step involves the floatation of the ground material in water baths to remove wood, pulp, and cork from the "worms" of rubber and resin. This is followed by a short wash in a bath containing detergent to keep the rubber "worms" from further aglomeration.

The final processing steps are chemical in nature, and are designed to remove resins and other undesirable contaminants from the rubber. First an acetone extraction is perfromed to remove about 95% of the resin and the water that has been carried along from the preprocessing (Vietmeyer, 1977). The acetone is recovered by distillation and reused.

The final step in the purification of rubber is to dissolve the rubber in hexane or cyclohexane. The rubber-hexane solution is then filtered to remove residual undesirables, including cork fiber and some dirt. The rubber is then recoagulated from the solution with hot wet steam and is then dried in ovens. The rubber thus produced is identical to Hevea rubber.

Much of the cost of the rubber produced in this fashion can be offset by the marketing of byproducts. The leaves of the plant stripped early on in the process are an excellent mulch material. In addition, the pulp produced is a good

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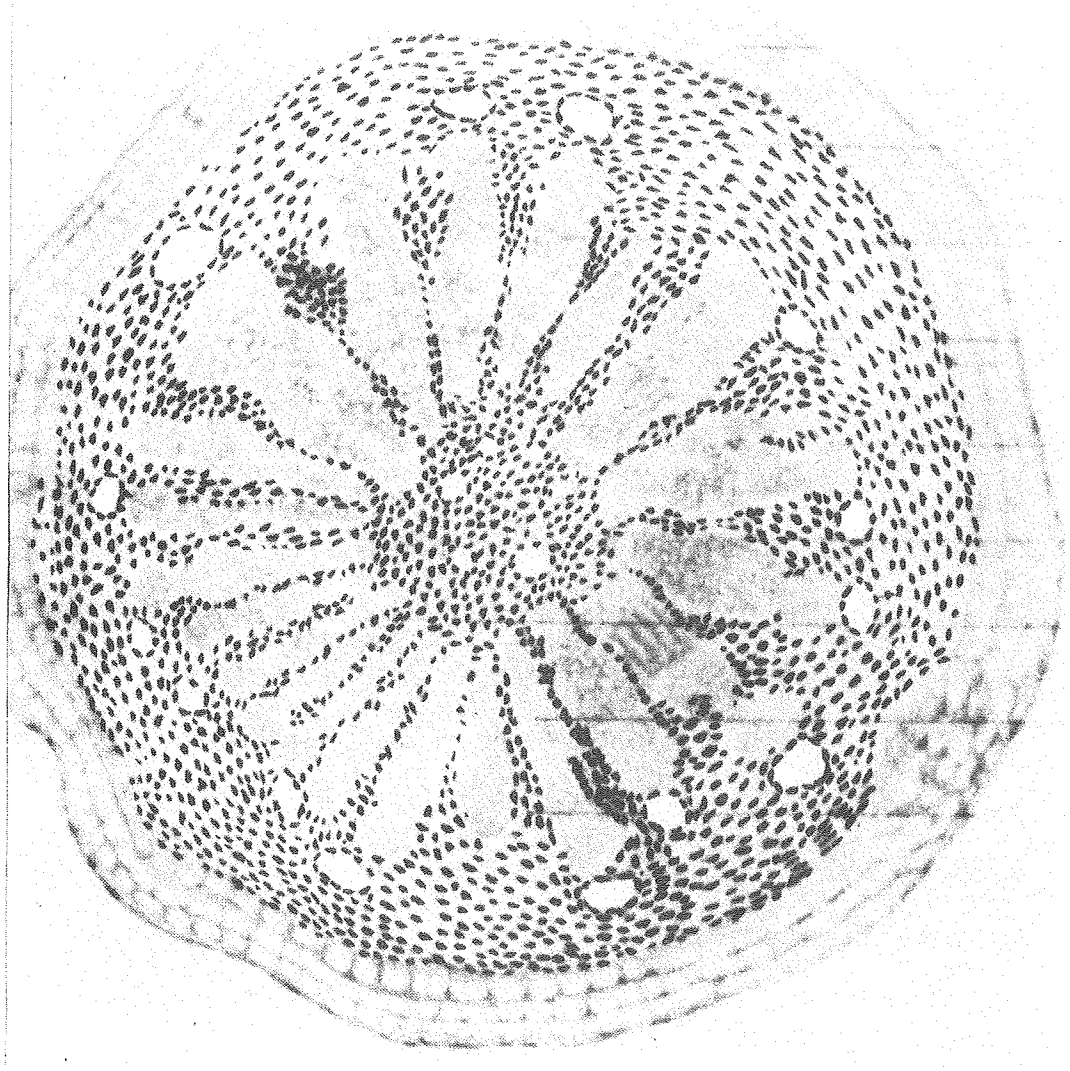
starting material for paper. Finally, the resins removed as contaminants have an economic value themselves. These resins are primarily monoterpenes, sesquiterpenes, and diterpenes (Vietmeyer, 1977). These materials can either be used as they are or as chemical feedstocks.

At this point it is impossible to state a precise figure for the cost of production of Guayule rubber, since there are no commercial Guayule rubber facilities in operation. It has been estimated however that the cost of producing Guayule rubber in 1946 was around $.25 a pound (Hammond & Polhamus, 1965). The cost of labor and materials has increased considerably since that time, but also a number of technological innovations have occurred to bring the price back into line. For comparison, the current price of Hevea rubber is $.29 a pound, but the world bank expects prices to increase to approximately $.60 per pound by 1980 (Vietmeyer, 1977).

C. General Morphology

Figure II-1 is a photograph of a Guayule bush. The plant is a shrub which grows perhaps to be 3 feet tall. It has a short central stalk, which rapidly branches out. Guayule has a very wide shallow fibrous root system with a large central tap root which usually looses its prominence to the lateral roots and their branches (Hammond & Polhamus, 1965). The rubber is found in the root and in the stem. The fact that the rubber is contained within these hard woody structures



Guayule stem cross-section. The stained rubber shows up as black dots, the resin ducts as white holes. (F.T. Addicott)

XBB 770-10750

FIGURE II-5

FROM (Vietmeyer,1977)

0 0 0 0 4 6 0 7 3 0 3

necessitates the milling and grinding operations described in the previous section.

Figure II-5 is a cross section of a Guayule stem which shows the location of the rubber. One somewhat unusual aspect of Guayule morphology is the existence of "resin canals." These are tube-like structures running the length of the plant from the leaves down to the roots and which lie just outside of the vascular system. These canals are extra cellular spaces, as opposed to the latex vessels in Hevea which are differentiated cells. The contents of the resin canals are a mixture of terpenoids and fats. The function of the resin canals is unclear.

The next section of this report is devoted to morphological material, with particular emphasis on our original work at the electron microscope level.

III. MORPHOLOGY OF GUAYULE--STRUCTURE AND ULTRASTRUCTURE

A. Introduction

The purpose of Section III is to present the morphology of Guayule at both the light and the electron microscope level, and to relate the plant's morphology to its hydrocarbon production. The general structures of the seedling stem and root and the adult root, stem, and leaf will be presented. The light level micrographs and microscopic drawings are all taken from an excellent monograph by Ernst Artschwager (1943). Artschwager was the senior plant anatomist on the World War II rubber project, and he shared the view of this author that the study of the anatomy of the Guayule plant could be very profitable with regard to improving the yields of hydrocarbon in the plant (Artschwager, 1943).

In this section, particular attention will be paid to the resin canals of the Guayule plant, and these structures will be compared with similar structures in other species of plant. Similarly, attention will be given to the structures of the cells immediately surrounding the resin canals in the Guayule plant. Of particular importance here are the organelles (plastids) found in these cells and suspected of being involved in the synthesis of the alkenes. Finally, we will direct our attention to the chloroplasts of the Guayule, since the autoradiographic results reported in a later

section of this work implicate these organelles in the early steps of hydrocarbon biosynthesis.

Similarly, several micrographs of phloem and surrounding tissue will be presented. This is because of the role of phloem in transport of materials from the leaf chloroplasts to the cells of the root and stem. These materials are very likely to include the precursors of rubber. Also, the vascular rays of phloem contain large amounts of rubber in the adult plant (Artschwager, 1943).

In contrast, very little emphasis has been placed on the tissues of xylem, epidermis, and ground cells of the cortex. These tissues contain little, if any, rubber.

B. Methods and Materials

In all our work on Guayule, specimen preparation involved a four-hour fixation in 2% glyuteraldehyde followed by an overnight post-fixation with 2% osmium in the cold. Dehydration was carried out by a graded series of ethanol solutions, starting with a 50% ethanol and water mixture and increasing at 10% intervals to 100 ethanol. Finally, the tissues were embedded in Spurr's medium and baked for twelve hours at 70°C. The plastic embedded sections were stained with Reynolds lead citrate and uranyl acetate.

Guayule seeds were first obtained from the National Seed Storage facility at Ft. Collins, Colorado. Later seedlings were produced from seeds supplied by Dr. John McFarland

of the United States Department of Agriculture Research Station in Salinas, Calfiornia. Dr. McFarland also provided sections of root and stem from mature Guayule plants for our work. Samples from other hydrocarbon producing plants exhibited in this section were provided by Dr. Bruce Bartholomew of the University of California Botanical Garden.

C. Seedling Structure and Ultrastructure

1. Seedling root. Figure III-1, due to Artschwager (1943), presents a cross section of a young root from a Guayule seedling. Somewhat later in the plant's development, resin canals will form from spaces created between the endodermal cells just outside of the phloem groups. The ontogeny of the resin canals will be discussed later on in this section.

Figure III-2 is an electron micrograph of a cross section of a seedling Guayule root. Evident here are the phloem bundles, the xylem in the lower right-hand corner, and two resin canals in the upper right-hand corner.

Figure III-3 is a somewhat magnified micrograph from the area of Figure III-2 to show the detail of a resin canal and the surrounding endodermal cells. As can be seen, these surrounding endodermal cells are living and possess a dense cycloplasm and many organelles. As previously stated, the resin canal itself is an extracellular space into which terpenoids and fats are secreted by the surrounding cells.

Figure III-4 is an enlarged view of the phloem in the

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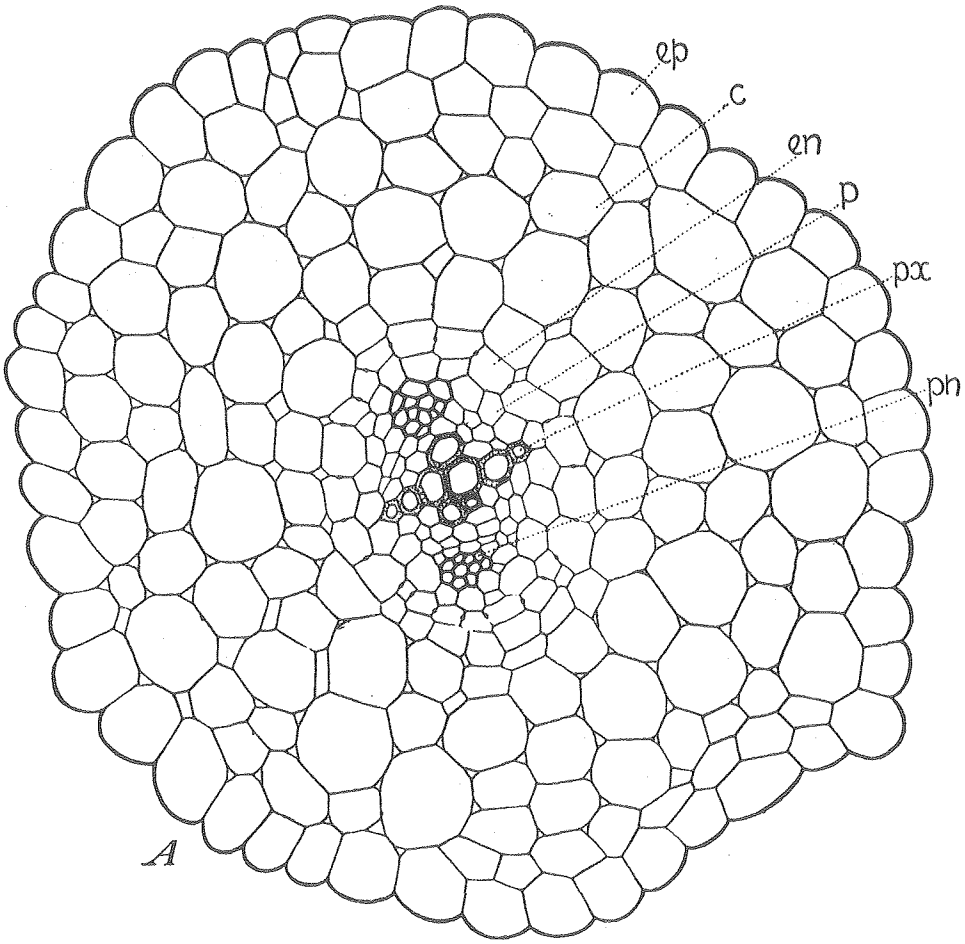

EP=EPIDERMIS C=CORTEX EN=ENDODERMIS P=PERICYCLE PX=PROTOXYLEM PH=PHLOEM

FIGURE III-1

FROM (Artschwager,1943)

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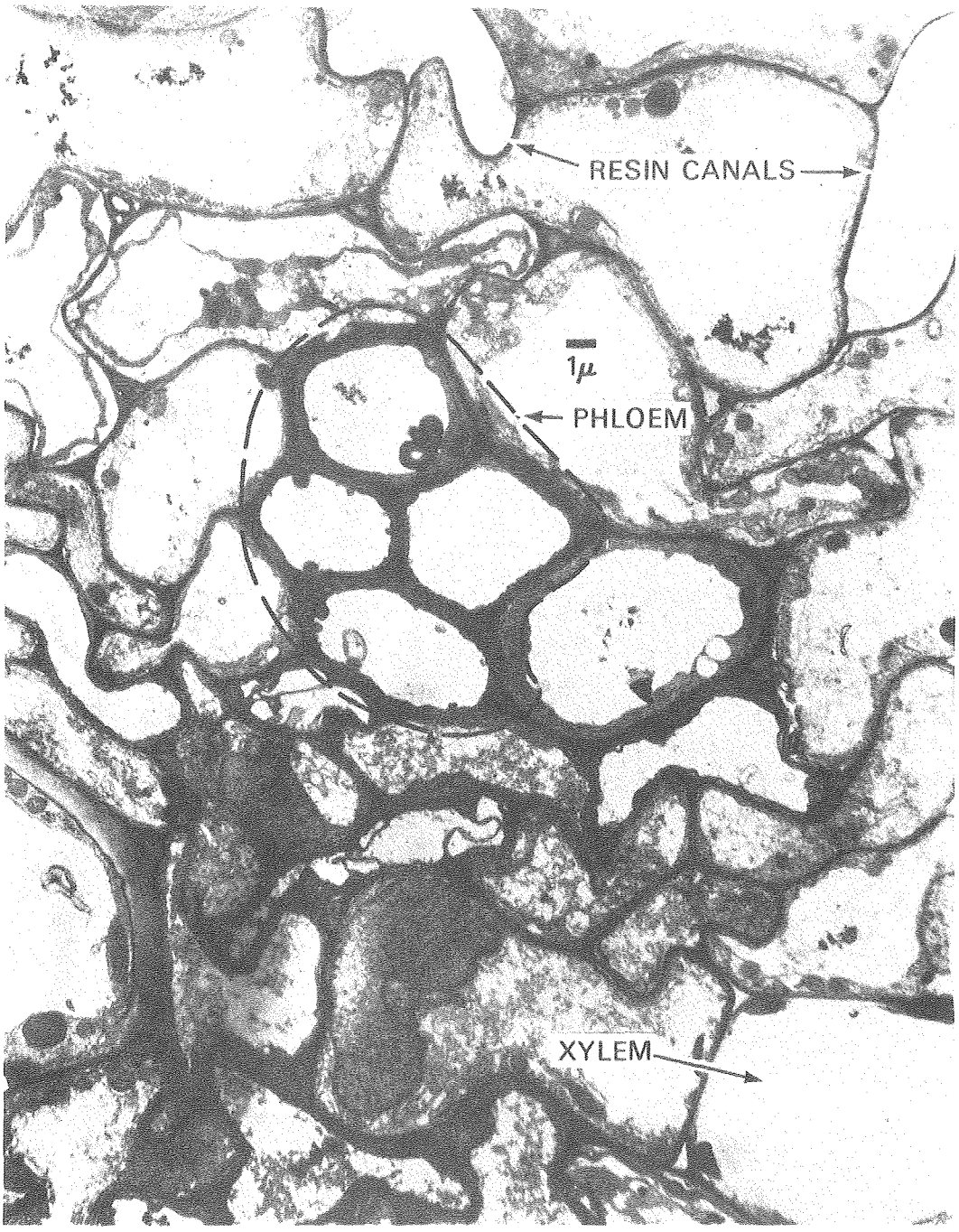

Micrograph 5. Parthenium argentatum.

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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FIGURE III-2

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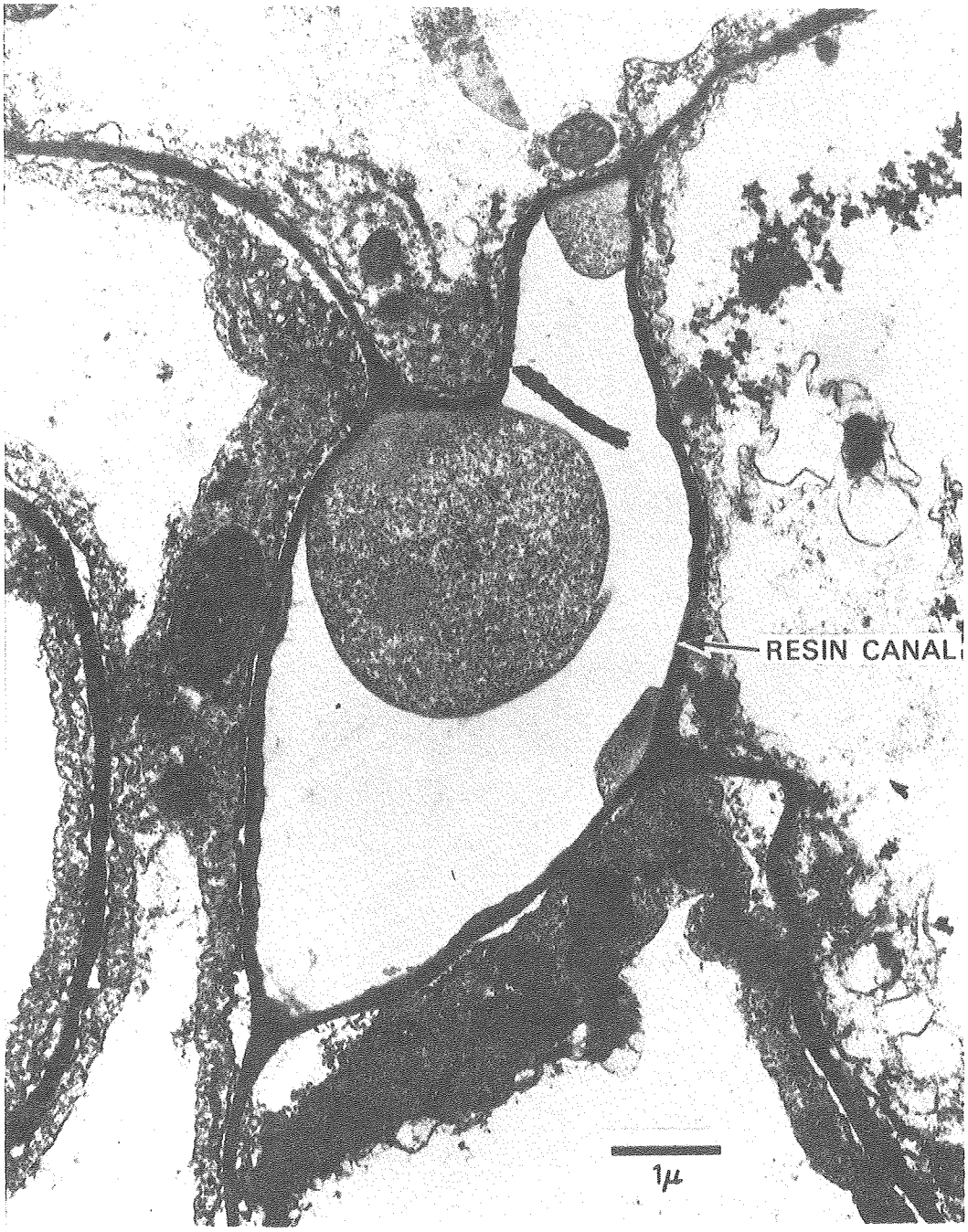

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Micrograph 7. Parthenium argentatum.

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.

FIGURE III-3

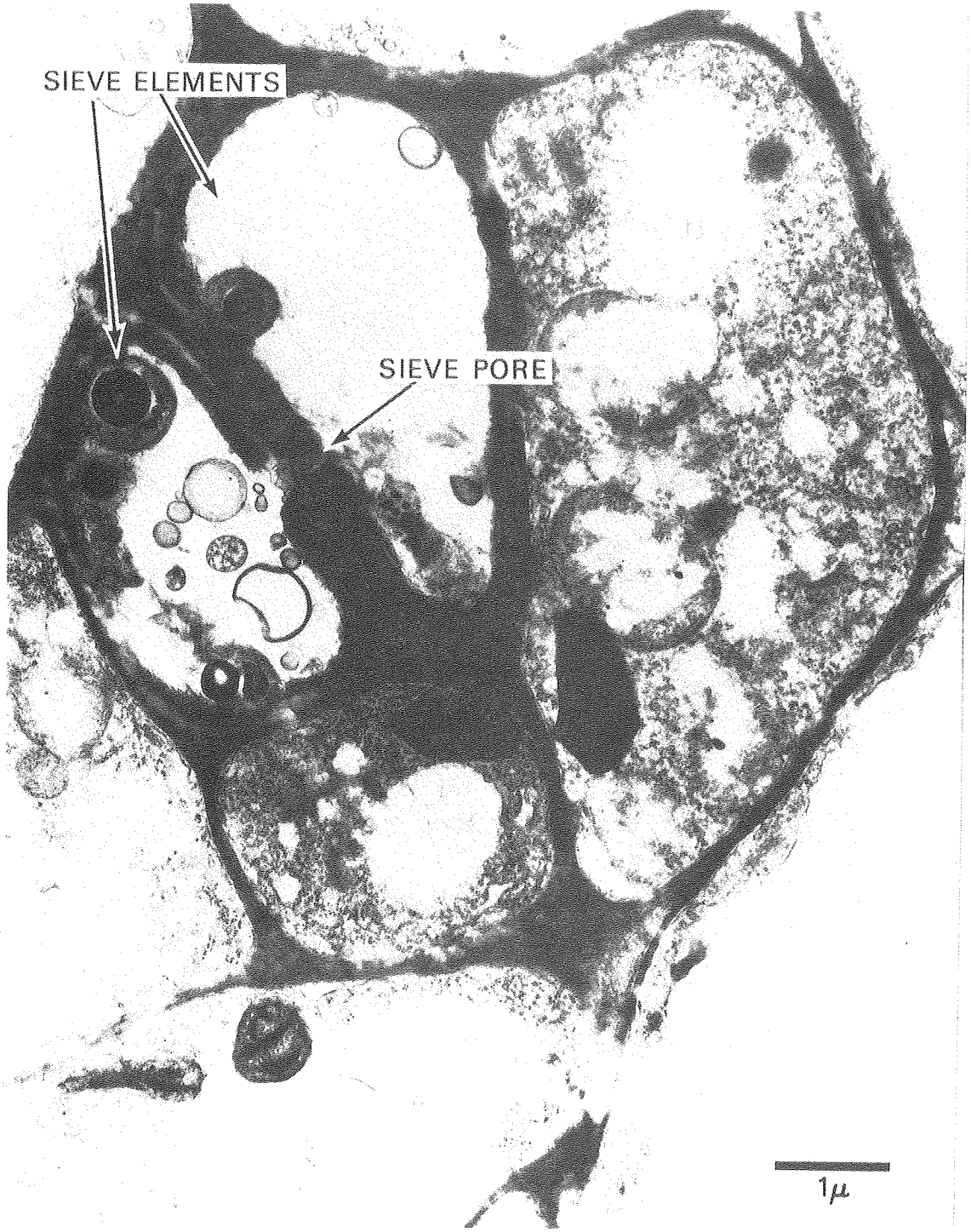

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FIGURE III-4

young root. Evident within the sieve elements are starch-containing plastids known as amyloplasts. Also seen is a sieve pore connecting two sieve elements.

2. Seedling stem. Figure III-5, also due to Artschwager (1943), is a cross section from the hypocotyl (lower stem) of a Guayule seedling. The resin canals and surrounding endodermal cells can be seen just outside the phloem groups.

Figure III-6 is an electron micrograph of the stem of a 2-week-old Guayule seedling. Figures III-7 and III-8 are enlarged views of a resin canal with surrounding cells and a vascular bundle respectively.

D. Adult Plant Structure

1. Adult leaf. This section presents the morphology of the adult leaf. Particular attention will be given to the chloroplasts within the cells of the leaf because they are the primary producers of all materials used in subsequent biosynthesis elsewhere, including the hydrocarbons. Also, material presented in the next section indicates that the chloroplasts are responsible for steps in the production of rubber considerably later in the biosynthesis than previously anticipated.

Figure III-9, due to Artschwager (1943), is an artistic rendering of the structure of the adult leaf (including details of the leaf hairs). As can be seen, resin canals are associated with vascular bundles in the plant, again positioned just outside of these vascular areas. Also shown in part C

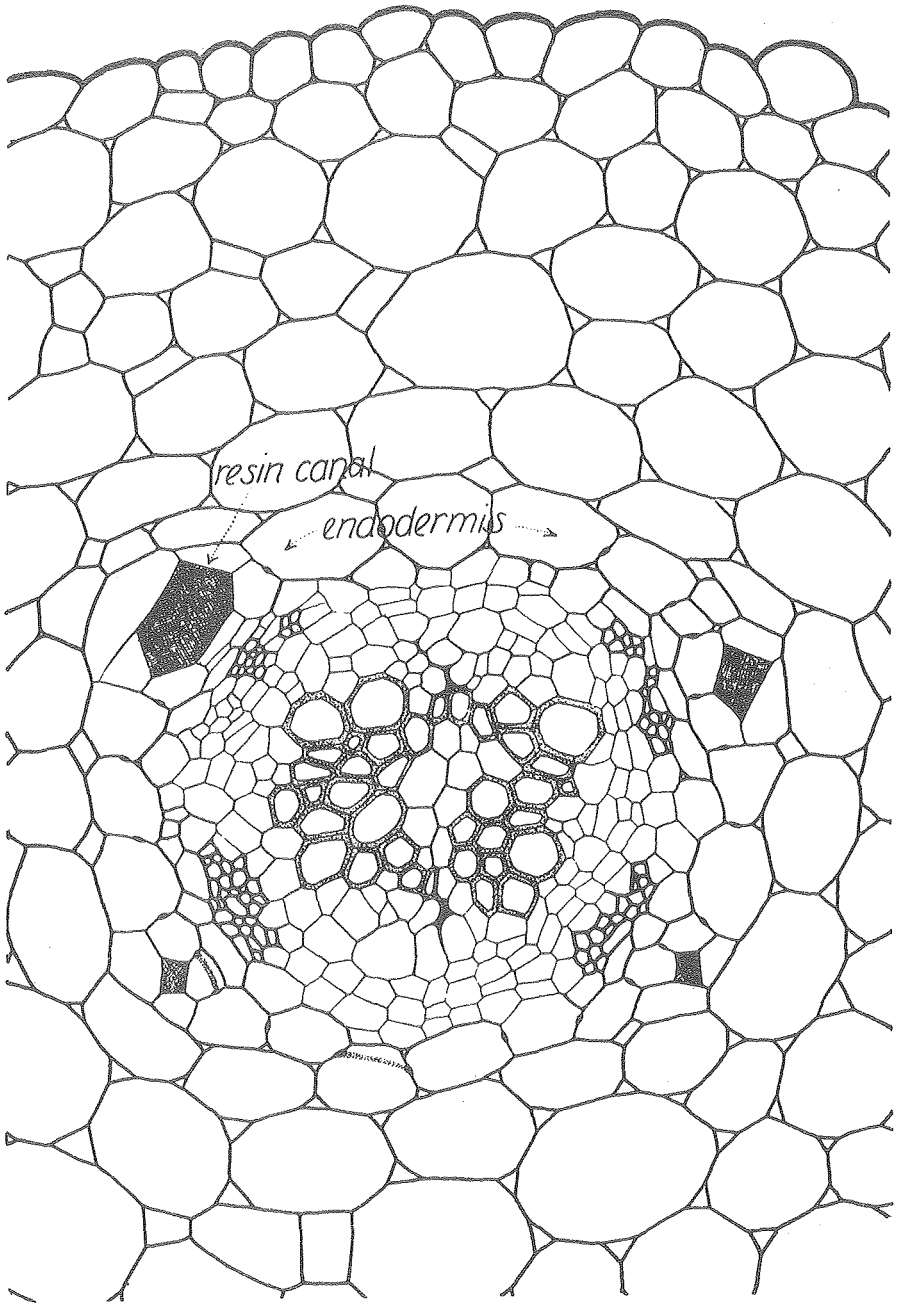

FIGURE 8.—Cross section through lower hypocotyl showing resin canals opposite the four phloem groups. The protoxylem points are several cell layers centrad from the pericycle. × 470.

FIGURE III-5

FROM (Artschwager, 1943)

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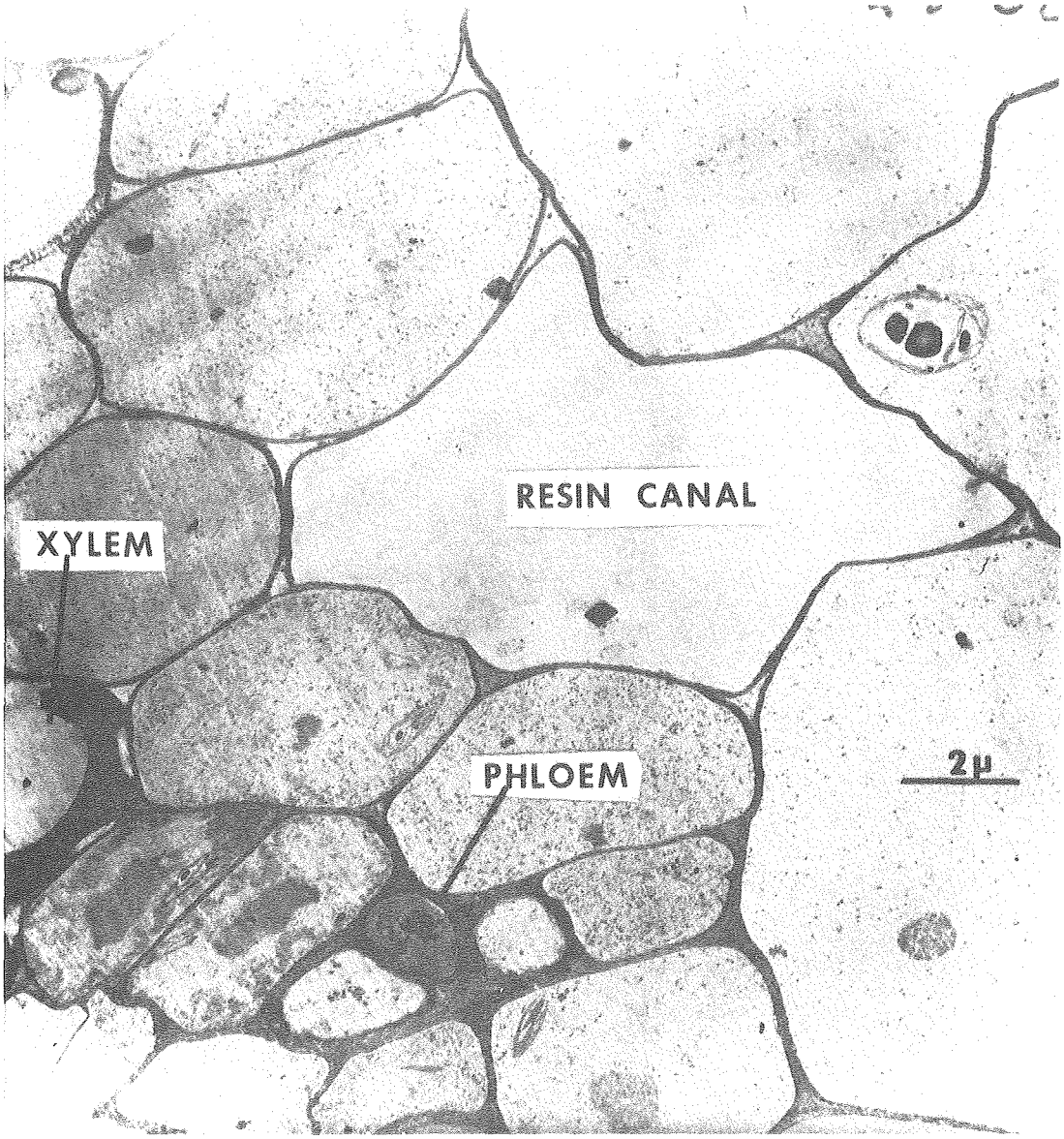

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FIGURE III-6

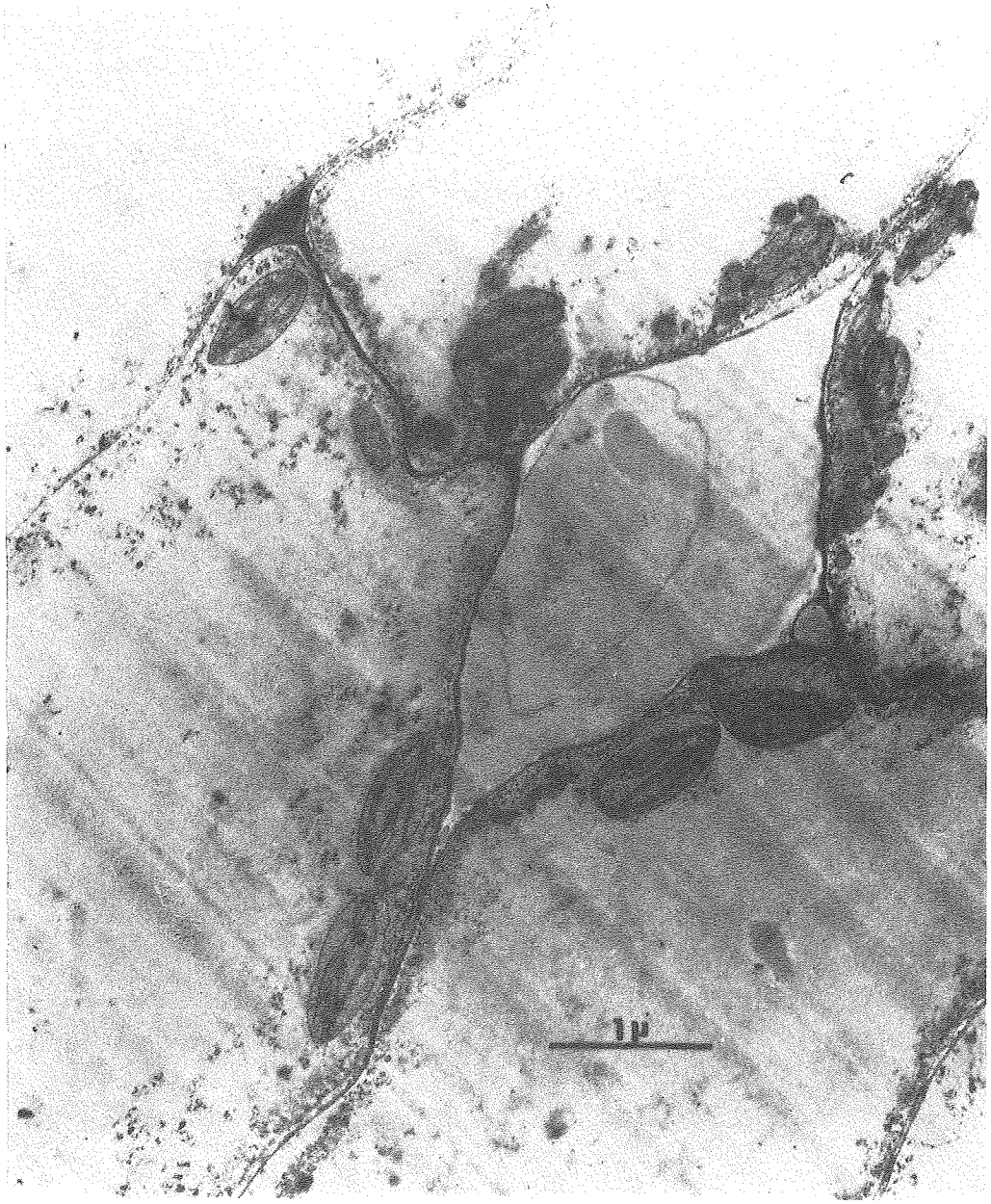

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FIGURE III-7

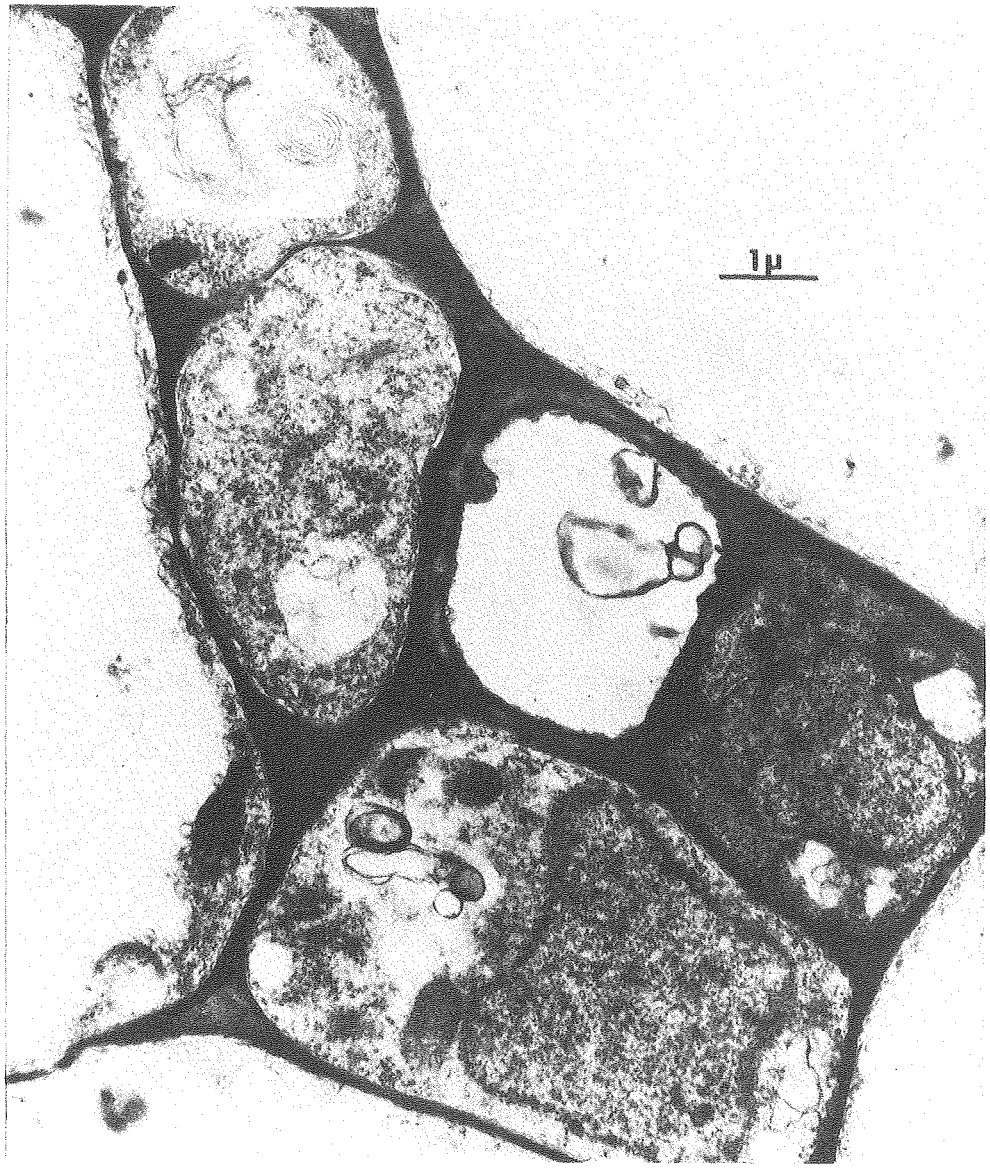

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FIGURE III-8

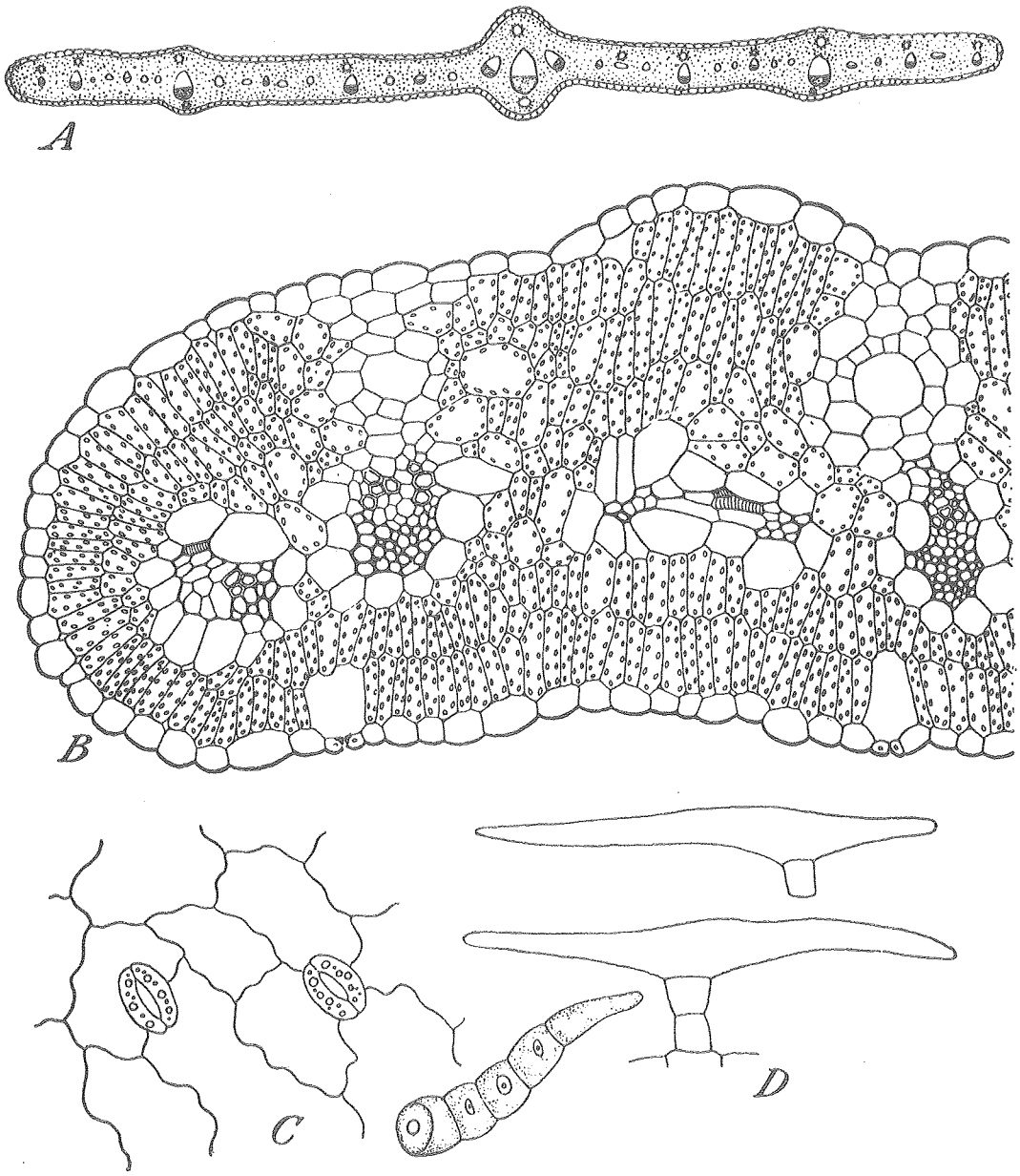

FIGURE 18.—*A*, Cross section of leaf; *B*, enlarged part of cross section to show detail in structure. × 190. *C*, Surface view of upper epidermis. × 200. *D*, Types of hairs. × 200.

FIGURE III-9

FROM (Artschwager, 1943)

XBL 7711-10381

of the figure are the stomates of the plant. These are the structures through which gasses are exchanged between the leaf and the environment. As suggested in Section IV of this paper, the stomates may close during the day, cutting off this exchange, requiring a mechanism to produce carbon dioxide during the high light intensity conditions in which Guayule usually grows.

Also shown in detail are the leaf hairs, which are T shaped with a long thin cylindrical cell mounted on top of a stalk of one or two cells.

Figure III-10 is a series of scanning electron micrographs taken of the leaf of the adult Guayule. These were taken by Professor Thomas Hayes of the University of California at Berkeley.

Figures III-10A and III-10B show the leaf hairs lying on top of a rather lumpy epidermis. Figure III-10C is a high magnification view of these leaf hairs showing a rather "warty" appearance of their exterior. Figure III-10D shows the edge of a leaf hair next to a stomate rising from the epidermis of the leaf.

Figure III-11 is a transmission electron micrograph of a section from the tip of one of the leaf hairs. It can be seen that the structure of the wall has at least two different layers with the very dark staining "warts" on the surface. The fact that these knobs are stained so darkly indicates they are osmophlic, and probably terpenoid in nature.

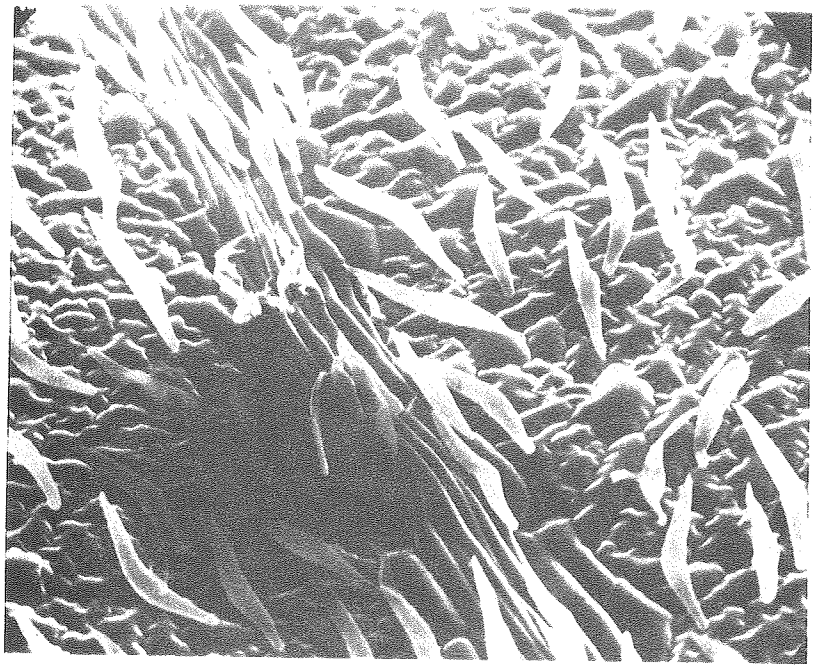

FIGURE III-10A

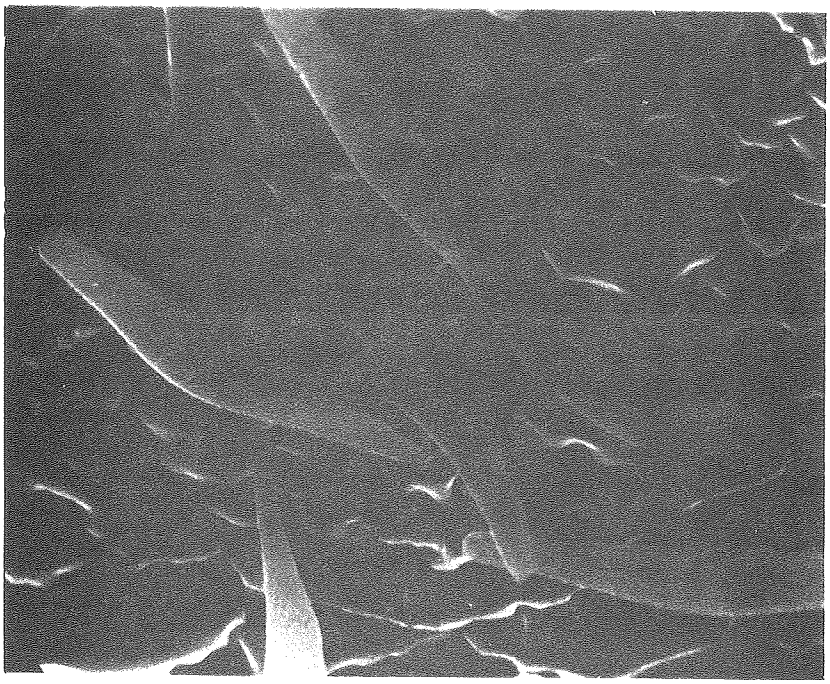

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FIGURE III-10B

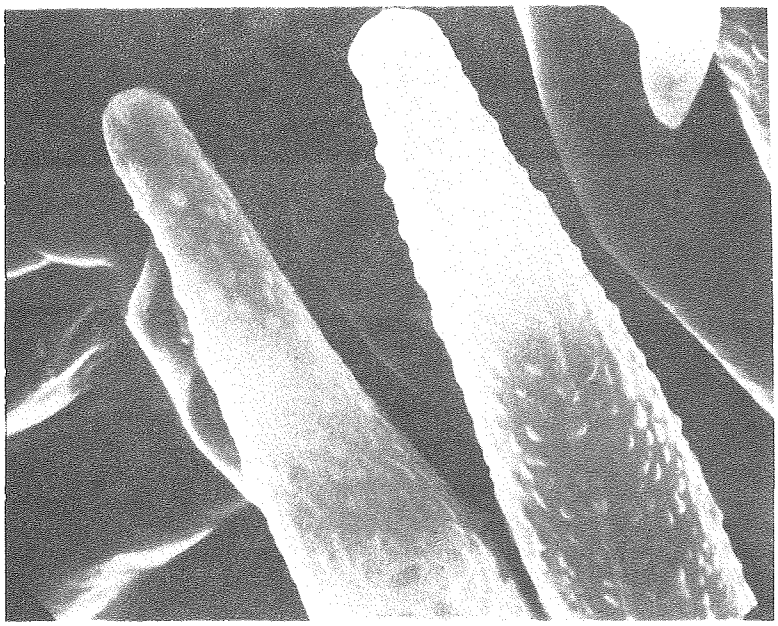

FIGURE III-10C

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FIGURE III-10D

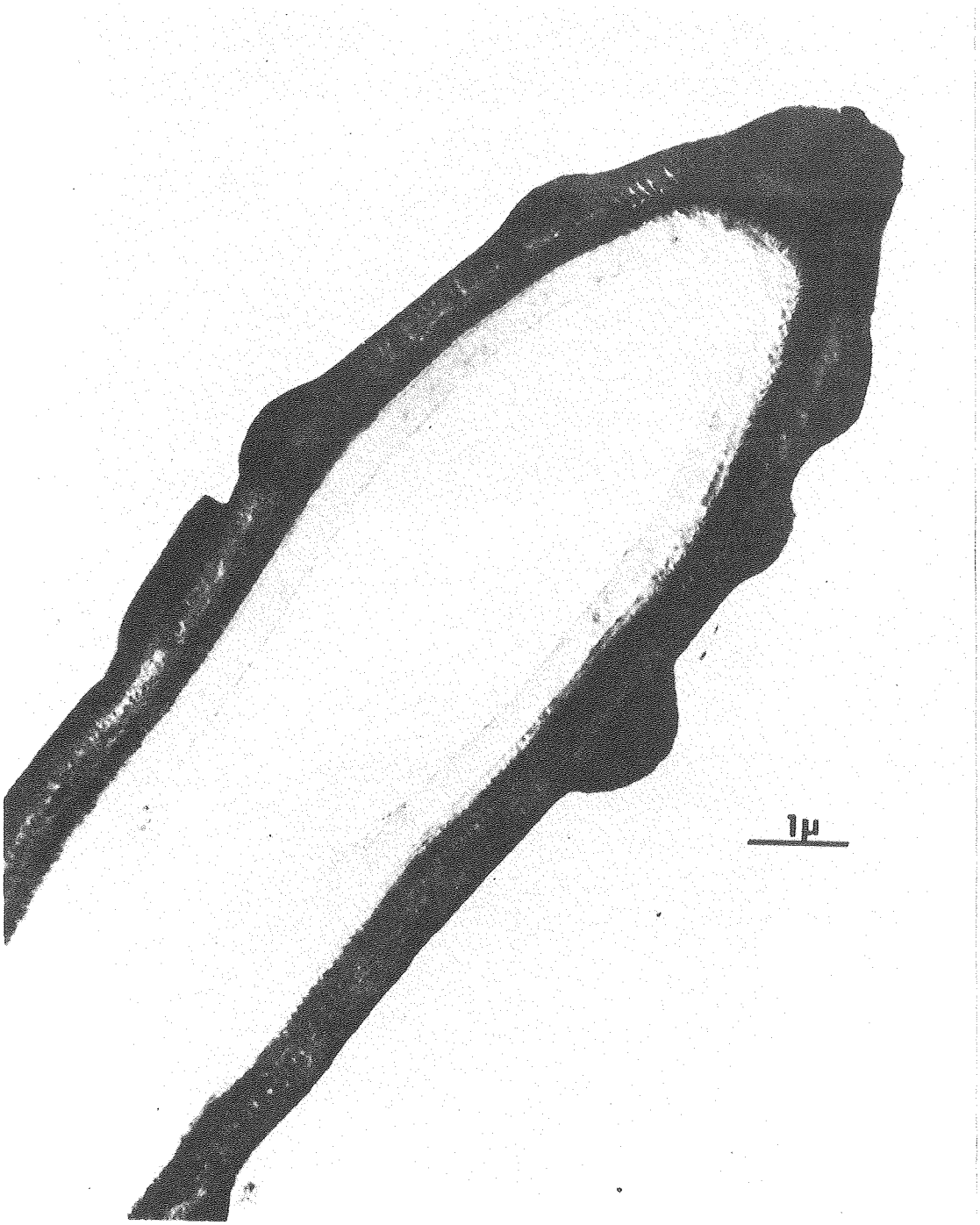

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FIGURE III-11

Figure III-12 is a transmission electron micrograph of the palisides layer of an adult Guayule leaf. Seen are the large extracellular spaces connected to the stomates for gas exchange with the chloroplasts. The chloroplasts are seen lying along the walls of the cells. Also seen are starch inclusions in the center of most of the chloroplasts and small black inclusions known as plastoglobuli.

Figure III-13 is another view of the same tissue showing once again the starch inclusions in the chloroplasts and the plastoglobuli. It can be seen in both Figure 12 and 13 that the Guayule chloroplasts do not have the nice "football" shape commonly associated with chloroplasts, but are considerably less regular.

Figure III-14 is an electron micrograph of a somewhat higher magnification of the same area showing the chloroplasts with their inclusions near mitochondria and other subcellular components. Seen are folds of endoplasmic retriculum in the upper areas of the micrograph. Figures III-15A and III-15B form a stereo pair at high magnification of part of a chloroplast. One can see starch inclusions, the grey smooth shapes, and plastoglobuli. Finally, just outside of the chloroplasts in the upper right-hand corner there is an interesting structure of endoplasmic retriculum. The value of stereo projections at this level should be evident by looking first at one of the micrographs and then comparing that with the detail seen when viewing both under a stereo viewer.

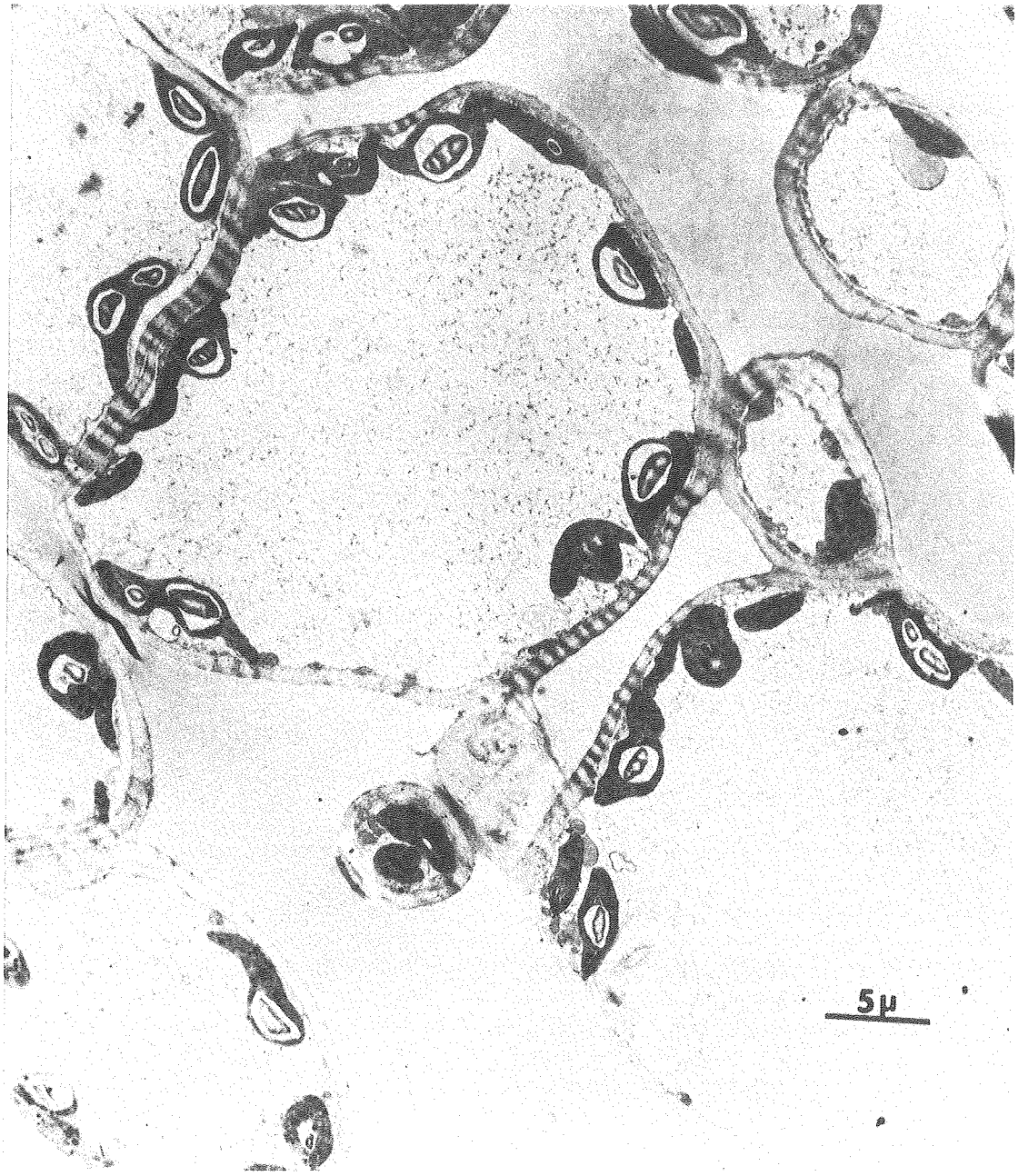

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FIGURE III-12

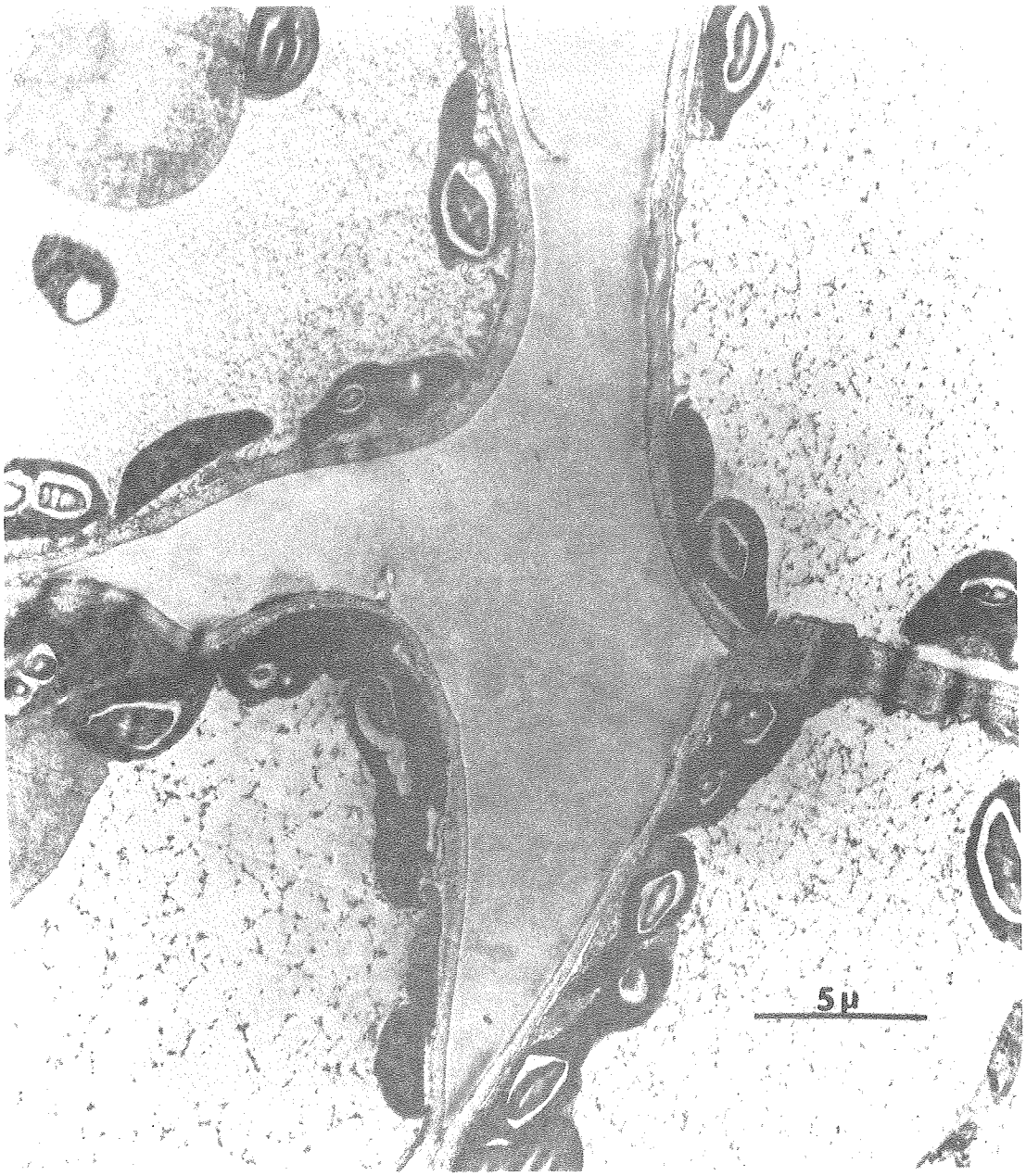

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FIGURE III-13

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FIGURE III-14

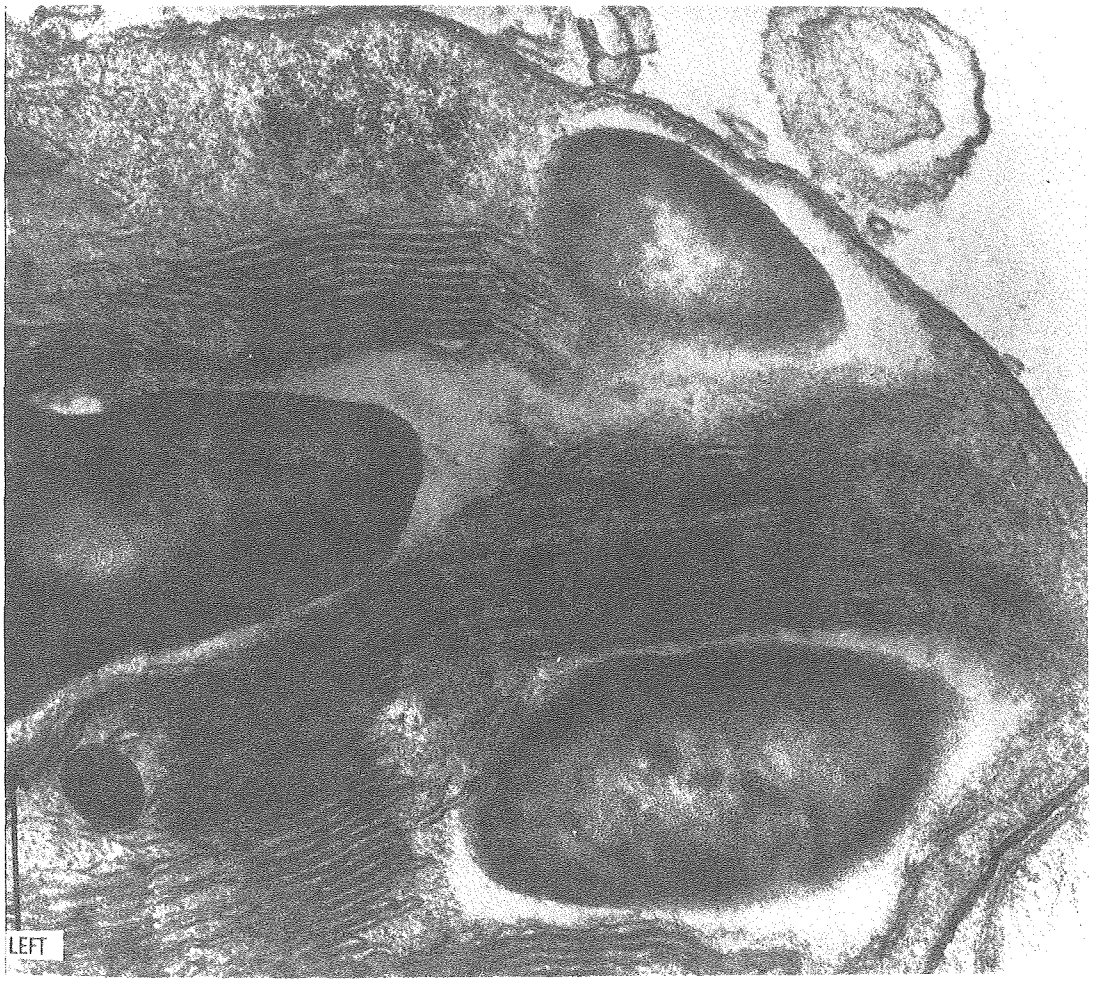

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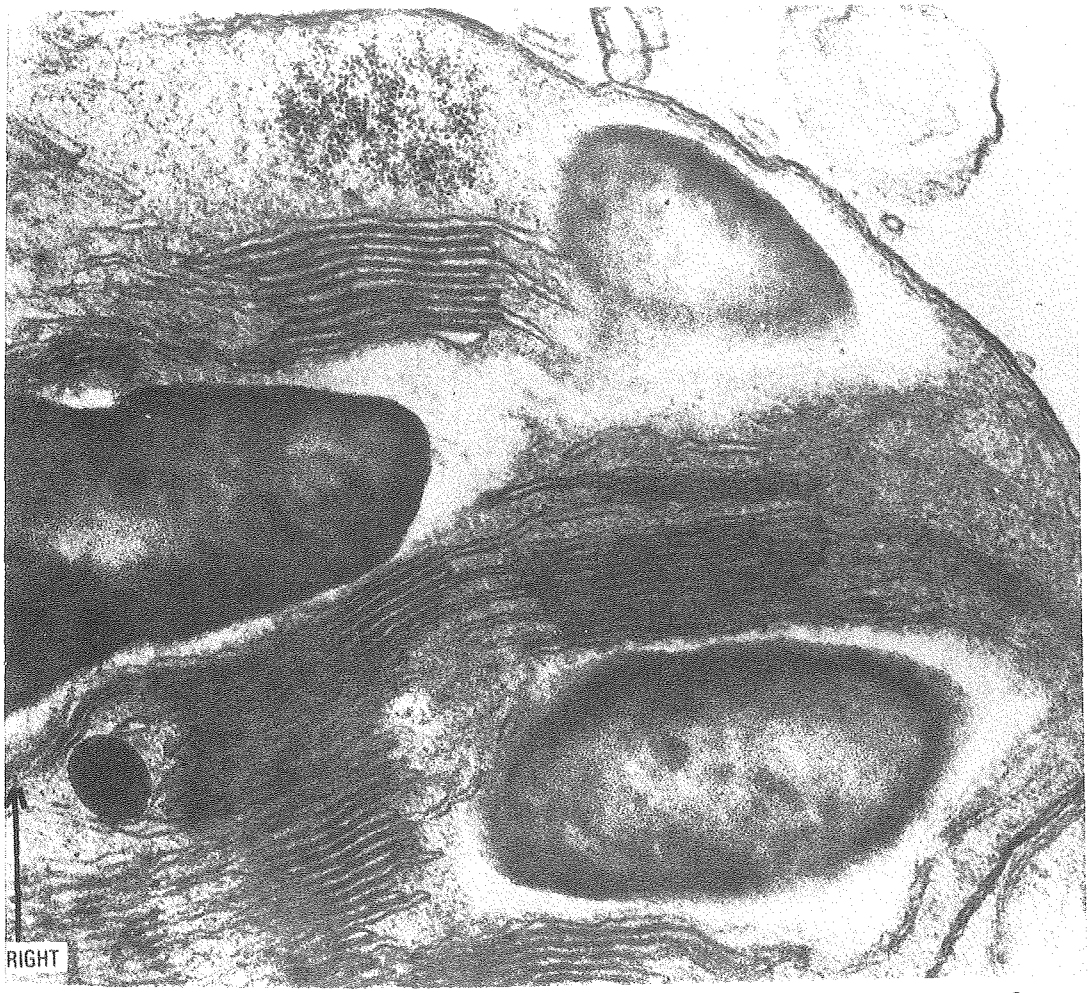
FIGURE III-15A



XBB 770-10879

FIGURE III-15B

XBB 770-10897

FIGURE III-16A

XBB 770-10898

FIGURE III-16B

Figures III-16A, and III-16B form another stereo pair of the tip of a chloroplast, again demonstrating the structures within the chloroplasts in three dimensions. Figures III-15 and III-16 were taken from thin sections (approximately 1000 Angstroms) using a tilt stage in our 100 KV AEI electron microscope.

2. Adult root. Figure III-17, due to Artschwager (1943), is a drawing of a light level micrograph of the tap root from a young adult Guayule plant. Here the resin canals are shown surrounded by endodermis. In later secondary growth, the resin canals are bounded by non-endodermal parenchymal cells. A similar drawing of a mature stem in the next section (Figure III-14) shows this secondary growth pattern.

Figure III-18 is an electron micrograph from the root of a mature Guayule plant found growing in Salinas. Across the center of the photograph is an adult resin canal that has been squashed transversely because of the growth of the root. Surrounding the resin canals are living rubber-containing cells (parenchyma). The dark, more or less spherical blobs with the "wet cake" appearance are the rubber. Also seen in the cells are a nucleus and organelles.

The adult root tissue was very difficult to properly embed and section. Also the level of preservation was found to be less than sufficient for high-resolution micrographs of structural details. The cells of adult stems store at least as much rubber as the roots, contain the plastids

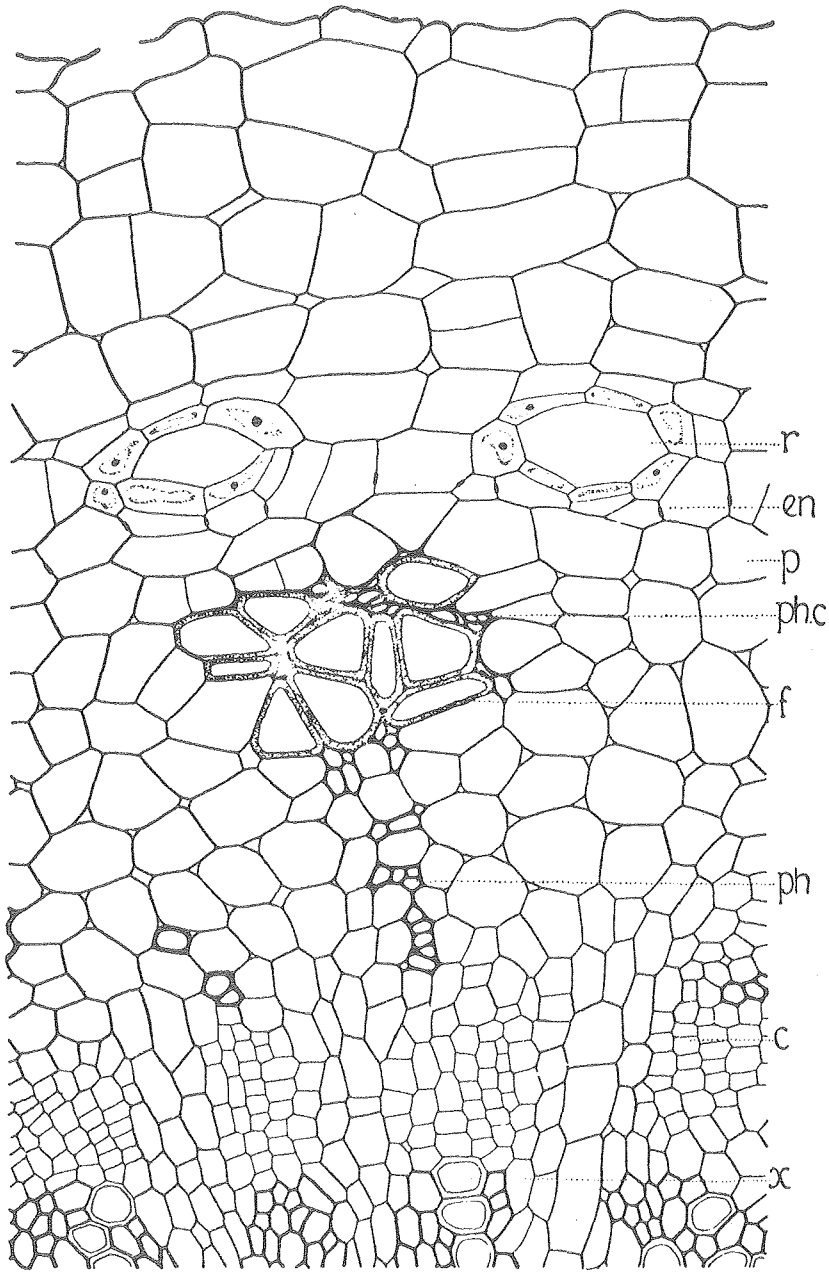
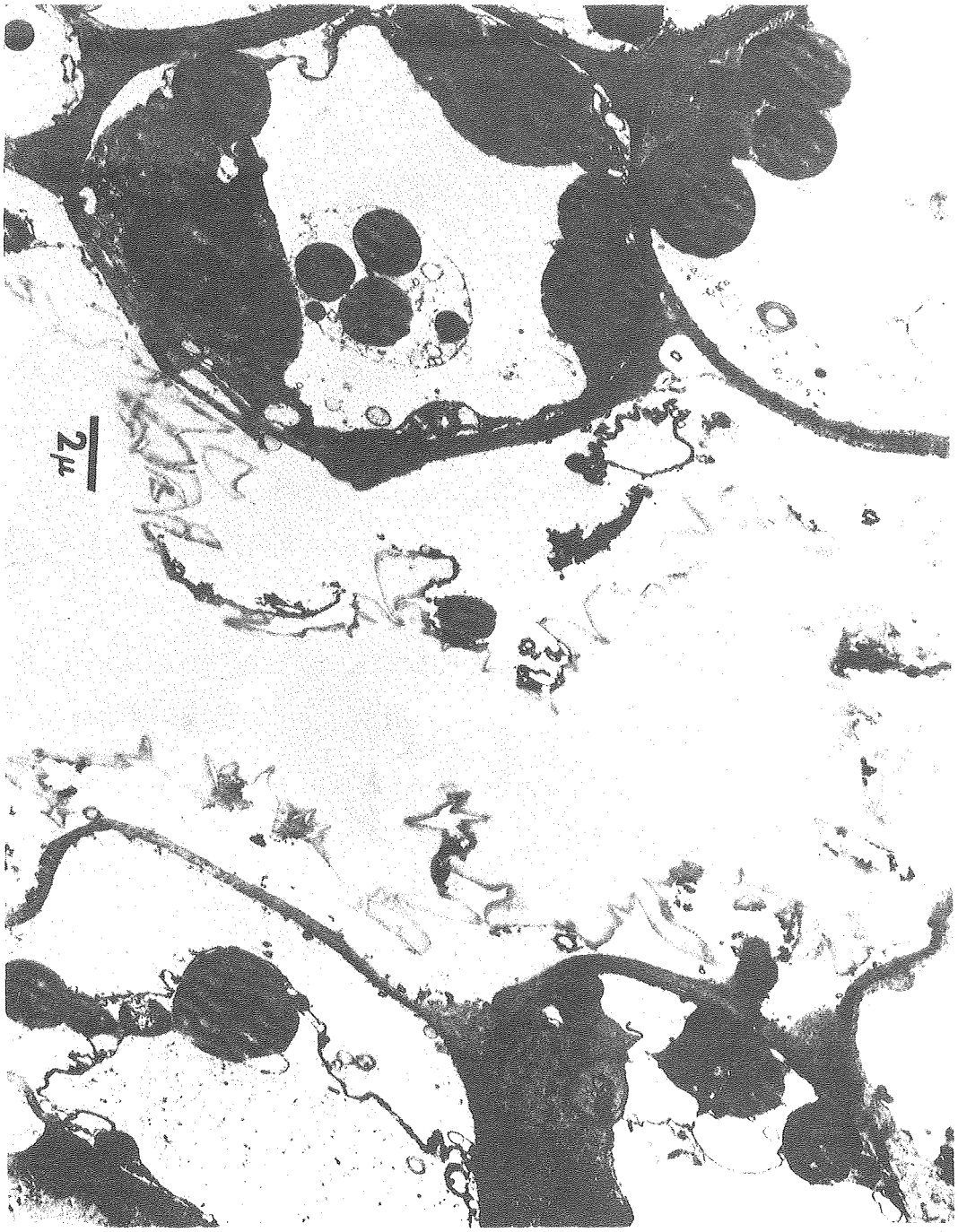


FIGURE 7.—Partial cross section of taproot 1.35 mm. in diameter, showing resin canals and group of fibers. × 410. *r*, Resin canal; *en*, endodermis; *p*, pericycle; *ph. c*, crushed primary phloem; *f*, group of primary fibers; *ph*, primary phloem; *c*, cambium; *x*, xylem.

FIGURE III-17

FROM (Artschwager,1943)

XBL 7711-10378



XBB 770-10747

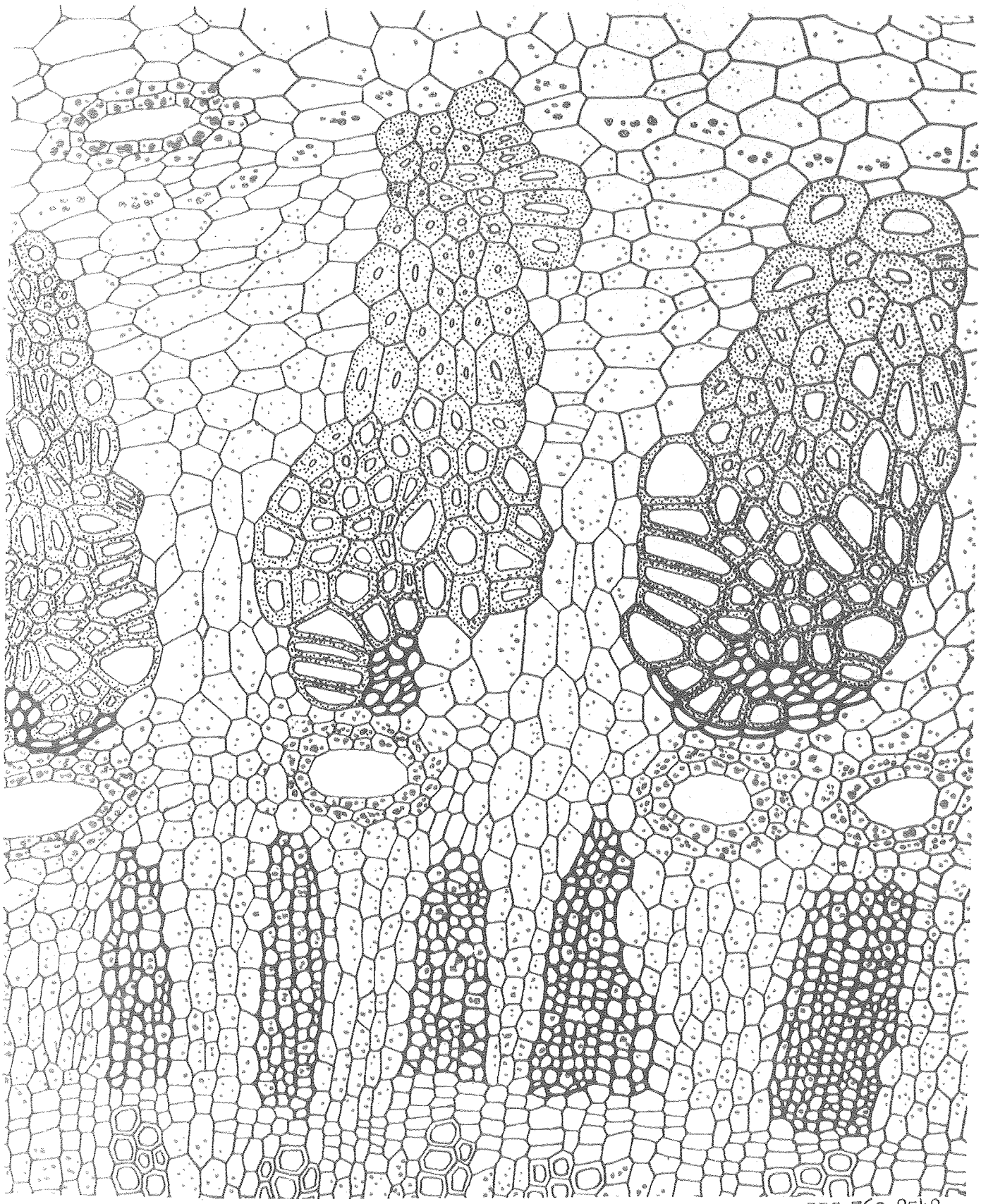
FIGURE III-18

suspected of involvement in rubber biosynthesis, and were much better preserved in our preparation procedures. Consequently, no further micrographs of the adult root will be presented, and the study of subcellular components suspected of involvement in later stages of rubber production will be presented only for the adult stem.

3. Adult stem. Figure III-19 (Artschwager, 1943) is a drawing of a light level micrograph of the stem of four-month-old Guayule plant. Also, Figure II-5 is a photograph of a four-month-old stem showing the larger-scale relationships of structures.

Figure III-20 is an electron micrograph of a cross section of the stem of an adult plant showing vascular tissue, resin canals, and ground tissue. Figures III-21 and III-22 show details of the phloem sieve elements and surrounding companion cells. Mature sieve elements have essentially no cytoplasm and serve only a transport function. They are supported by the neighboring companion cells which transport the essentials of life to the sieve elements by means of channels through the cell walls called plasmodesmata. The companion cells are rich in mitochondria which correlates with their supportive metabolic function. The micrographs illustrate these features.

Figures III-23 and III-24 are electron micrographs of resin canals of a mature Guayule stem. Here the surrounding tissue is made up of non-endodermal parenchymal cells



BBC 769-8548

Semidiagrammatic drawing of cross section of base of actively growing 4-month-old shoot. × 175. Orange color indicates rubber; blue color, starch.

FROM (Artschwager, 1943)

FIGURE III-19

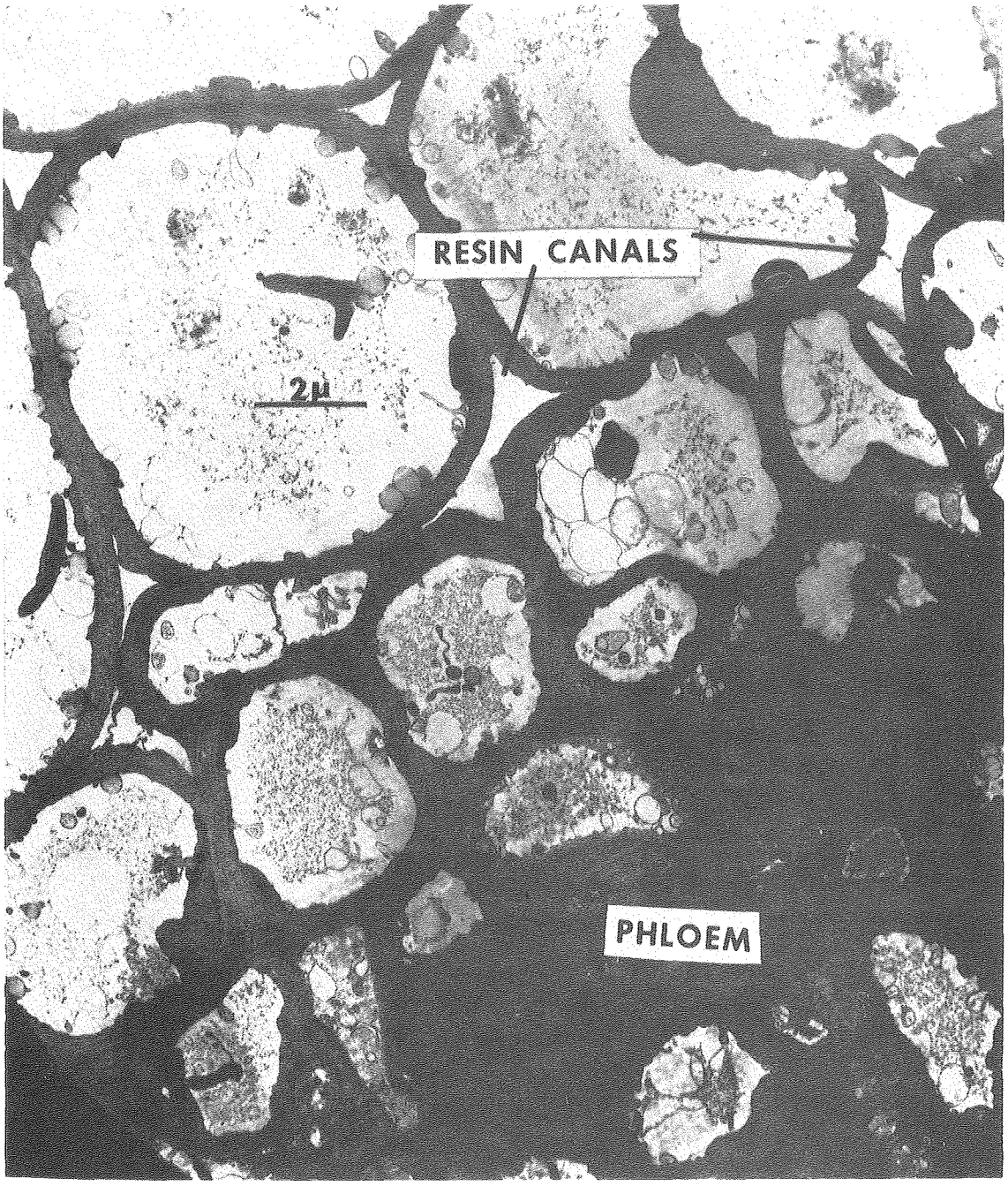


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FIGURE III-20

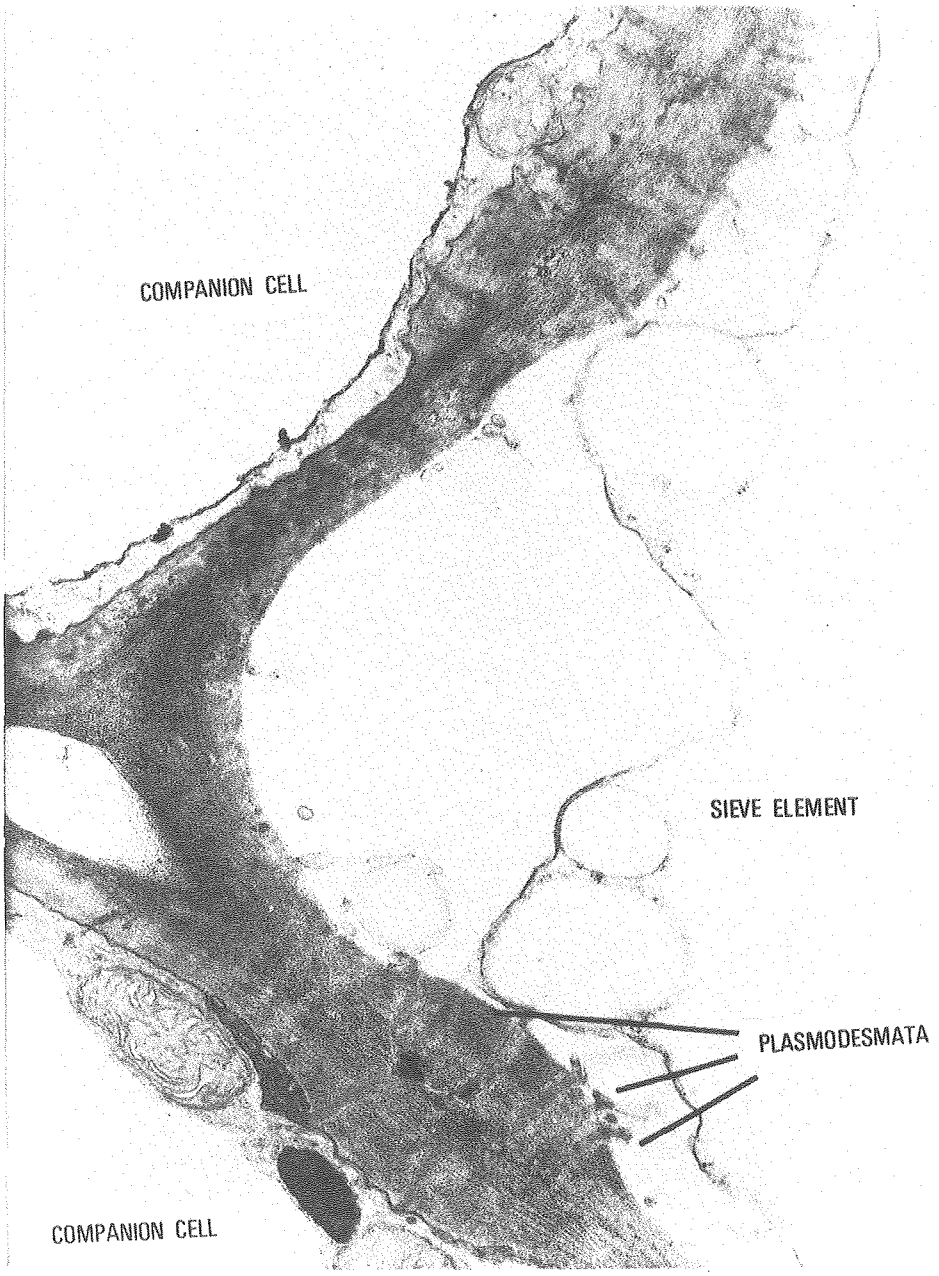


FIGURE III-21

XBB 770-10748

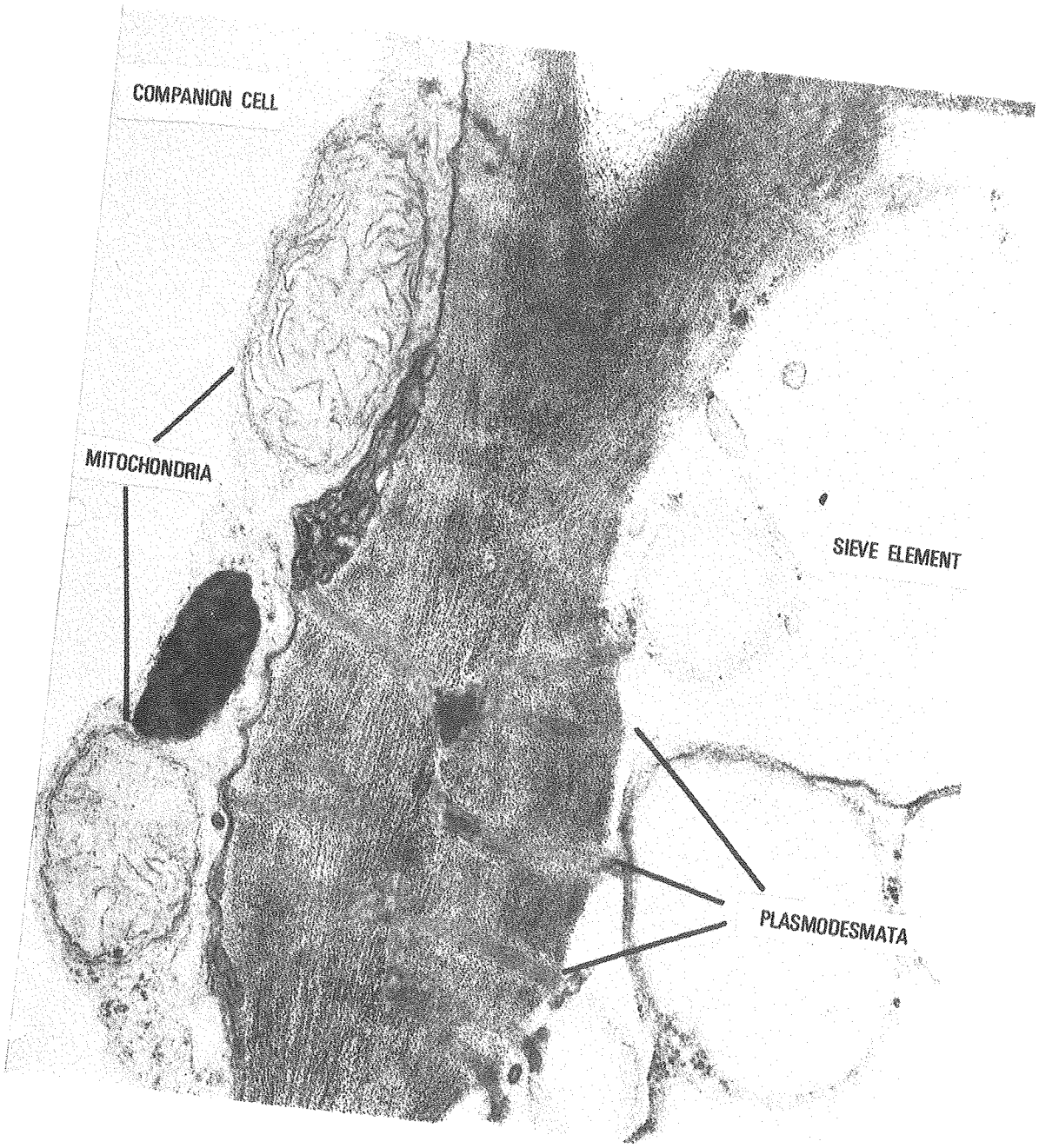
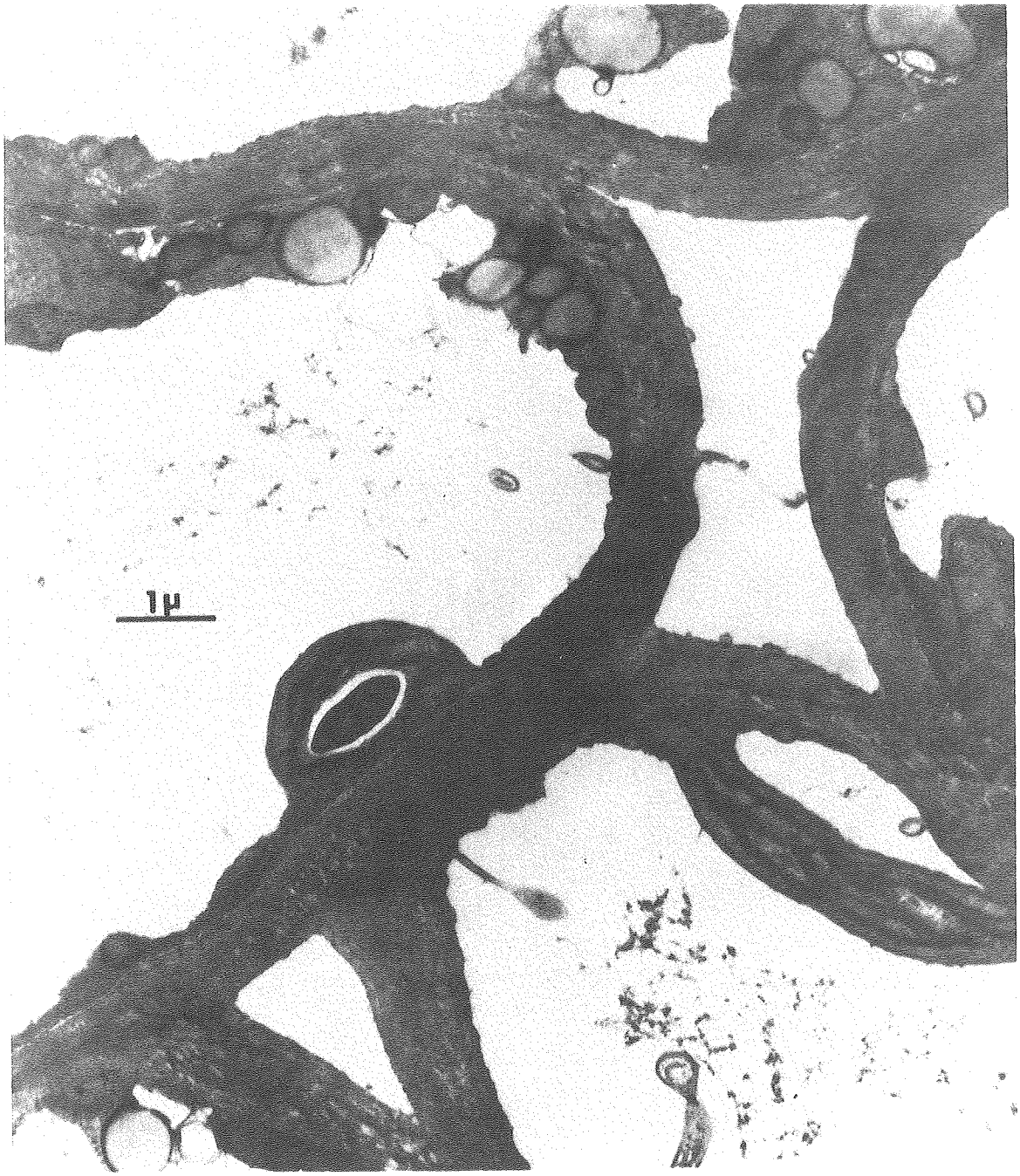


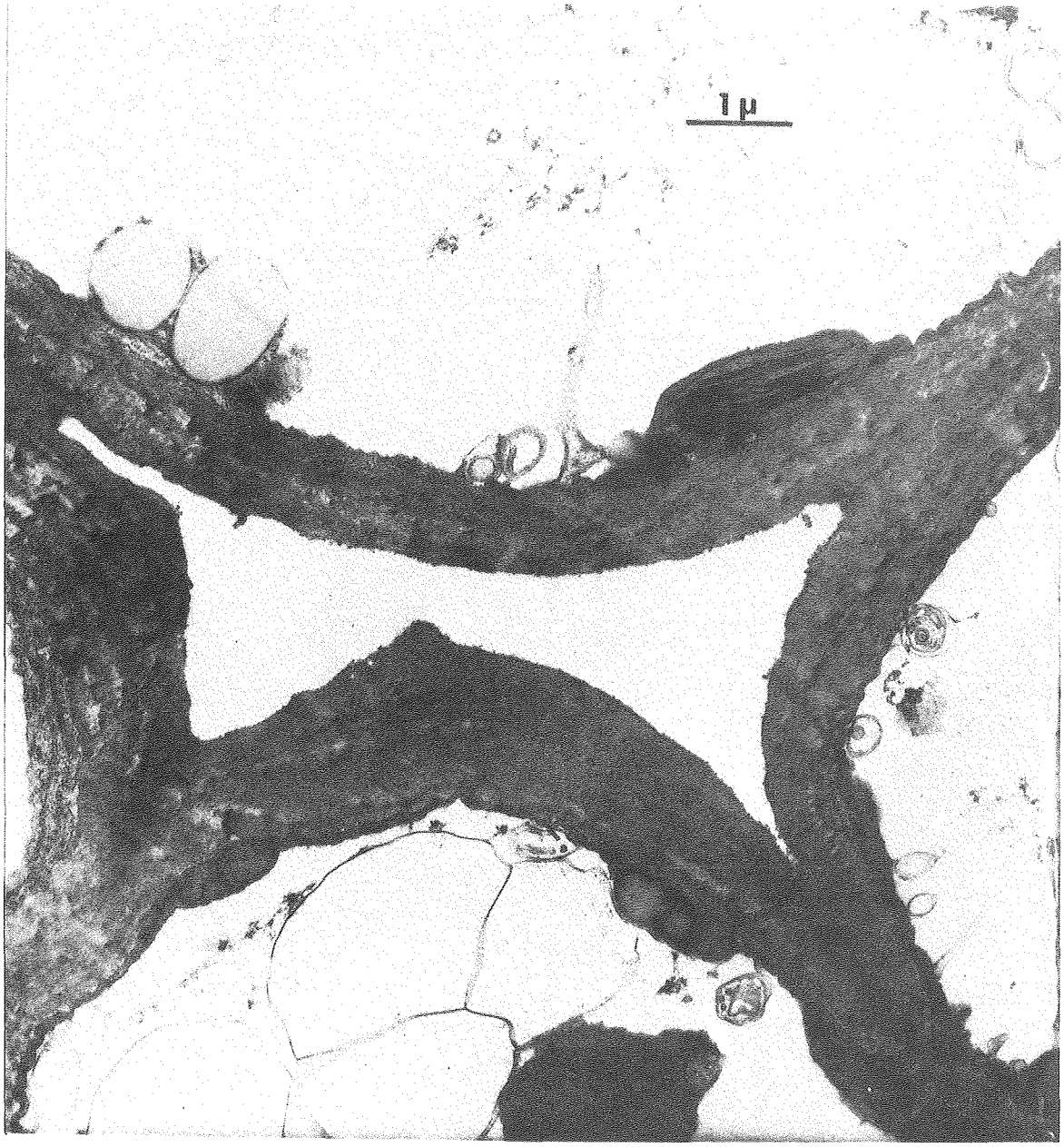
FIGURE III-22

XBB 770-10749



XBB 770-10903

FIGURE III-23



XBB 770-10908

FIGURE III-24

0 0 0 3 4 6 0 7 3 2 1

rather than the endodermis as with the primary resin canals of the seedling.

E. Subcellular Elements Suspected of Involvement in Rubber Biosynthesis

1. Seedlings. Tissue from Guayule seedlings is better preserved and easier to work with than adult tissues. Also, seedlings have been used almost exclusively in our biochemical tracer experiments, described in Section IV. Consequently, seedling tissue has been most intensively studied. This section presents micrographs of organelles found in the root cells of Guayule seedlings.

Figures III-25 through III-28 are of an organelle which bears a marked similarity to the Frey-Wyssling body found in the latex vessels of *Hevea brasiliansis* (Dickenson, 1964). The organelles shown are common in the cells surrounding the resin canals in the seedling root, and contain densely staining spherical inclusions which are similar in appearance to the plastoglobuli of the chloroplasts and which may be terpenoid in nature. These organelles are members of the family of plastids, a group which includes chloroplasts and the pigment (terpenoid) producing chromoplasts. They are identified as plastids by the characteristic double membrane which surrounds them, the network of internal membranes, and the presence of plastoglobuli (Gunning & Steer, 1975:98).

Figures III-29 through III-32 are stereo pairs of serial sections of another plastid found in the roots of

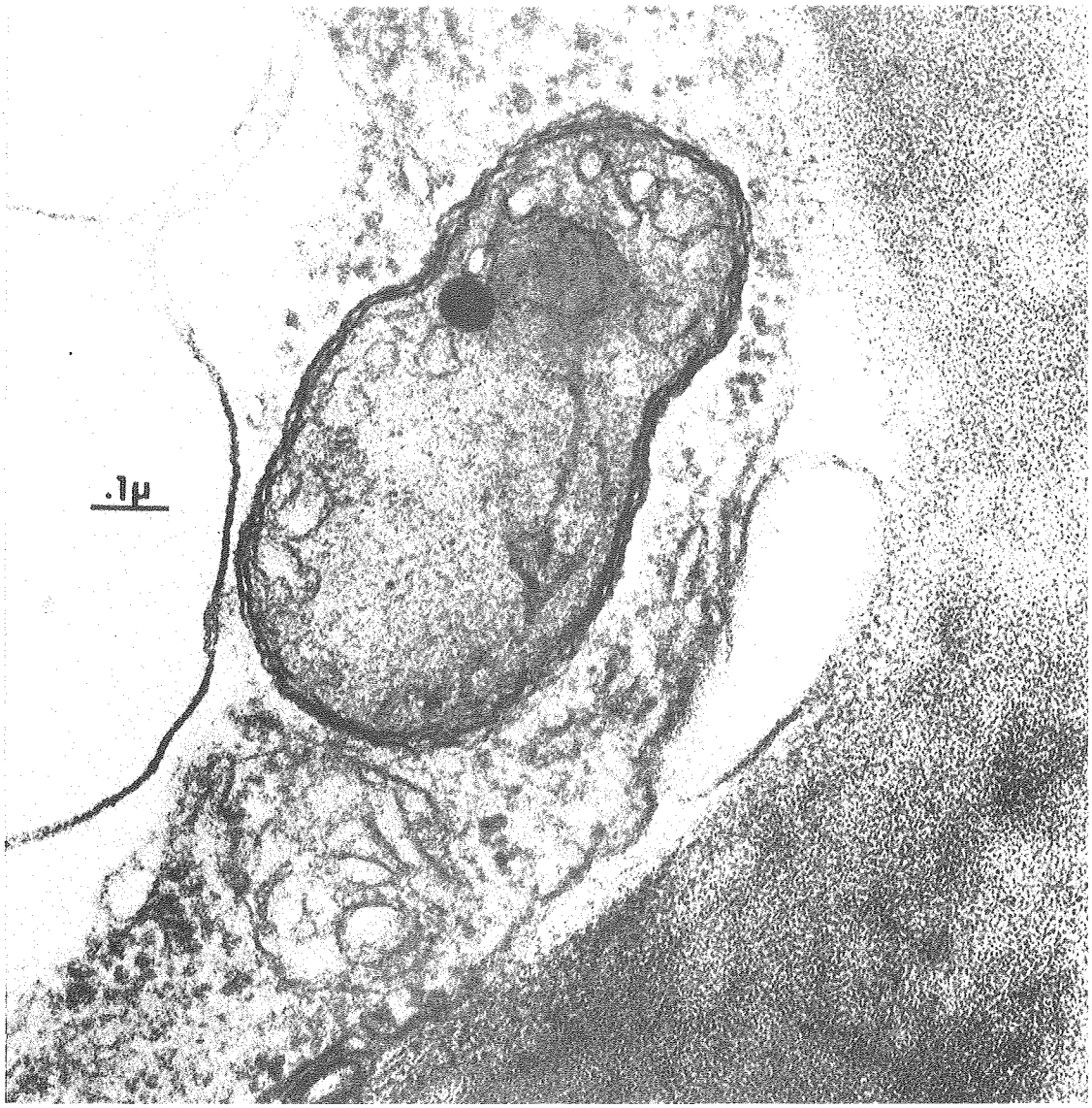


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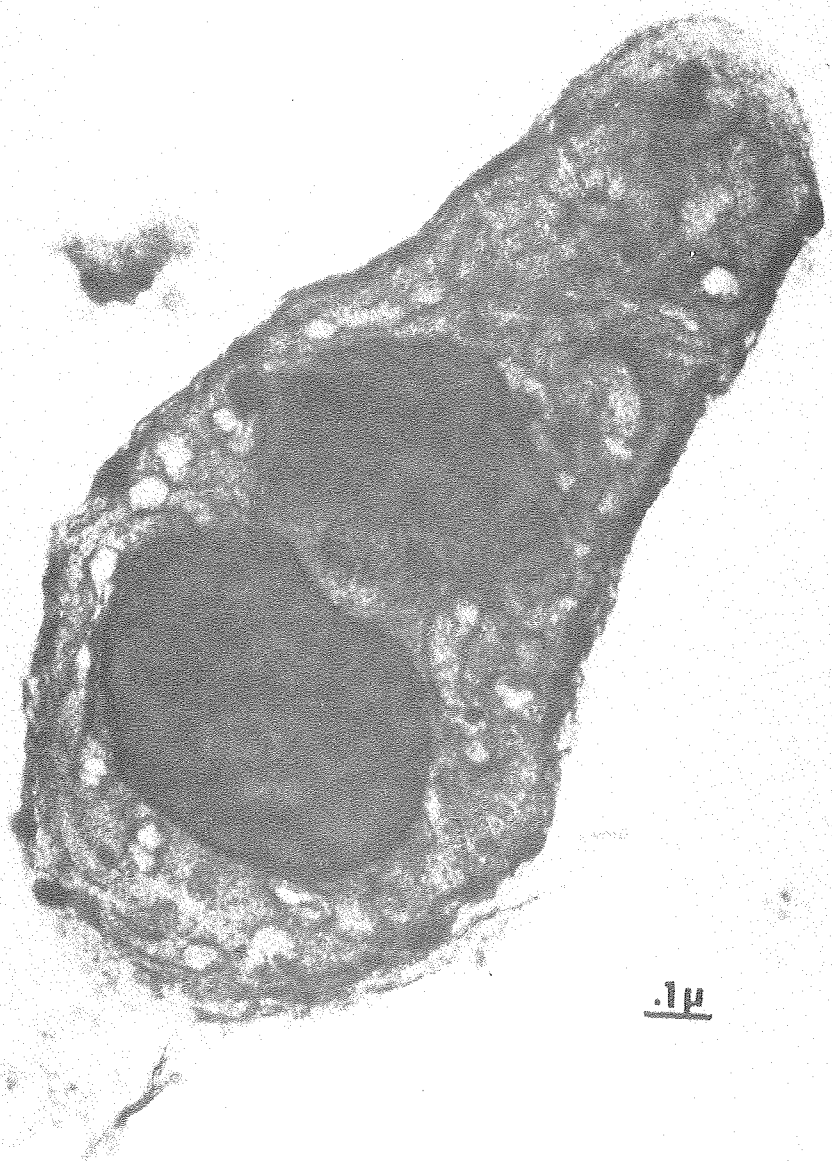
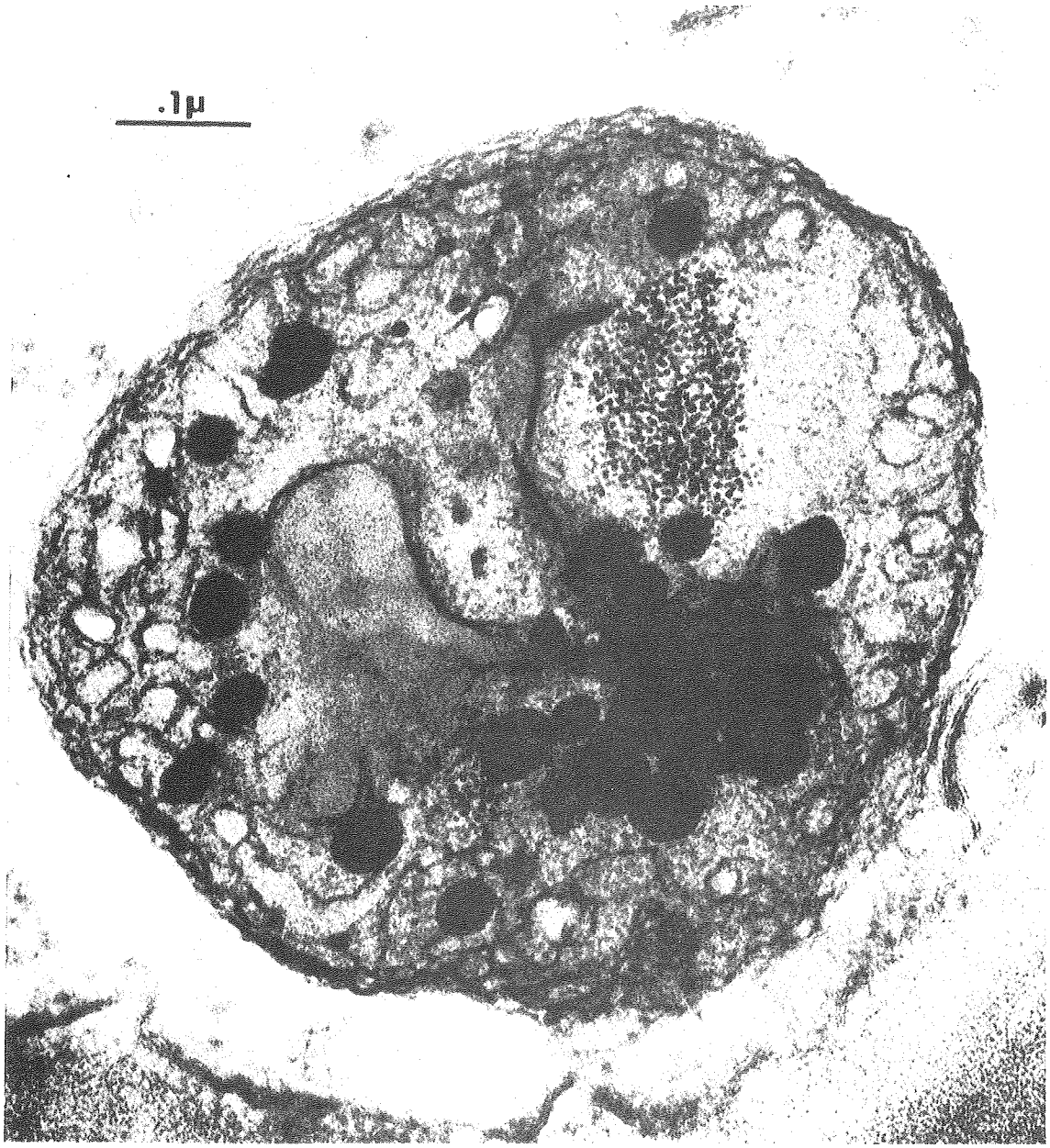
FIGURE III-25



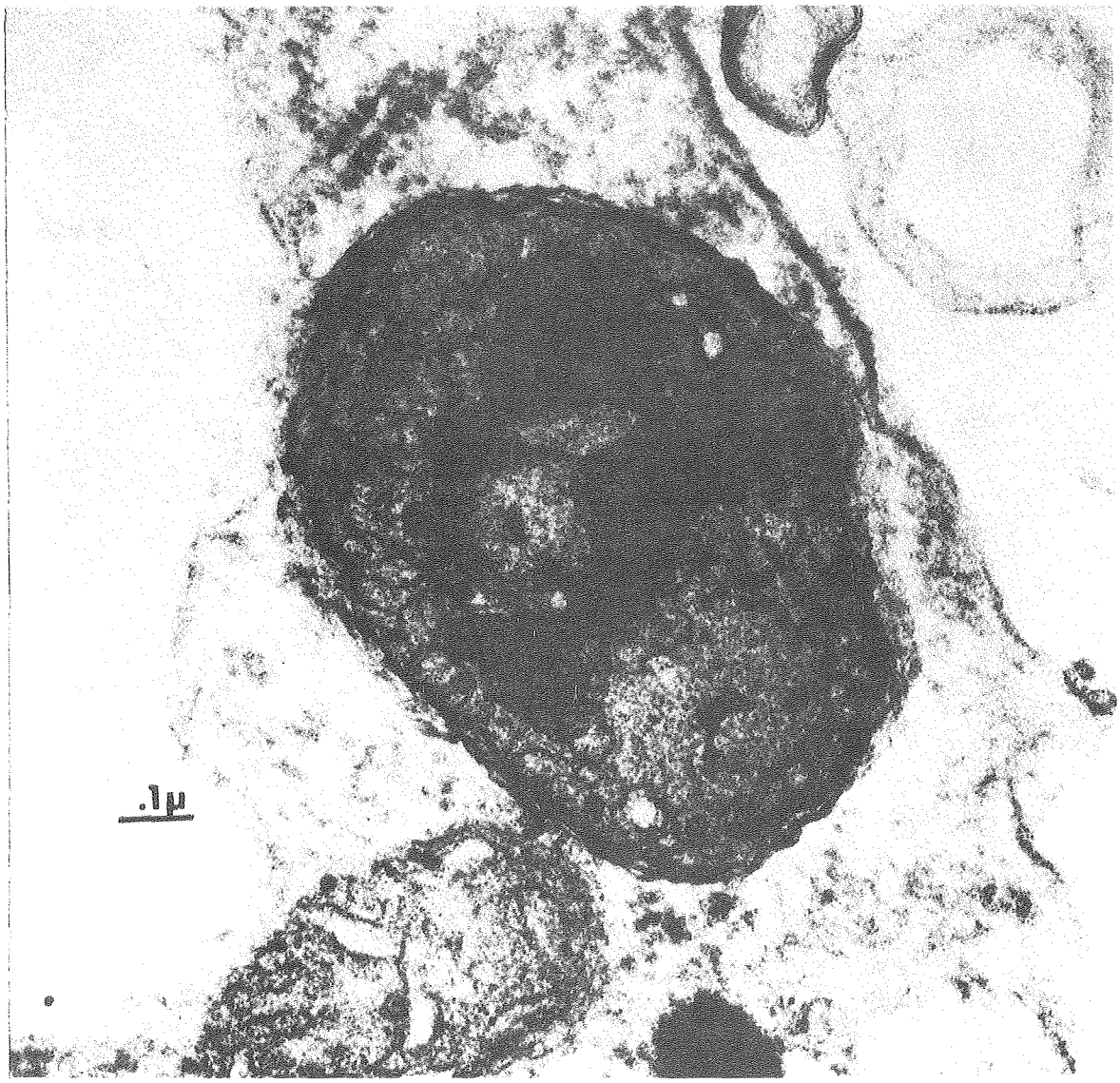
XBB 770-10889

FIGURE III-26



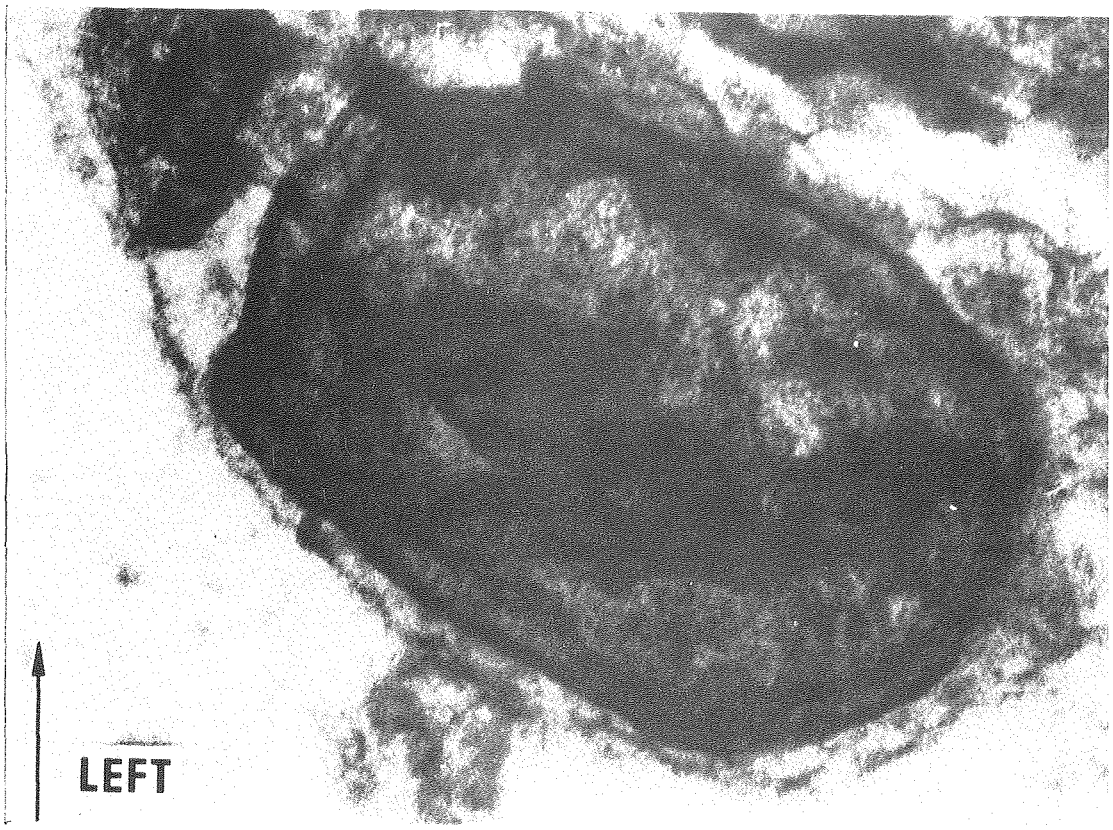
XBB 770-10894

FIGURE III-27



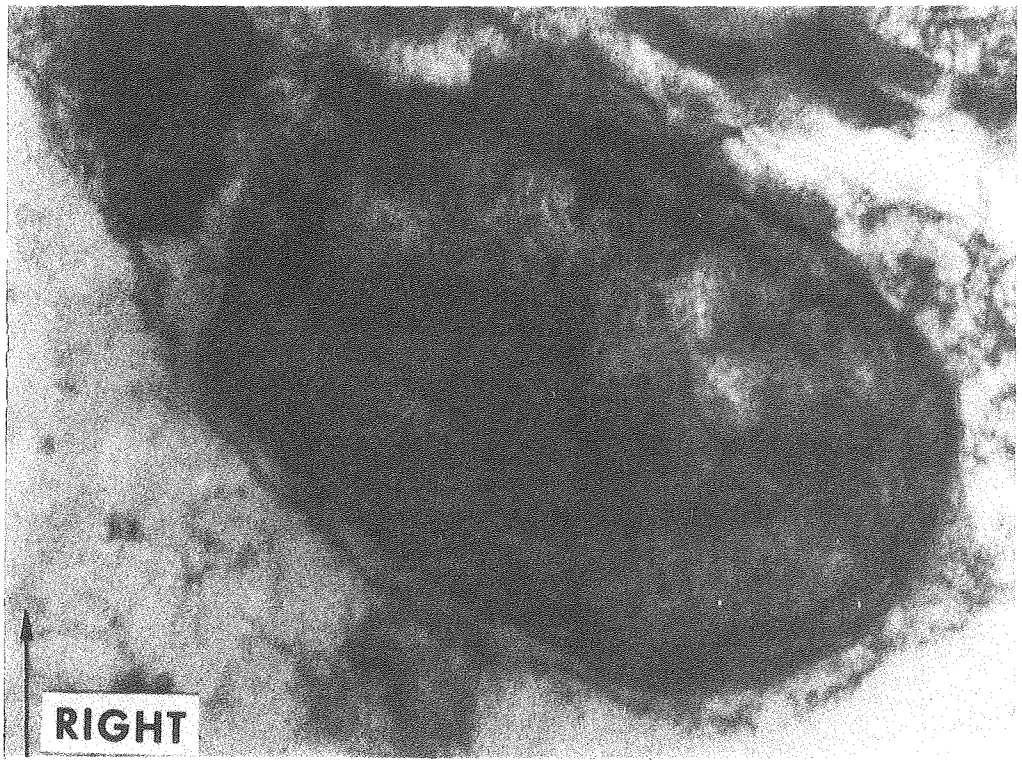
XBB 770-10893

FIGURE III-28



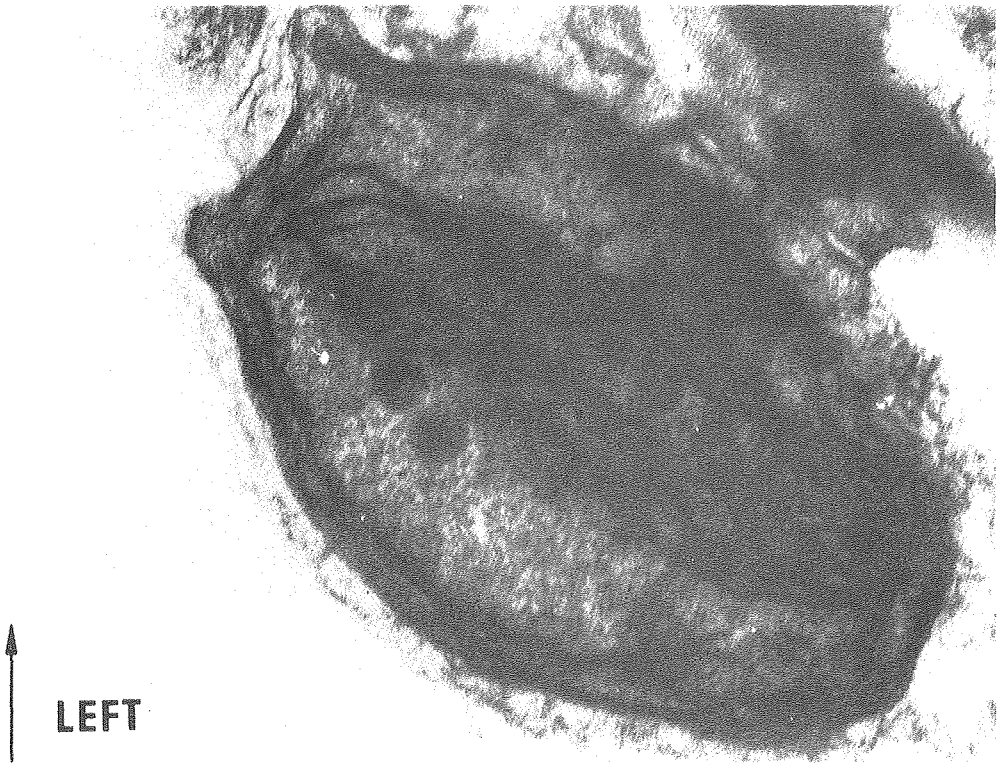
XBB 770-10881

FIGURE III-29A



XBB 770-10882

FIGURE III-29B



XBB 770-10884

FIGURE III-30A

0 0 0 0 4 6 0 7 3 2 5

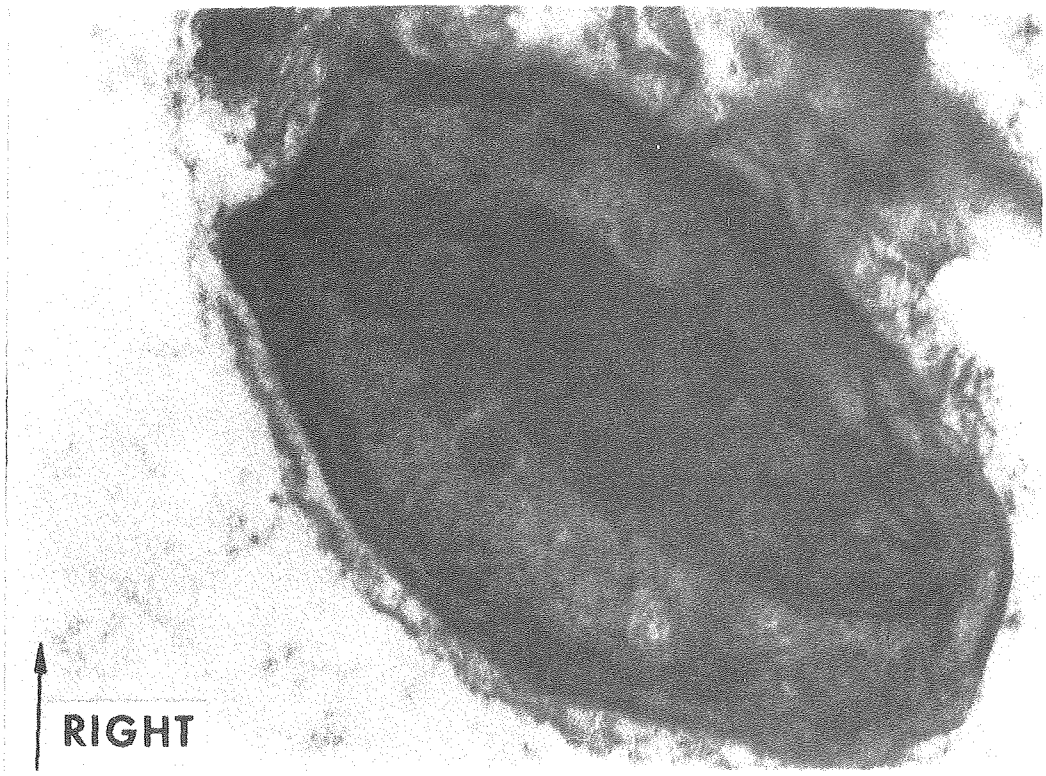


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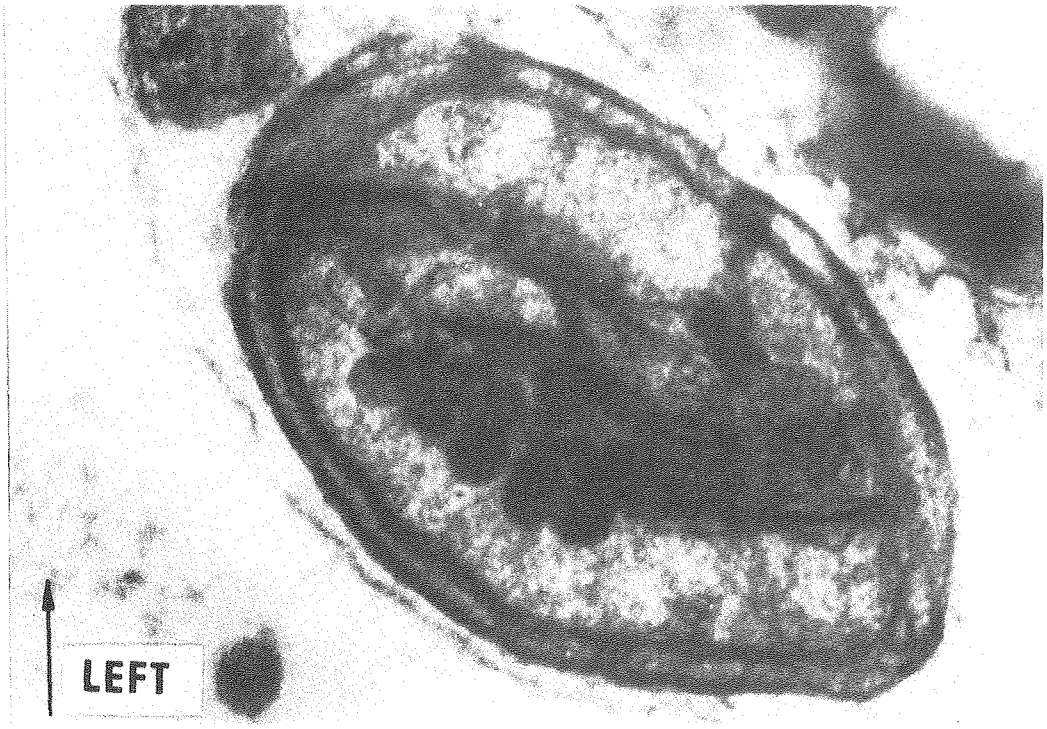
FIGURE III-30B



XBB 770-10887

FIGURE III-31A

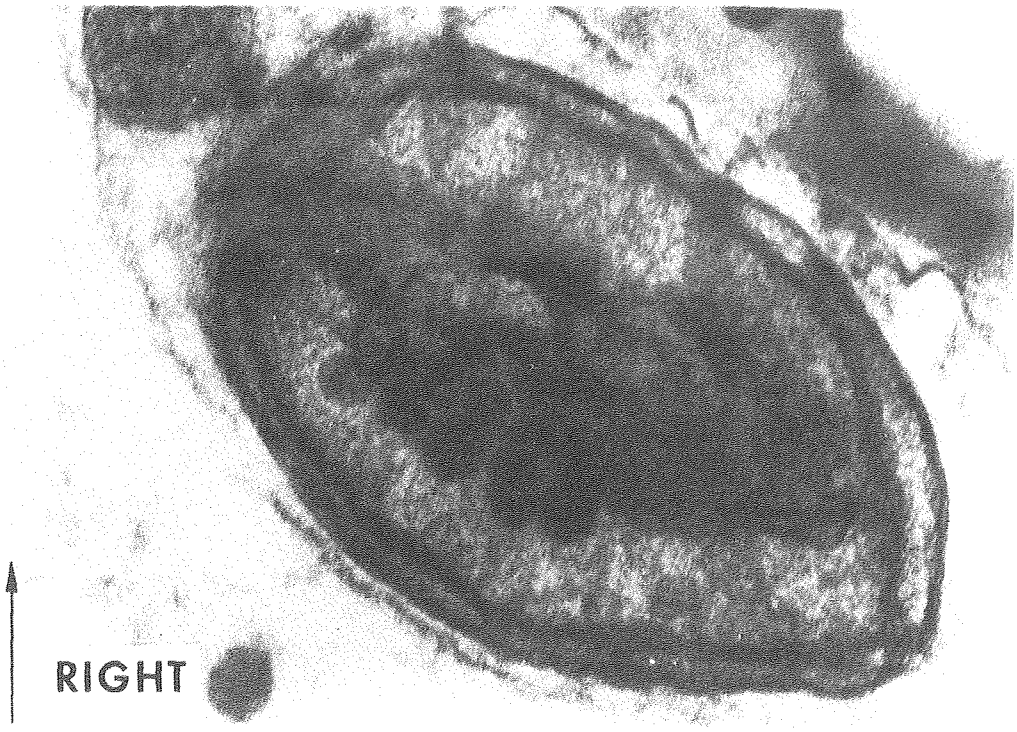


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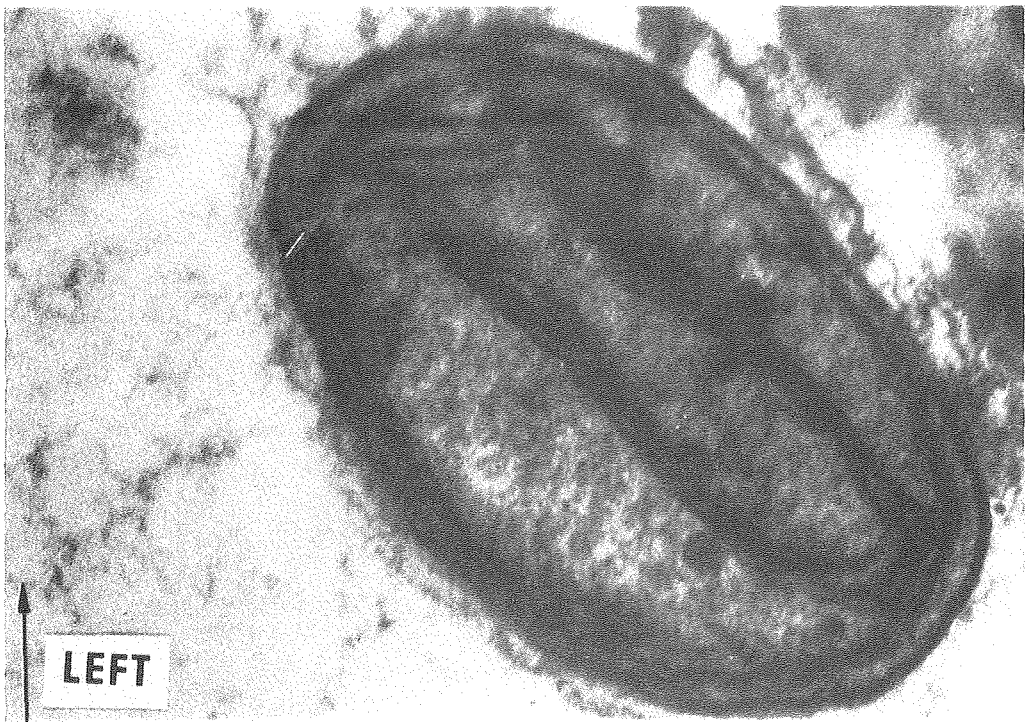
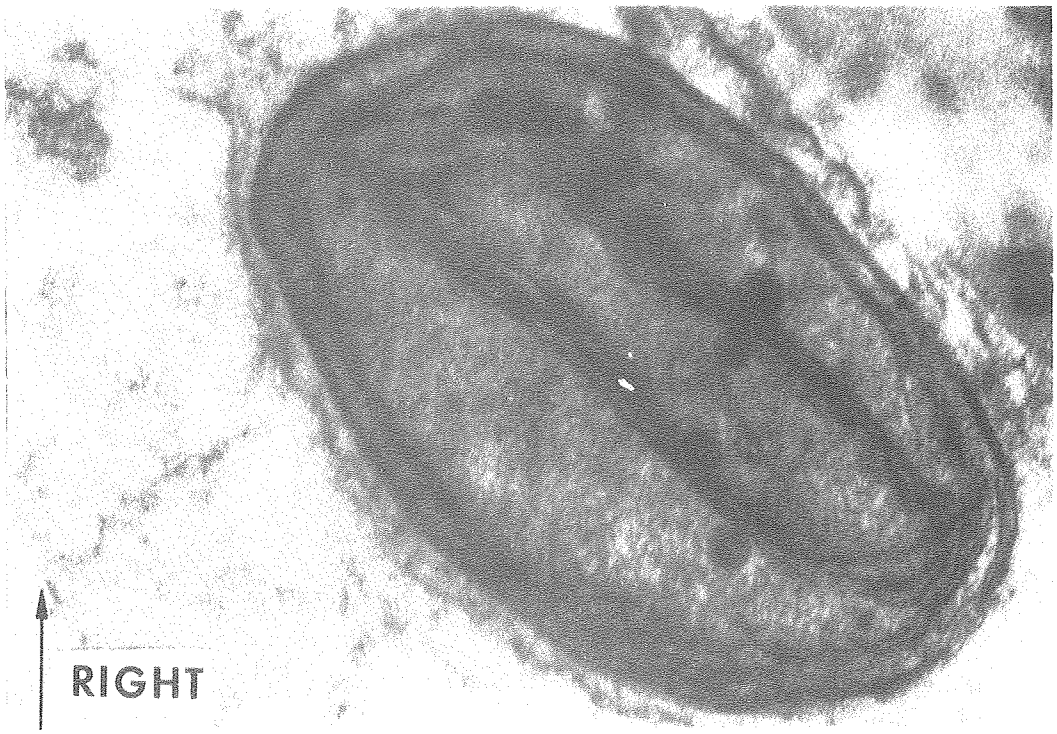
FIGURE III-31B



XBB 770-10886

FIGURE III-32A



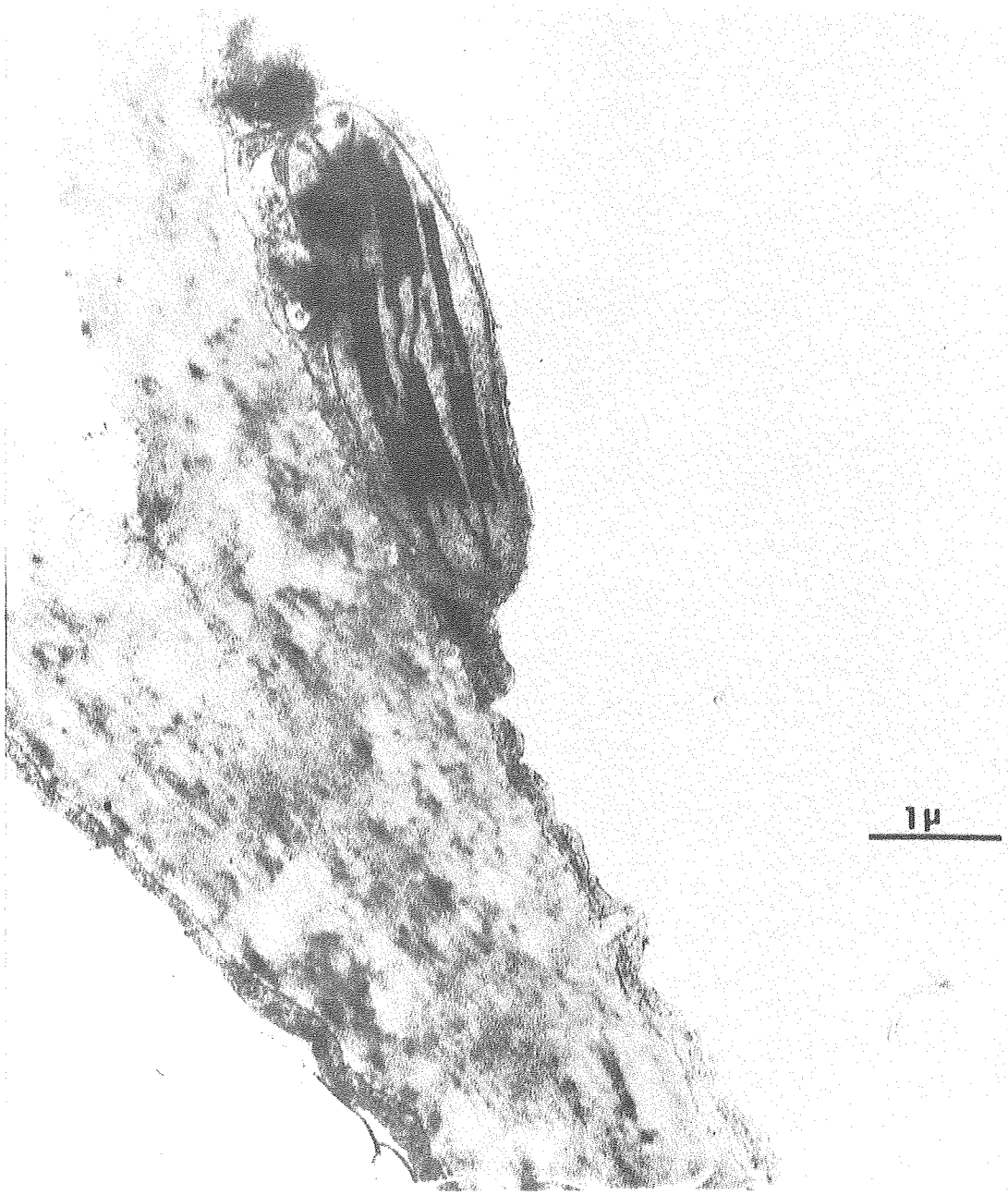
XBB 770-10885

FIGURE III-32B

Guayule seedlings. The sections in this series are about .3 micron in thickness, and the micrographs were obtained using a Hitachi 650 KV high-voltage electron microscope. This organelle also contains densely staining particles which resemble plastoglobuli. Also, these plastids resemble chloroplasts, and suspicion of their involvement in rubber biosynthesis stems from results contained in Section IV which implicate the chloroplasts in the early stages of hydrocarbon biosynthesis.

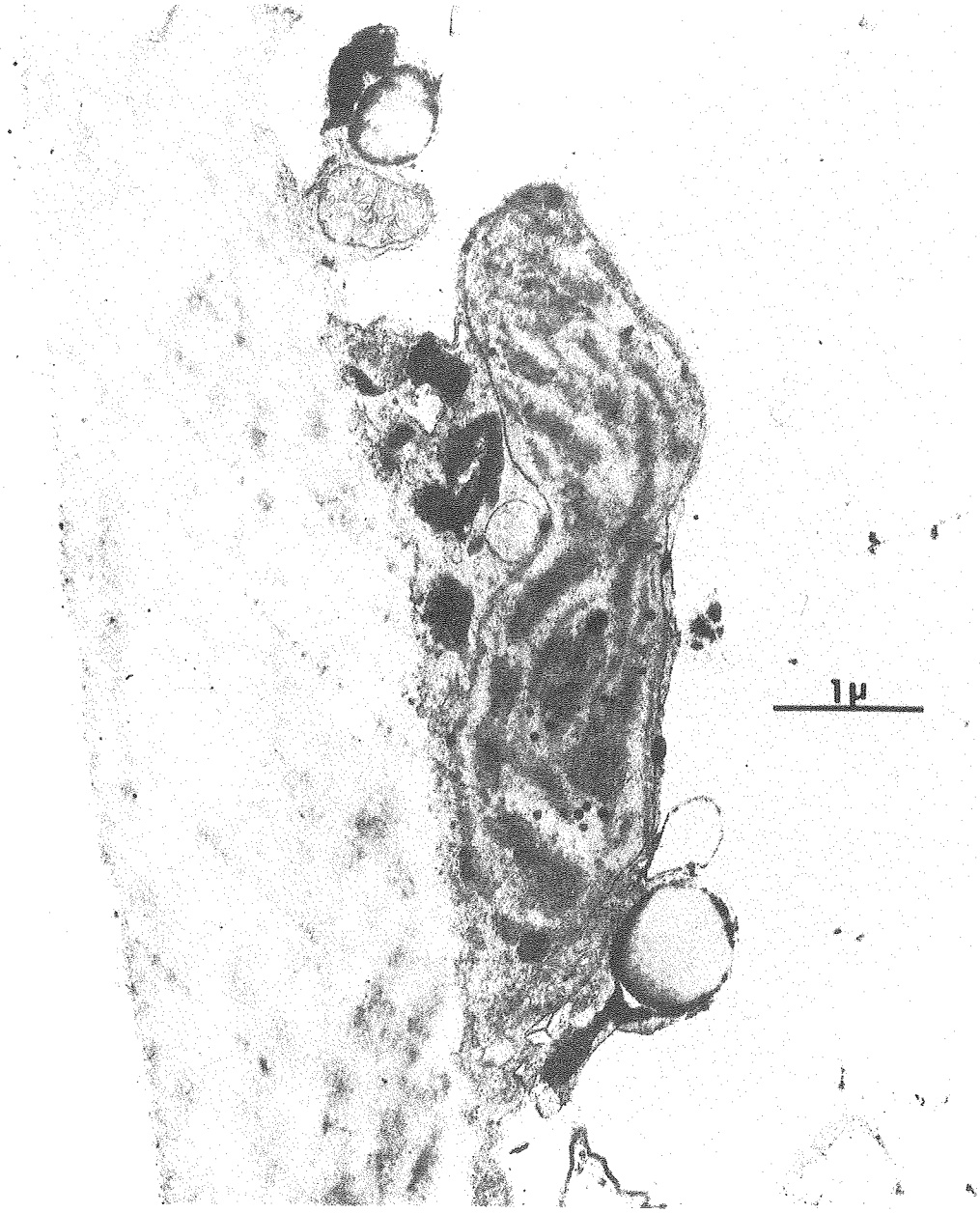
2. Adult organelles. The adult stem tissue of Guayule is better preserved by our techniques than the root tissue, so the micrographs presented in this section are from cells near the resin canals of the stem. The stems and roots of Guayule store about equal amounts of rubber, and it seems likely that the same mechanisms and structures exist in both tissues.

The predominant organelles found in the cells surrounding the resin canals of the mature Guayule stem are chloroplasts and plastids with diffuse interiors which may be chromoplasts. Chromoplasts are closely related to chloroplasts, and the development of chromoplasts from chloroplasts has been observed in the cells of fruits and flowers (Gunning & Steer, 1975:120). Chromoplasts lack photosynthetic ability and are responsible for pigment (terpenoids) formation in fruits and flowers (Gunning & Steer, 1975:120). Figure III-33 is an electron micrograph of chloroplasts contained in one of the cells bordering a resin canal in the mature Guayule stem.



XBB 770-10905

FIGURE III-33



XBB 770-10906

FIGURE III-34

Figure III-34 is of one of the plastids which has been tentatively identified as a chromoplast.

The fact that chromoplasts are responsible for terpenoid formation (pigments) in other tissue suggests that the plastids of Guayule are responsible for at least some of the steps in rubber biosynthesis. The plentiful distribution of the plastids (presented in Section III-E1) in the seedling root further argues for the role of these plastids in hydrocarbon production in Guayule. Additionally, autoradiography results presented in Section IV of this thesis implicate the chloroplasts of the Guayule leaf in the biosynthesis of terpenoids. All of these results taken together support the view that terpenoid production in Guayule is by means of plastids.

F. Discussion

Since the resin canals and their associated and surrounding cells are most closely and obviously associated with hydrocarbon production in Guayule, the next few paragraphs will be spent describing the general class of structures, secretory ducts, within which resin canals fall. It will be noted now that the cells surrounding the secretory ducts are thought to be responsible for synthesizing the products secreted. Since rubber storage in Guayule is primarily associated with cells near the resin canals and since the resins are primarily composed of lower molecular weight versions of rubber, a knowledge of this class of structures in general is desirable.

According to Schnepf, specialized intercellular cavities for the purpose of collecting secretions are common in green plants (Schnepf, 1974). The secretions that are transported to these spaces are hydrophilic substances such as sugars and starches, or in the case of Guayule and, for example, pine trees, lipophilic terpenoids.

In the case of plants like Guayule which secret a terpene, it has been found that the cells surrounding the secretory duct either contain specialized plastids, or large amounts of smooth endoplasmic reticulum, or both (Schnepf, 1974). These components are suspected of being involved in the synthesis and transport of the terpenes. In Guayule, there is not too much evidence for an endoplasmic reticulum production mechanism, but plastids are found regularly in the cells around the resin canals. Section III-E presents several electron micrographs of these plastids.

In Guayule, the cells surrounding the primary resin canal are endodermal with parenchymal cells lying directly surrounded by non-endodermal parenchymal cells which derive from the vascular cambium in each annual growth period. The ring of endodermis which contains the primary resin canals is pushed outwards by the annual production of phloem and xylem derived from the cambium. The endodermal cells continue to divide and grow during this process to maintain a continuous ring of endodermis of ever-increasing circumference.

The canals themselves, as previously mentioned, are

0 0 0 0 4 6 0 7 3 2 9

extracellular spaces formed by the pulling back of the surrounding cells from their corners. This sort of formation is known as schizogenous formation (Artschwager, 1943). The ontogeny and functioning of secretory ducts in several plants have been studied with both the light and electron microscope (cf. Wooding & Northcote, 1965; Fahn & Evert, 1974; Schnepf, 1969a; Schnepf, 1969b for example).

Secretory ducts, that is internal extracellular spaces to receive secretion, are not the only or even the primary method of storage of terpene products in plants. The most commonly occurring of such storage devices is the latex vessel, common to Euphorbias and several other families of the plant kingdom. In the early stages of our work on hydrocarbon production in green plants, we examined the latex vessels from two different plants, one of which was the Hevea rubber tree. Appendix A of this thesis contains electron micrographs of these two different kinds of latex vessels.

The primary distinction between latex vessels and resin canals is that the latex vessels are composed of differentiated, sometimes living, cells while the resin canals are extracellular spaces. For example, the latex collected when tapping the Hevea tree is rich in mitochondria, which demonstrates the living nature of these vessels. The micrographs presented in the appendix illustrate this feature.

Several micrographs of chloroplasts have been presented. This is in recognition of the chloroplasts' role as the source

of all materials used in subsequent biosynthetic steps and also in view of the results of Section IV which implicate the chloroplasts in intermediate steps of rubber biosynthesis.

Micrographs have also been presented of organelles of the root and stem tissues of Guayule. These organelles were selected because of their physical location in cells near the resin canals. Additionally, these same cells are the primary sites of rubber storage in the plants, and this further implicates the contained organelles in hydrocarbon biosynthesis.

Further, one of the organelles presented bears a close resemblance to the Frey-Wyssling body found in Hevea latex vessels (Dickenson, 1964), while another closely resembles the chloroplasts which have been implicated in the early state of hydrocarbon biosynthesis (Section IV). Finally, all of the organelles presented contain densely staining spherical inclusions which may be terpenoid in nature. Hopefully, research which continues that described in Section IV will identify which of these organelles is involved in rubber biosynthesis in Guayule.

0 0 0 0 4 6 0 / 3 3 0

IV. BIOCHEMICALLY BASED EXPERIMENTS

A. Biochemical Pathway of Hydrocarbon Synthesis

The electron microscope presents an opportunity to investigate biological structures in very fine detail. A modern electron microscope can usually resolve 20 Angstroms in standardly prepared and stained biological materials. However, in order to use this tremendous resolving power, one must have a precise idea of what to investigate.

The nature of the work reported here is an investigation into hydrocarbon production in green plants, with primary emphasis on rubber production in Guayule. Thus the exact structures we are wishing to examine are those involved in rubber production in Guayule. Since these structures are unknown and precisely the ones we wish to determine, the biochemical experiments described in this section were chosen in an attempt to discover which subcellular structures are involved in rubber production in Guayule. Figures IV-1 through IV-4 present the biochemical basis of our experiments.

Figure IV-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 in the role of mitochondria.

FIGURE IV-1

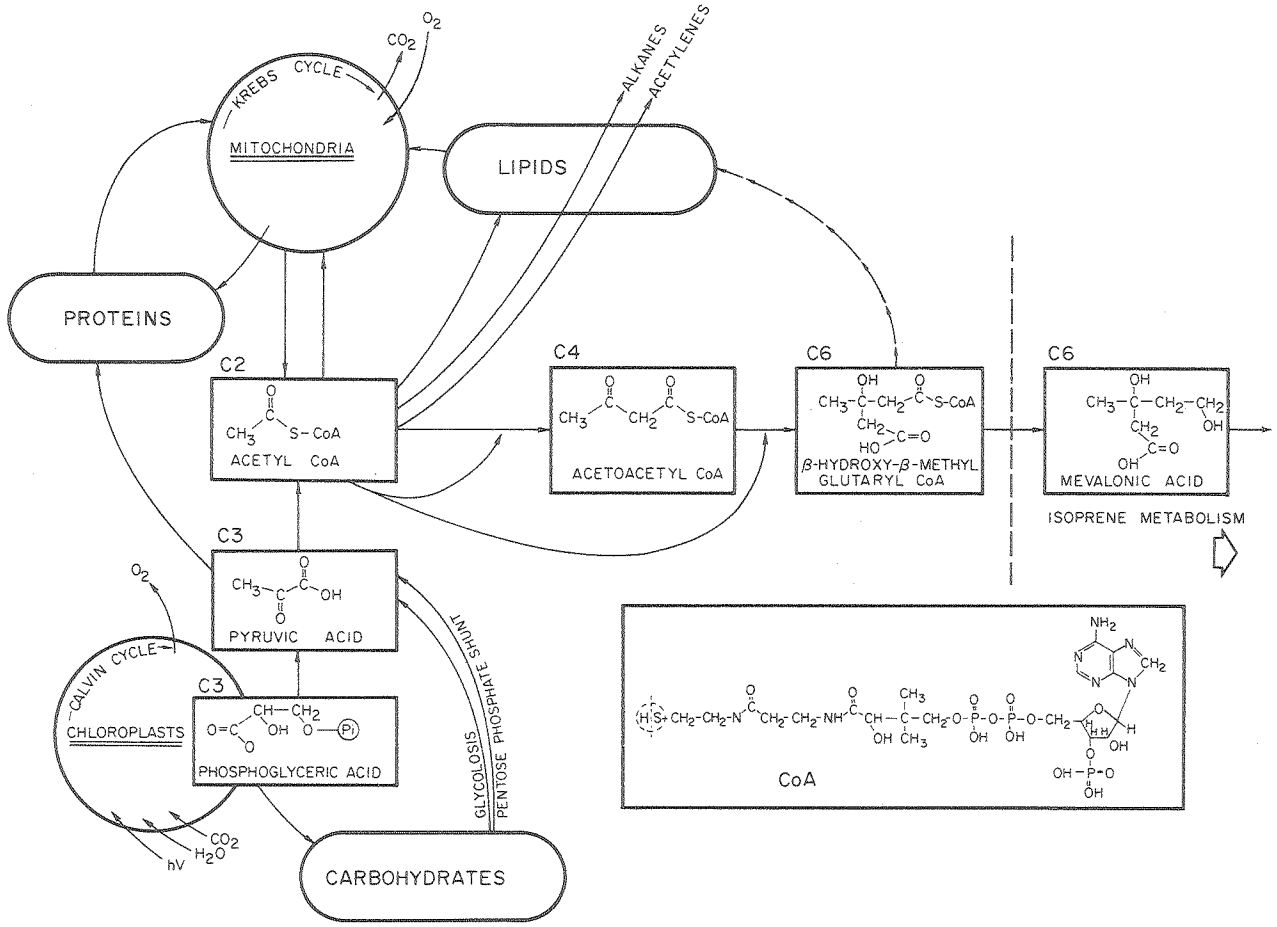


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FIGURE IV-2

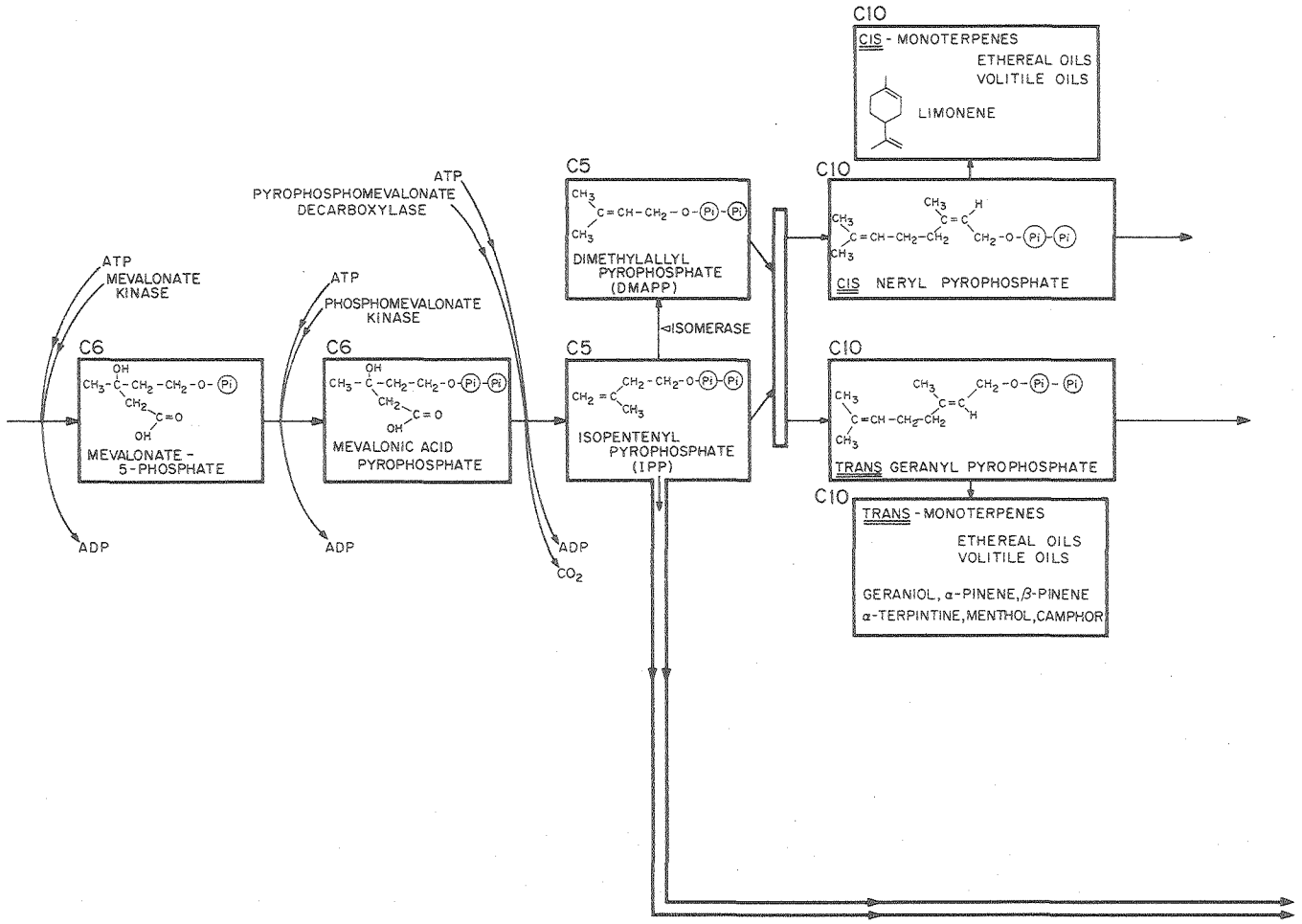

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FIGURE IV-3

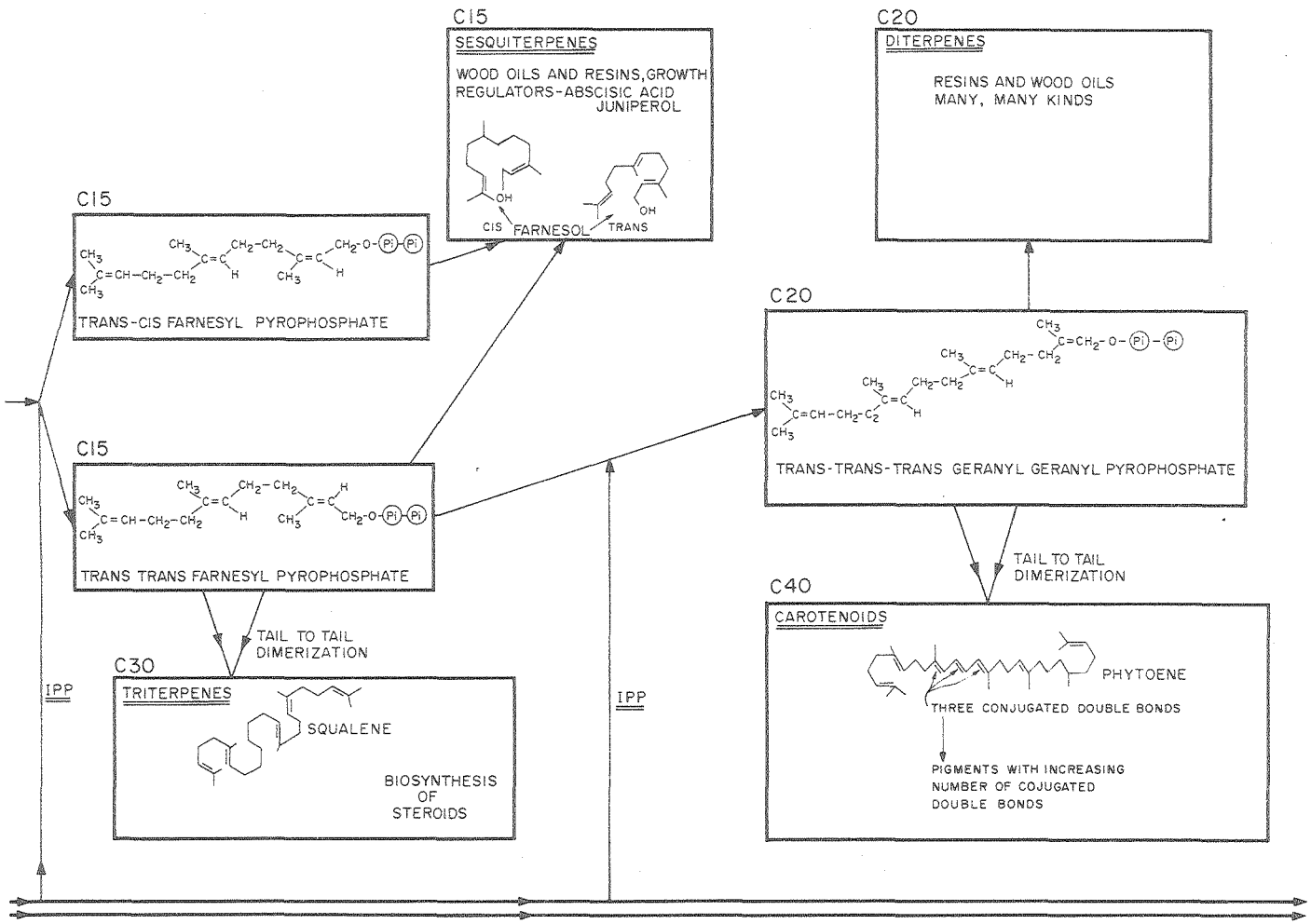

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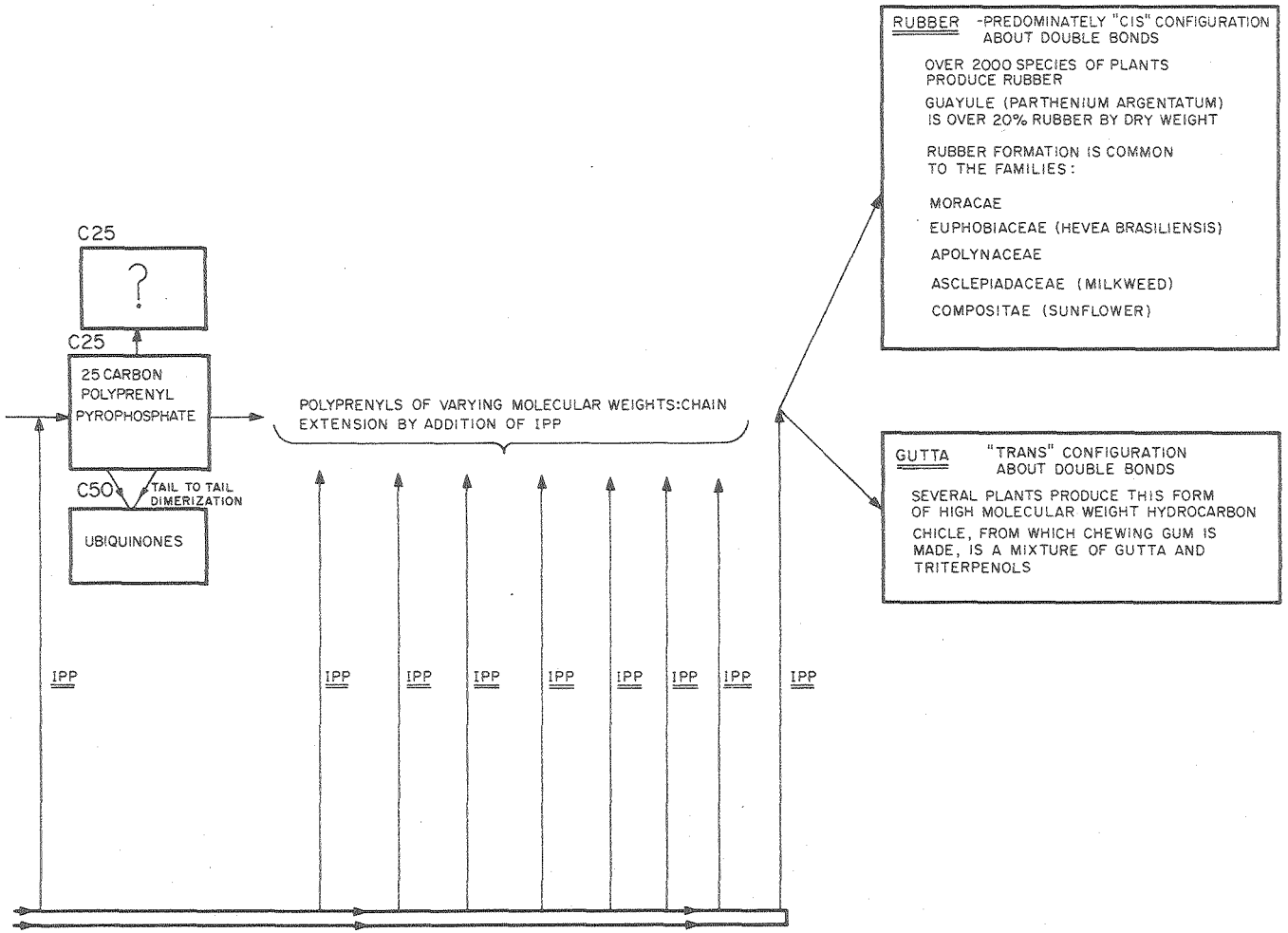
FIGURE IV-4



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0 0 0 0 1 0 0 0 6 2 2

As stated our current research is directed toward understanding the production of alkenes--particularly rubber. However, plants also produce alkanes and acetylenes, and their generation through the lipid metabolism pathway is shown in Figure IV-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 Figure IV-1. However, mevalonic acid, produced from beta hydroxy beta methyl glutaryl CoA, is involved only in the terpene (alkene) metabolism.

Referring to Figure IV-2, mevalonic acid is twice phosphorylated to produce mevalonic acid pyrophosphate (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 (Figure IV-3). From these are derived 15-carbon sequiterpenes including wood oils, resins, and some growth regulators. Also, two of the 15-carbon

0 0 0 0 4 6 0 7 3 3 3

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 geranyl pyrophosphate. 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 Figure IV-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 vivo_. The initiation of rubber synthesis is a mystery so far.

Interestingly, the first steps in determining the biosynthetic pathway for the formation of rubber were accomplished using Guayule. In a series of papers in the 1950s (Bonner & Arreguin, 1949; Arreguin & Bonner, 1950; Arreguin & Bonner, 1951), James Bonner and his colleagues presented their results which linked acetate as a precursor of rubber in Guayule.

There later followed a large number of papers (Bandurski & Teas, 1958; Park & Bonner, 1958; Archer et al., 1963; McMullen & Sweeney, 1966; Hepper & Audley, 1969) which primarily

worked with Hevea rubber production mechanisms to unravel the total synthesis as shown in the figures. An excellent review of the history of the unraveling of the biosynthetic pathway, particularly in the Hevea rubber trea, is found in the review paper by Archer and Audley (1967).

Beytia and Porter have a recent review of the biochemistry of polyisoprenoids in general (Beytia & Porter, 1976). The biochemistry of polyprenols is reviewed by Hemming (1969), and the biochemistry of the carotenoids is reviewed by Goodwin (1966, 1969). Most recently, work has been aimed at determining and isolating the enzymes and enzyme systems responsible for terpenoid biosynthesis (Loomis & Bataile, 1963; Downing & Mitchell, 1974; Green & Baisted, 1972; Holloway & Popjak, 1968).

B. Mevalonic Acid Tracer Experiments

1. Introduction. The biochemical flowcharts suggest that mevalonic acid be administered to the plant and that we attempt in some ways to follow the metabolism of this chemical within the plant using the electron microscope. Our experiments using mevalonic acid fall into two main categories.

Technically the most simple approach is to administer various concentrations of MVA and to see what effects are created in the plant by these administrations. With these experiments one hopes to see either some structural change in the plant because of extraordinarily high loading of MVA, or

possibly the build-up of some biochemical product. The details of these experiments and the results follow later in this section.

A more sophisticated approach is to use radioactively labeled MVA and to follow the biochemical pathway using the technique of autoradiography. The experimental setups for these tests as well as the results are detailed later on in this paper. The autoradiographic technique itself works essentially as follows.

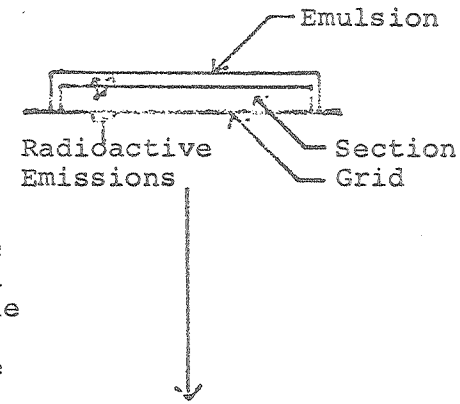
Radioactively labeled precursor is administered to the plant through its roots for a specified period of time. There may or may not be a "cold chase" of unlabeled precursor after this. The plant is killed, fixed, embedded in plastic, and thinly sectioned for examination in the electron microscope. A layer of photographic emulsion is applied on top of the section, and the tissue is then set aside while the disintegrating atoms of the label exposed the emulsion. After a 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. Figure IV-5 graphically depicts the procedures in the case of tritium label administrations to our Guayule seedlings.

In our experiments, we took advantage of the existence on the Berkeley campus of a 650 KV high-voltage electron microscope. The power of this instrument enabled us to cut

FIGURE IV-5

Timed dose of tritium labeled rubber precursor.
Timed "chase" of unlabeled precursor.

The plant is killed, fixed, and prepared for electron microscope examination. Thin sections of tissue are placed on a fine mesh grid and coated with photographic emulsion. They are then set aside for 3 to 6 months while radioactive emissions from the metabolized label expose the emulsion directly above.



Finally, the photographic emulsion is developed and the composite emulsion/tissue specimen is examined in the electron microscope.

biological sections of approximately .3 microns for use in our autoradiography experiments. The high-voltage microscope allows the examination of these thicker materials, roughly three to four times as thick as those sections that can be viewed in a conventional electron microscope. The thicker sections contain proportionally more of the radioactive label, and this reduces the duration of the exposure necessary to produce significant photographic grain formation. The autoradiographs in a later section of this chapter demonstrate the results.

2. Biochemical Experiment #1--the administration of mevalonic acid. Figure IV-6 presents the experimental setup for our mevalonic acid experiments. Guayule seedlings were germinated and grown for 10 days in a nutrient solution ("Schultz-Instant" liquid plant food, 1 dropperful per quart water). For the full 24 hours of the eleventh day, part of the seedlings were administered mevalonic acid at 1.09×10^{-3} molar concentrations, others received five times that concentration of mevalonic acid, while standards received no mevalonic acid. At the end of the 24 hours, all the plants were sacrificed and fixed in osmium. Samples from the leaf, stem, and root were processed for examination in the electron microscope. No differences were seen in the tissue except for the chloroplasts of the leaves.

Figures IV-7 through IV-9 are electron micrographs of the chloroplasts in the leaves of the plants exposed to no

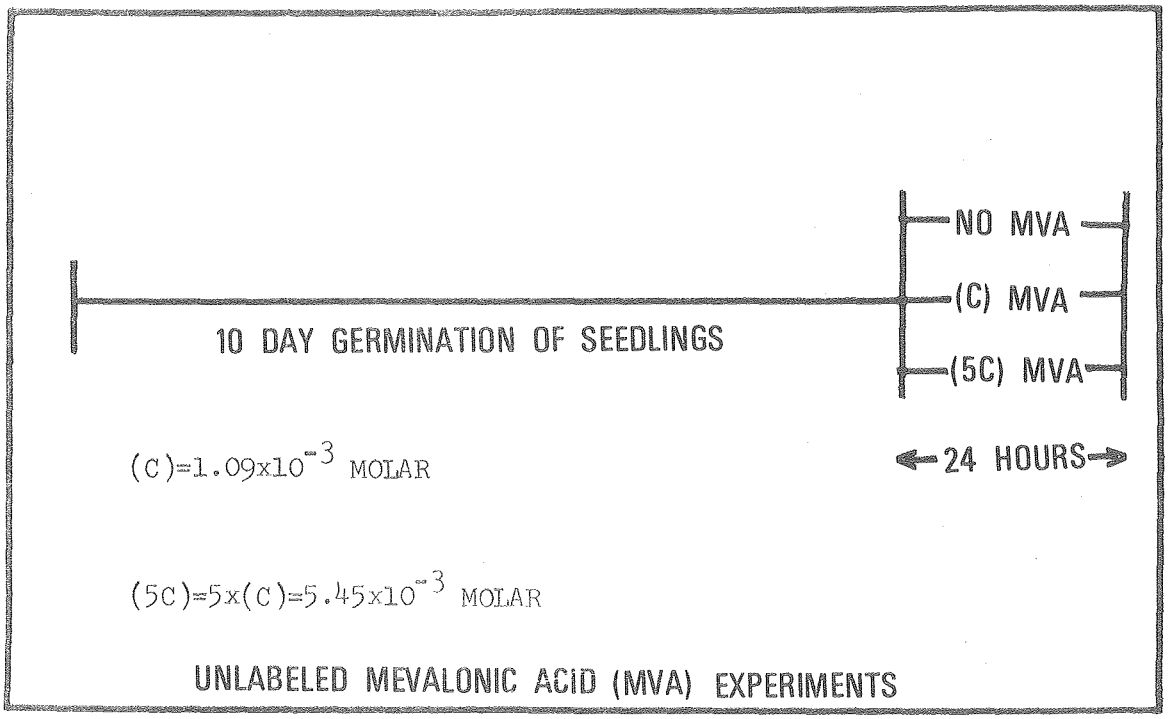


FIGURE IV-6

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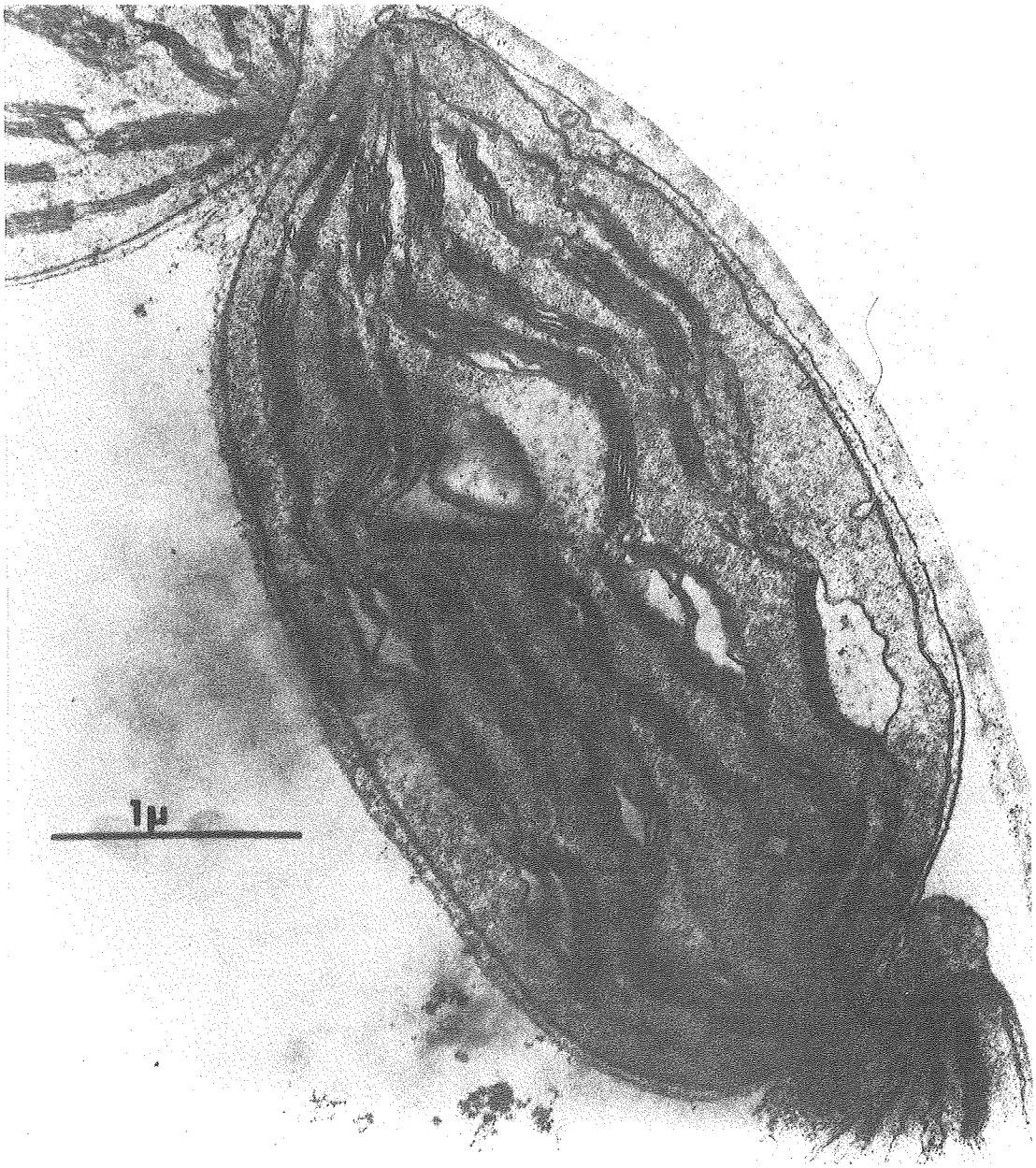

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FIGURE IV-7

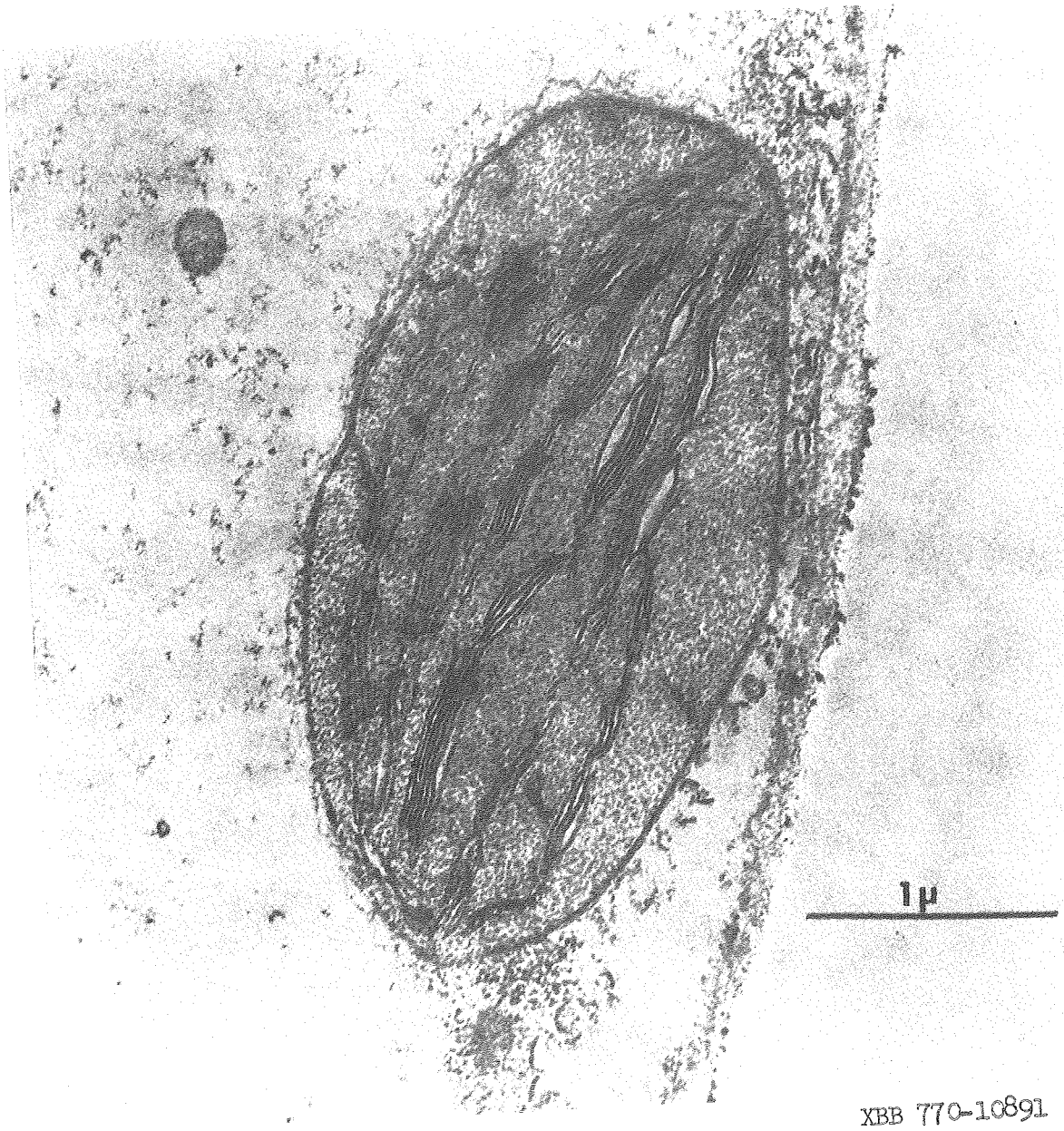

FIGURE IV-8

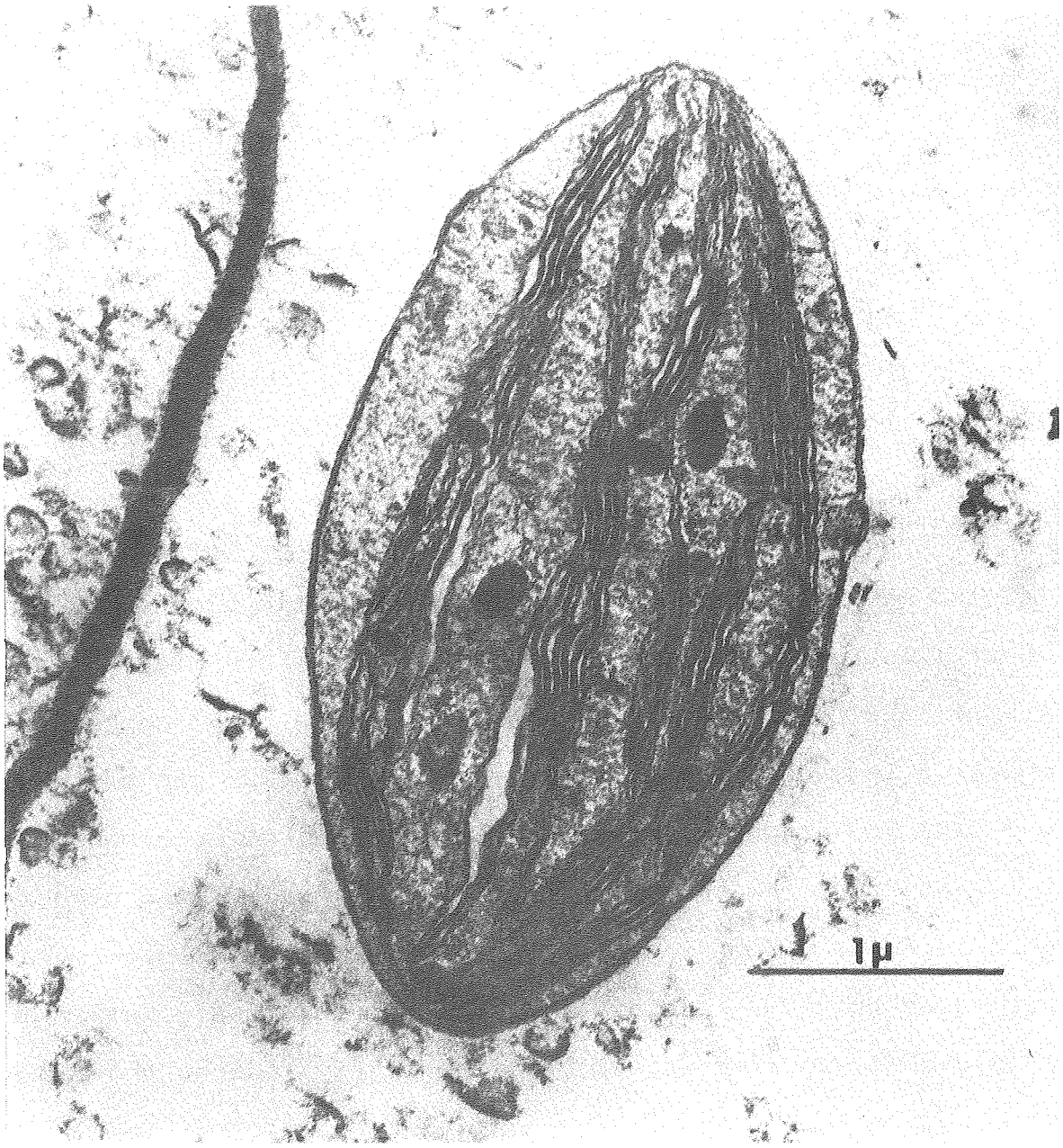

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FIGURE IV-9

mevalonic acid, the standard concentration of mevalonic acid, and five times that concentration respectively. Figures IV-7 and IV-8 are essentially identical, while in Figure IV-9 large dark inclusions are seen within the chloroplasts. The analysis of this result will wait until the end of this section, but these results do indicate mevalonic acid is finding its way to the chloroplasts, and in sufficient concentration so that the chloroplasts are unable to process and export this chemical as fast as it is coming in.

3. Biochemical Experiment #2--autoradiography of labeled mevalonic acid. Referring back to the biochemical flow chart (Figures IV-1 and IV-2), it is clear that mevalonic acid can be labeled in two distinct ways. The carboxyl carbon which is removed at the conversion of MVA to IPP can be labeled, and thus the fate of this removed carbon can be determined. Alternatively, the backbone chain can be labeled with either carbon-14, or one of the hydrogens in the backbone can be replaced by tritium. The advantage of tritium is a considerably higher specific activity which greatly reduces the amount of exposure time necessary.

Finally, the backbone tritium labeled mevalonic acid may either be administered for a long period of time, for example 24 hours, or it can be administered in short pulses followed by varying durations of cold chase. All three of the above experimental designs were used, but success in isolating a structure involved in hydrocarbon biosynthesis was

achieved only with the pulse and chase experiments.

In all the autoradiographic experiments reported here, seeds were germinated on wet filter paper and the freshly sprouted seedlings were transferred to small plastic vials containing a nutrient solution ("Schultz-Instant" plant food, one dropperful per quart of water). Waxed paper (Parafilm) was used to seal the tops of the vials and support the seedlings in position with the roots dangling in .6 ml volume of nutrient solution and the stems upright above the top of the vial. A hundred-watt light bulb placed approximately one foot above the seedlings supplied light and warmth for the growing seedlings.

Carbon-14 experiment: Ten-day-old Guayule seedlings grown in the nutrient solution were supplied with carbon-14 labeled mevalonic acid (1.09×10^{-3} molar, specific activity 6 millicuries/millimole) with the carboxyl carbon having the label, for 24 hours. The plants were then killed, fixed, and prepared for electron microscope autoradiography as well as light level autoradiography. After 6 months of exposure, these carbon-14 labeled experiments had produced very little grain formation. This is due to the low specific activity of the carbon-14. Thus this experiment was inconclusive, and to be conclusive would probably require an exposure time of several years. This was not attempted.

Long duration tritium-labeled mevalonic acid experiment: The first tritium labeled mevalonic acid experiment

was to give 10-day-old seedlings 24 hours of tritiated mevalonic acid (1.09×10^{-3} molar, specific activity 382 millicuries/millimole). The plants again were sacrificed, fixed, and prepared for autoradiography. After 3 months, there was significant grain development over almost all areas of the plant. This demonstrated that the autoradiography technique had potential, but produced no real knowledge. However the information on concentrations and exposure times developed in this experiment led to the successful pulse and chase experiments described in the next section.

This experiment was also valuable in that we determined to what extent the labeled metabolites were leached from the tissue during preparation for electron microscopy. The preparation involved soaking the tissue in water solutions of fixative as well as alcohol solutions for dehydration. Figure IV-10, obtained from scintillation counts of the preparation fluids, show that virtually no label was removed during preparations. Apparently, the osmium fixation effectively tied the labeled metabolites in place.

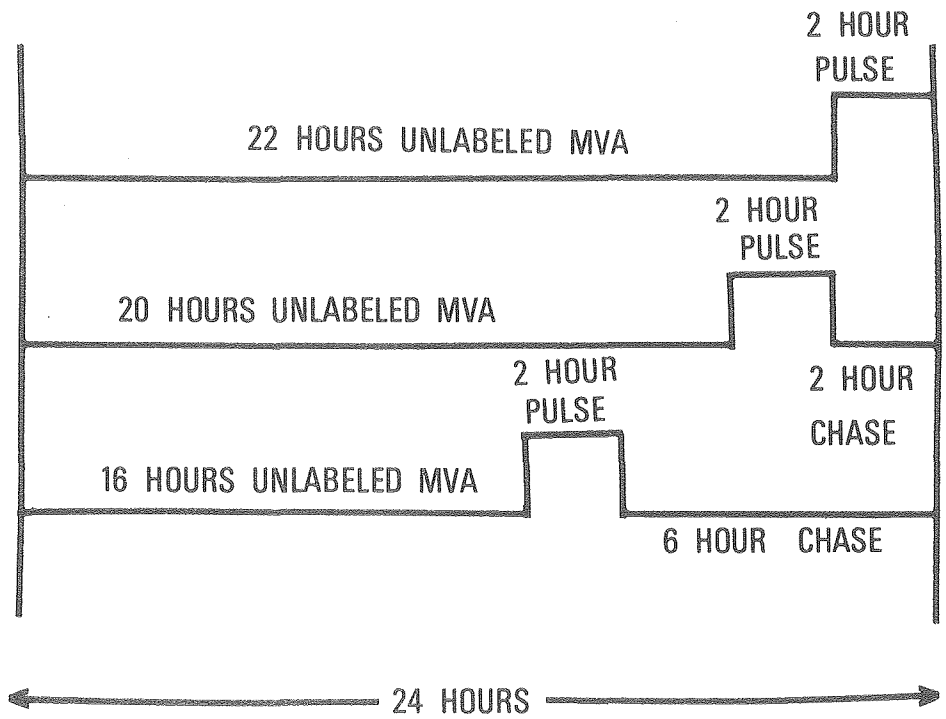
Tritium-labeled pulse experiments: Figure IV-11 graphically depicts the experimental setup for our pulse and chase experiments with tritium-labeled mevalonic acid. Again 10-day-old seedlings grown in 16 ml of nutrient solution were used and then mevalonic acid was administered to the nutrient solution within which the roots of the plants were suspended. All the plants were given 24-hour exposures of mevalonic

DISTRIBUTION OF LABEL AFTER PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

FRACTION OF LABEL	SEEDLING
In nutrient solution after 24 hours	.16
Extracted during Osmium fixation	$\sim 2\times10^{-4}$
Extracted during Ethanol dehydration	$\sim 10^{-3}$
In fixed and dehydrated material	$.83^{+}$

FIGURE IV-10

XBL 7711-10430



PULSE CONCENTRATION=1.09×10^{-3} MOLAR

SPECIFIC ACTIVITY=382 MILLICURIES/MILLIMOLE

PULSE AND CHASE EXPERIMENTS WITH TRITIATED MEVALONIC ACID (MVA)

FIGURE IV-11

XBL 7711-10429

acid (1.09×10^{-3} molar). However, some of the plants received a two-hour pulse of tritium-labeled mevalonic acid after 22 hours of unlabeled mevalonic acid, whereas others received a two-hour pulse after 20 hours followed by a 2-hour "cold chase," and a final group received a 2-hour pulse after 16 hours followed by a 6-hour cold chase. At the end of 24 hours, all the plants were fixed and processed for electron microscope autoradiography. All the tritium-labeled MVA was given at 1.09×10^{-3} molar concentration, specific activity of 382 millicuries/millimole.

Sections from the root, stem, and leaf of the plant were prepared for autoradiography and examined after a 3-month exposure. All showed grain development to some extent in all of the pulse experiments. However, clean concise results were obtained only regarding the chloroplasts in the leaf of the plant. Figures IV-12 through IV-16 demonstrate these results.

Figure IV-12 is an electron microscope autoradiograph taken of the material which received a 2-hour pulse of labeled mevalonic acid and then was immediately killed and fixed. The distribution of photographic grain then reflects the early stages of rubber biosynthesis. As can clearly be seen, the grain is distributed fairly uniformly over the cells of the leaf.

Figures IV-13 and IV-14 are autoradiographs of the leaf which received a 2-hour cold chase after a 2-hour labeled pulse. These micrographs then represent a somewhat later

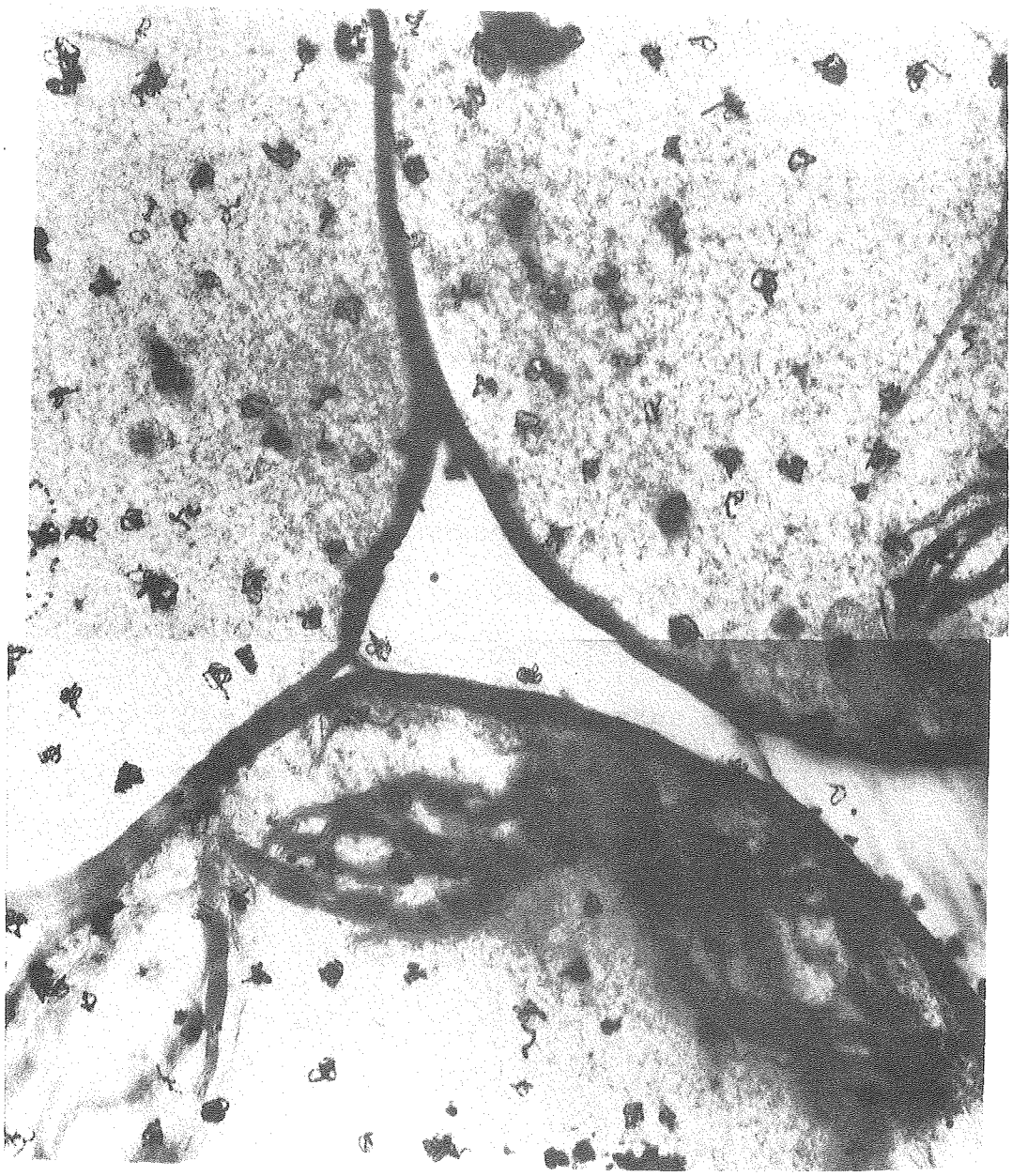


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FIGURE IV-12

XBB 776-5737

FIGURE IV-13

XBB 770-10890

FIGURE IV-14

XBB 776-5736

FIGURE IV-15

XBB 770-10978

FIGURE IV-16

0 0 0 0 4 6 0 / 5 4 3

stage in the metabolism and the grain is primarily located over the chloroplasts.

Figure IV-15 and IV-16 are the result of the experiment with the 6-hour cold chase after the 2-hour pulse. At this time there is considerably less grain and that is now distributed primarily over areas near but outside of the chloroplasts. These autoradiographs clearly demonstrate that the chloroplasts have a role in the biosynthesis of rubber between mevalonic acid and polyisoprenoids. The implications of these results is detailed in the discussion section to follow.

C. Results and Discussion

The results of the pulse and chase tritiated mevalonic acid experiments implicate the chloroplasts in a biosynthetic step or steps along the way between mevalonic acid and polyisoprene. The results of the high-concentration unlabeled mevalonic acid experiment corroborate this result. Since the autoradiographic technique does not provide information as to what chemical species has the label, but only the physical location of the label, the exact nature of the biosynthetic step occurring in the chloroplasts is uncertain. However, review of earlier experimental results on Guayule coupled with information regarding specialized respiration metabolisms in other green plants suggests a probable synthetic step involved.

In one of the above-mentioned references by Bonner (Arreguin & Bonner, 1950), it was found that stem tissue taken from the Guayule plant and grown in callus culture was incapable of producing rubber unless extracts of leaves were added. It was also found that defoliated Guayule plants produce no rubber. Bonner found that extracts of the leaves, when added to the callus cultures, rapidly promoted rubber growth. Since there is no rubber formation in the leaf of the plant itself, it seems clear that the chloroplasts are producing some lower molecular weight precursor of rubber which is transported to the stem and roots for further processing. I suggest that the early stages of rubber formation occurring in the chloroplasts are associated with a specialized respiration mechanism. To explain this calls for a brief digression into a discussion of specialized photorespiration metabolisms used by plants.

Under certain conditions, notably 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 chloroplasts, and always reduces the net efficiency of carbon fixation (Calvin, 1975). There are at least two known methods in which plants minimize this effect (Hatch & Slack, 1971). Some plants, notably 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 plasmodesmata, have been found between these two types of cells in many plants, using the electron microscope.

Carbon dioxide is first fixed in mesophyll chloroplast by condensation with a three-carbon phosphoenol pyruvate (PEP). The four-carbon product, oxaloacetate (OAA), is then converted to malate or asparate. 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 three-carbon backbone remaining is converted to PEP and then shuttled back to the mesophyll cells.

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

There is another specialized system for supplying carbon dioxide to the chloroplasts in the succulent family. Here there is only one type of chloroplast, and the mechanism consists of a daily storage and release cycle for carbon dioxide. At night, the plants open the stomates of the leaves

to accept carbon dioxide from the atmosphere. The carbon dioxide is condensed with an acceptor to form malate, and this is stored until daytime. During the day, the plant closes its stomates to avoid water loss, and the necessary carbon dioxide is supplied to the chloroplasts from the malate storage pool (Hatch & Slack, 1971).

All of the above has been recited to show that plants have evolved various strategies for storing and generating carbon dioxide for times of high stress. Guayule's natural environment is an area of high sunlight (which could promote photorespiration), high temperature and low moisture. This means that the plant leaves will close their stomates during the day, thus restricting water and also carbon dioxide flow. Consequently, the hypothesis that Guayule has developed a carbon dioxide strategy is not unlikely.

If rubber production in Guayule is indeed associated with a strategy of producing carbon dioxide in times of need, then the evidence from our autoradiographic pulse experiments would seem to indicate that the step occurring in the chloroplast is the conversion of the 6-carbon mevalonic acid pyrophosphate to the 5-carbon isopentenyl pyrophosphate. Carbon dioxide released is then used by the plant during conditions of high sunlight intensity and low exchange of carbon dioxide with the atmosphere because of closed stomates. Obviously, in nature mevalonic acid is not applied to the roots of the plant. Presumably, in nature Guayule produces mevalonic acid

0 0 0 3 4 6 0 7 3 4 5

by means of the pathway shown in Figure IV-1.

If our hypothesis that the biochemical step occurring in the chloroplasts is the conversion of the 6-carbon mevalonates to the 5-carbon IPP is correct, we are presented with the fortuitous circumstance that the mevalonate has been fixed in place by osmium. Mevalonic acid (and its prosphoralated derivatives) is a small molecule, soluble in water and with no double chemical bonds. Generally the fixation mechanism of osmium can only be expected to stabilize chemical species which contain double bonds, such as unsaturated fats and terpenoids. Thus the apparent fixation of the mevalonates by osmium is unexpected and very fortunate.

A possible alternative to the above hypothesis is the immediate conversion of MVA to IPP, DMAPP, or some higher molecular weight terpene, followed by diffusion of the terpene product to the chloroplasts. Since the terpenes have double bonds, fixation of these labeled metabolites by osmium is quite reasonable. However, since it is known that IPP molecules are directly added to growing rubber molecules (Figure IV-4), this alternative seems at odds with the observed flow of label to chloroplasts.

To distinguish between these alternatives, it is hoped in the future to combine our autoradiographic techniques with a parallel biochemical assay to determine the nature of the chemical associated with the label in the chloroplasts. We also hope to continue with our autoradiographic efforts to

identify further the structures in the stem and in the root of the plant responsible for more advanced steps in the formation of rubber.

0 0 0 0 4 6 0 7 3 4 6

V. THREE-DIMENSIONAL RECONSTRUCTION PROBLEM

A. Statement of the Problem

It is obvious that the physical world is intrinsically three dimensional, and that to understand a real physical or biological object means to understand its three-dimensional structure and relationships. Occasionally an object will have a cylindrical or spherical symmetry which reduces the understanding of the structure to an understanding of a two- or even one-dimensional shape. Usually though, this is not true, and especially in the biological world where irregular, twisty, and fuzzy shapes abound.

These considerations are especially relevant for structures in the microscopic world, since in either light level microscopy or electron microscopy, structure is studied through the examination of thin sections. These thin sections present a two-dimensional view of a slice of an inherently three-dimensional object, and this produces great problems in interpretation. A general and time-honored approach to the solution of this problem is to examine serial thin sections and then to reconstruct the three-dimensional shape of the object by (in some sense) stacking the serial sections on top of one another in the appropriate order. The stacking can be accomplished either physically with wax or plastic models of the fixed biological sections, or by an artistic representation

or computer reconstruction. A recent paper by Ware and Lo Presti (1975) gives an excellent review of the history of this art form. The next few paragraphs are primarily an abstractive review of that paper.

According to Ware and LoPresti, the first reconstruction from serial sections were formed by the German anatomist His in 1880 (Ware & LoPresti, 1975). This was a reconstruction from serial sections of a human embryo, the reconstruction being rendered by an artistic drawing. Until the considerably later development of techniques making use of micrography (film), a scientist hoping to produce reconstructions from serial sections had to also be an artist.

In 1883, Borne published a procedure for constructing solid models from wax plates cast of the appropriate thickness (Ware & LoPresti, 1975). By 1937, celluloid and plastic had pretty much replaced wax as the material of choice for physical three-dimensional reconstructions (Ware & LoPresti, 1975). The problem with physical model building is that, besides being terrifically time consuming, the physical models are intrinsically incapable of being distributed on any mass basis. Consequently, the search went on for better methods to present three-dimensional structures within the framework of a two-dimensional page.

Recently, stereo pairs of projections of the reconstructed image have become the method of choice for presenting three-dimensional structures determined from serial section

reconstruction. These methods generally involve the abstraction of contours or other data from the actual micrographs of serial thin sections, then a computer processing step to align and "stack" the serial section contours. Finally, the computer generates stereo pairs of projections of the reconstructed image, and prints of these projections are submitted to journals or are presented at meetings as stereo slides.

There are a large number of workers in the field performing three-dimensional reconstruction using computer graphic systems today. Several of these authors are listed in the work by Ware and LoPresti, along with abstracts of their work. The most closely associated with that described in this section, and which has inspired much of our effort, is that performed by Peachey et al. (1974).

B. Description of Our System

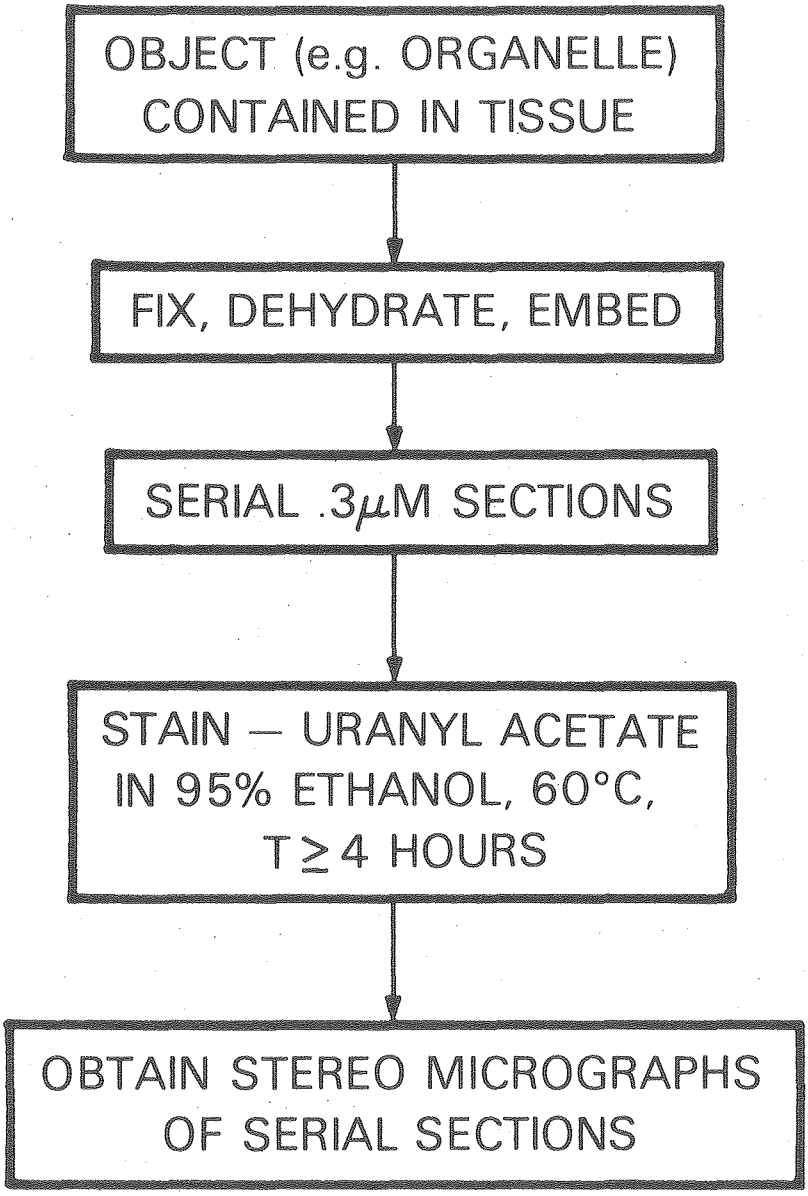
As stated above, the three-dimensional shape of microstructures is generally obtained by reconstruction from serial thin sections. In the case of electron microscopy, a thin section is taken as having a thickness of 500 to 1000 angstroms. This requires the abstraction and alignment of contours from approximately 50 to 100 serial sections to obtain the three-dimensional structure of an object only 5 microns in diameter. If one increases the thickness of the section to reduce the number of serial sections, one ordinarily loses resolution because the structure of the objects

changes within the thickness of the section and this confuses the reconstruction of the shape. We have recently developed a method to use stereo pairs of micrographs of thicker (around .3 microns) sections to facilitate the reconstruction of three-dimensional objects. Figures V-1 and V-2 illustrate the steps involved in producing these three-dimensional constructions.

Following Figure V-1, we have an object, for instance an organelle, contained in the tissue. To view this object we must first fix, dehydrate, and embed the tissue in plastic. The next step is to place the plastic embedded block of tissue in a microtome and cut serial sections, generally around .3 microns in thickness. This section thickness is chosen because it is about as thick as one can work with, using the high-voltage microscope available to us. This microscope produces a top accelerating voltage of 650 kilovolts.

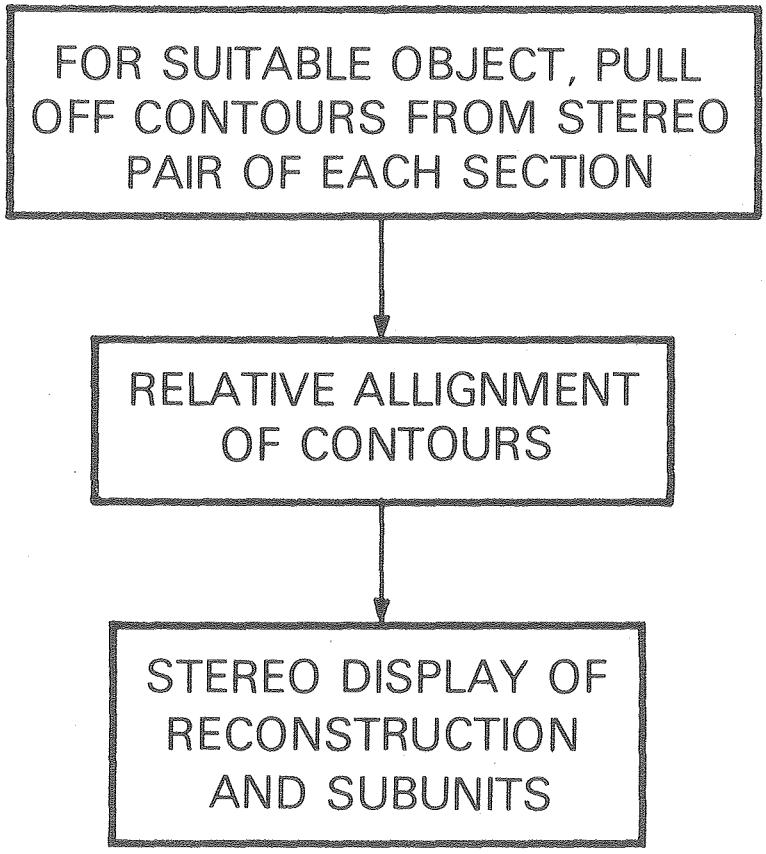
As shown in the figure, the sections are stained in 2% uranyl acetate dissolved in 95% ethanol at 60°C for some time greater than 4 hours, usually overnight. This method for staining was developed by Locke and Krishnan (1971). These conditions of alcohol solution of stain coupled with higher temperatures for longer times are necessary to ensure that the stain can penetrate the entire thickness of the section. Uniform staining is particularly important since stereo micrographs are obtained which show the three-dimensional structure of the section.

As indicated in Figure V-1, the next step is to obtain



PREPARATION AND MICROSCOPY

FIGURE V-1



COMPUTER ASSISTED RECONSTRUCTION

FIGURE 4-2

stereo micrographs of the serial sections. Figures III-29 through III-32 are stereo pairs of serial sections obtained in the fashion described. One can note good uniform quality of stain throughout the depth of the section. One can also note that much detail is present, enough detail to make these sections unsuitable for the reconstruction process described next. The steps of the reconstruction procedure are outlined in Figure IV-2.

Figures V-3 and V-4 are photographs of the machine we have adapted to generate contours within the depth of the section. The machine is an ER55 stereoscopic projector lent to us by the U.S. Geological Survey. The machine was originally intended to produce contour topographic maps from aerial photographs, but is adapted very easily to our purposes of producing contours from stereo pairs of thin sections. The only necessary modification of the machine was to change the size of the photographic plate holder to fit our electron microscopy plates.

As shown in the figures, the working parts of the machine consist of two overhead 55 mm projectors which focus images of our electron microscope stereo plates onto a reflecting screen on a table top. The screen is viewed through the rotating slot viewer fixed to the front of this screen. The overhead projectors flicker on and off at a constant rate, with the right and left projectors each on for alternate halves of the cycle. The rotating slot viewer opens the view

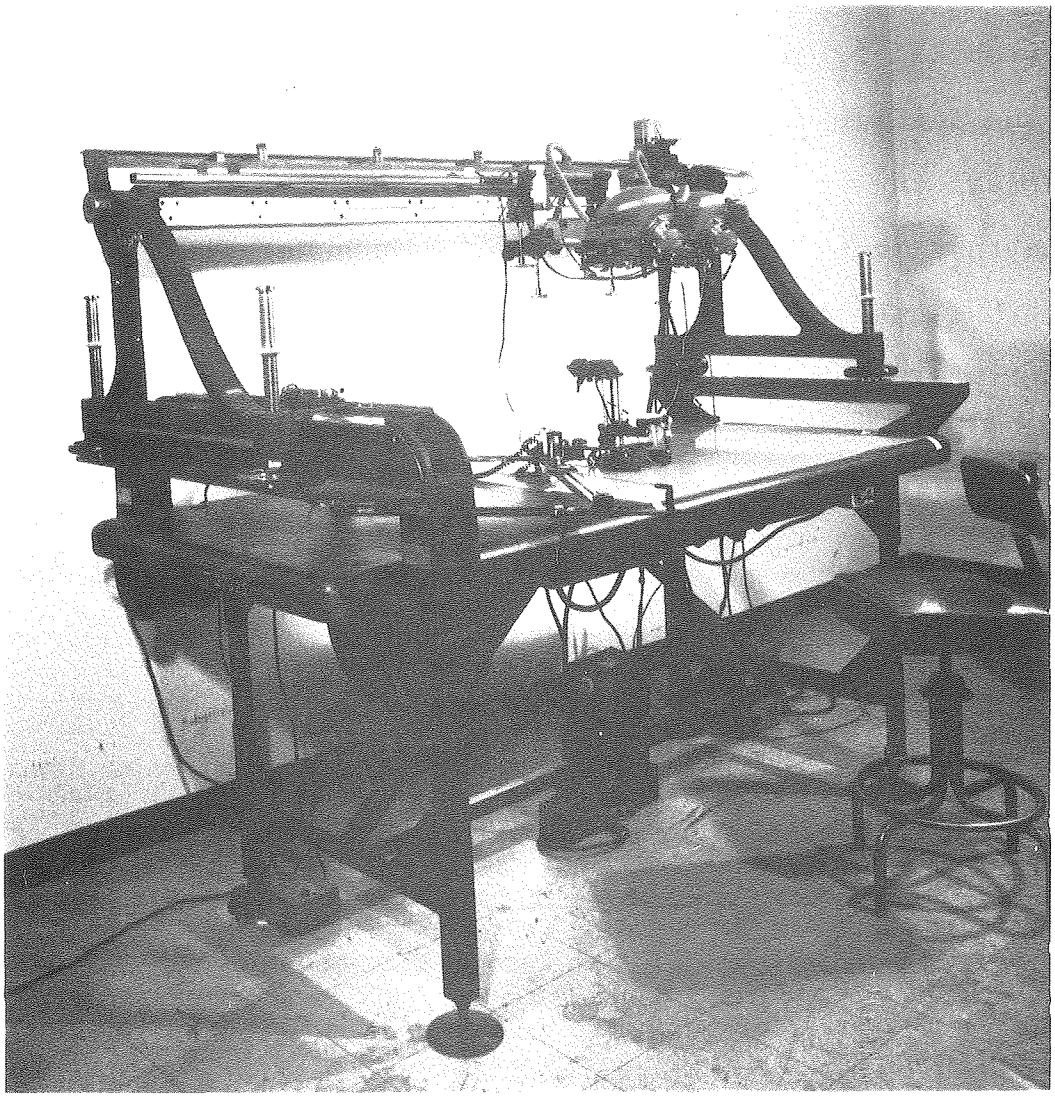


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FIGURE V-3

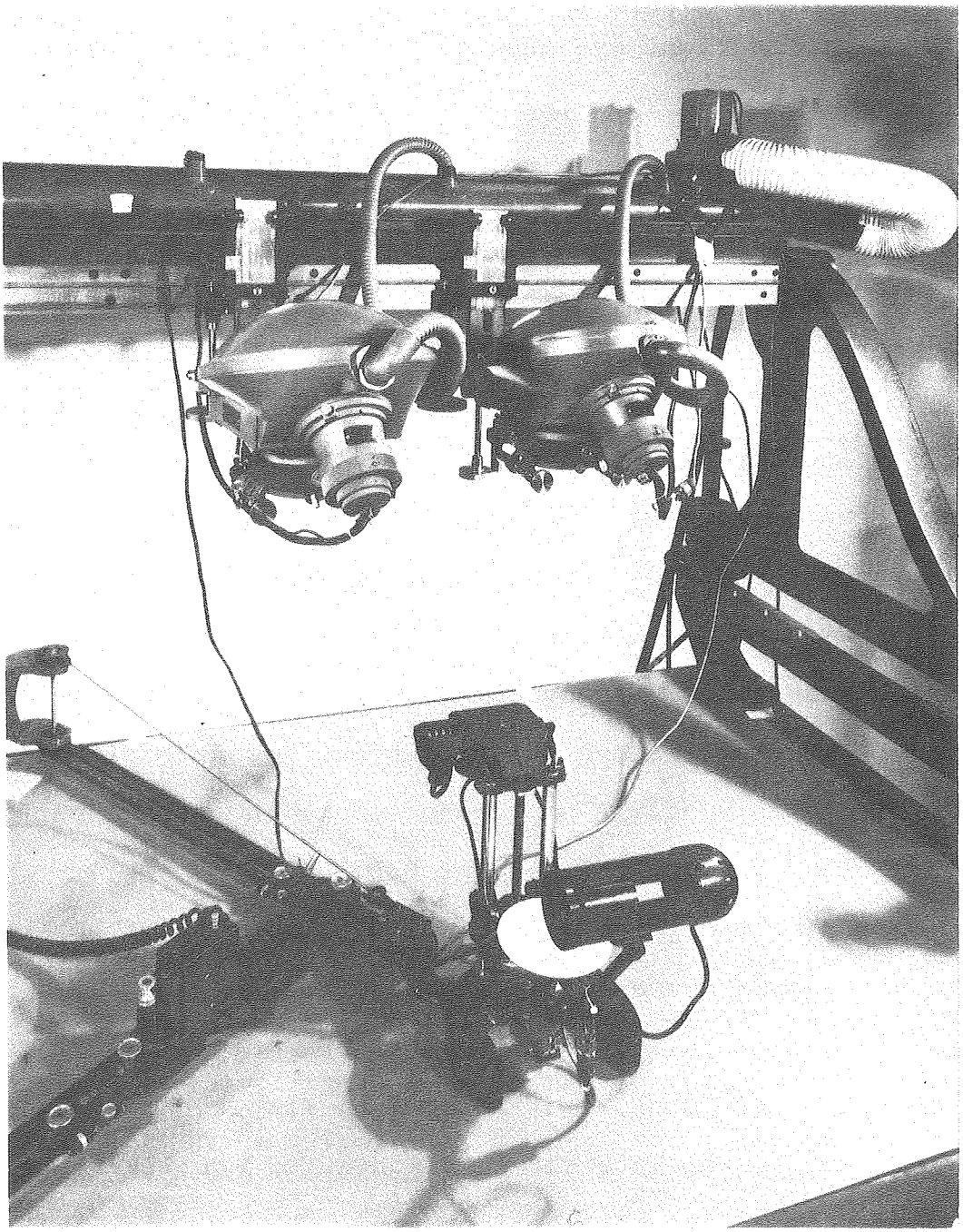

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FIGURE V-4

to first the right eye and then the left eye in synchrony with the projectors. Thus the right eye sees only the image from the right projector and the left eye only that from the left projector. The flicker rate is fast enough so that a three-dimensional stereo image is produced.

In the middle of the screen is a white dot produced by a light under the screen and a pinhole in the screen. By adjusting the vertical position of the screen the floating spot can be made to (virtually) move up and down within the depth of the stereo image. One obtains contours from a chosen depth within the section by placing the dot at some virtual height, and then tracing out the intersection of the shapes in the stereo representation with an imaginary plane at the level of the spot. A pen connected to the screen performs the actual drawing of the contours.

The mechanism which controls the vertical position of the screen is mechanically attached to a device which displays the height of the screen. Thus if one obtains readings for the "top" and "bottom" of a section, simple calculations yield the appropriate screen heights for equally spaced contours from within the section.

Though this operation is simple in principle, it becomes difficult and confusing in the context of transmission micrographs. The machine was originally designed, as stated above, to perform contour map topography. In that application the object to be contoured is solid, and thus only one surface is traced. In our transmission electron micrographs,

several surfaces may lie on top of one another within the depth of the section. This confusion can produce sometimes insurmountable difficulties in tracing out the contours. However we have been successful in certain structures, and these results are presented later on.

For .3 micron section, we have been taking contours at the top and bottom of the section as well as two equally spaced contour levels within the depth of the section. Thus four contour levels are produced from each section.

A standard problem in reconstruction is the relative alignment of the contours obtained from different serial sections. Fortunately, in our material the micrographs of the organelle to be reconstructed also contained a number of outside identifying features. These outside features, such as a cell wall or other organelles, allow for the optical alignment of contours from one section with those of the next.

More precisely, the optical alignment of the micrographs of the serial sections is accomplished as follows. The "left" member of the two micrographs which form a stereo pair of the middle section is taken as the reference orientation. On this plate are placed three widely spaced non-colinear dots with a fine-tipped fiber pen. The reference plate is placed on a light table and one at a time the "left" members of the pairs from each of the other serial sections are placed on top of the reference plate. In this way, the

features of the reference plate are seen simultaneously with the features of the top plate.

The top plate is then moved about to achieve alignment of features common to both plates, such as cell walls and large organelles. When properly aligned, three dots are marked on the top plate, the dots corresponding in position to those of the reference plate.

Before the contours of each section are taken, the positions of the three dots in the projected image are marked on the same sheet of paper onto which the contours will be traced. Thus each contour to be digitalized is associated with the three dots that specify the correct orientation of the contour. The dots are then used to properly align the contours for the device which digitalizes the contours. The digitalization procedure is described later in this section.

The procedure for aligning the micrographs of the serial sections only need be performed on one member of each stereo pair since the orientation of the second member is determined by the first and the axis of tilt between the two members of the pair. The choice of using the "left" or "right" member for alignment procedure is arbitrary.

The contours, once obtained and aligned, are digitalized and inputted into the computer for a reconstruction. The method of digitalizing the data and inputting it to the computer, and the computer algorithm used to generate the stereo displays will be briefly described next. Appendix C

to this thesis contains the details of the computer techniques. Appendix B contains a discussion of the pitfalls of working with stereo pairs.

The contours are digitalized by drawing over them with a Graf-Pen digitizer. This device works by producing a spark whenever the tip of the pen is depressed on the line denoting the contour. The noise of this spark is picked up by perpendicularly arranged microphones above and to the side of the drawing area. The arrival time of the sound to each microphone from the spark allows the computer to calculate the location of the spark itself. Thus the graphical data is converted into a set of coordinate data stored within the computer.

The digitalized data very often need correction. This need arises because extraneous points occur (often due to loud talk or objects being dropped while the data are being taken with the spark pen). Additionally, while tracing more than a dozen or so contours it is not uncommon for the operator to miss certain aspects of the contours. Consequently it is necessary to review the contour data for editing.

This editing operation is accomplished by displaying the contours on a Tectronix 4014 screen, and then adding, subtracting, or moving points around until the contour is "right." The appendix of this section contains a description of computer programs which allow the execution of these editing operations on the Tectronix 4014.

Finally, the edited contour data are input to a computer algorithm which is designed to produce stereo reconstruction of the object. The appendix of this paper contains a detailed description of this program, and the next few paragraphs briefly describe its operation.

The heart of the algorithm is a routine (INIT3D) which projects the lattice points of a rectangular array with minimum coordinate (0,0,0) and maximum coordinate (n,n,n) onto the plane normal to the eye of the observer. To produce our stereo pairs, we choose to view the array from the viewpoints at ±7° from the 45° line which runs from (0,0,0) to (n,n,n). The computer outputs 35mm negatives displaying the reconstruction.

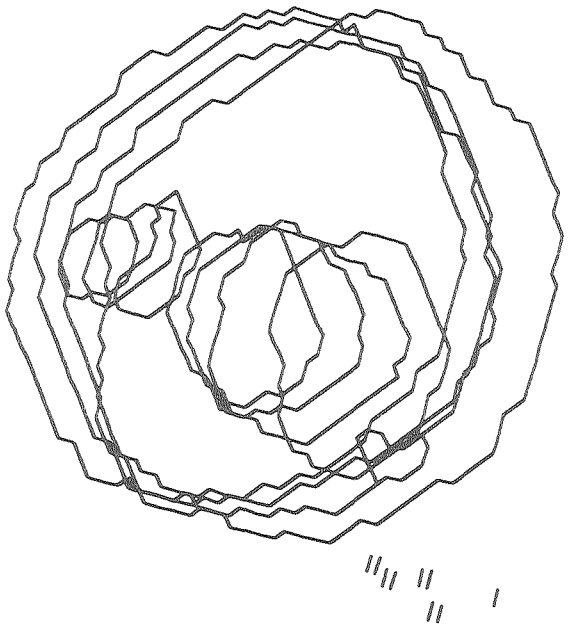
There are currently any number of algorithms available for producing two-dimensional projections of three-dimensional objects. We developed this one to represent very general shapes. Most of the systems designed for this kind of work trade off the ability to present more general shapes for certain gains in speed and cost. Our routine is capable however of presenting closed contours, open contours, solid areas, or any other data that can be inputted into the computer as (X,Y,Z) data points. A more complete description of the computer routines used is given in Appendix C of this thesis, along with references to the original programs from which our routines were developed.

The final presentation form of our reconstruction is

0 0 : 0 4 6 0 / 3 5 3

designed to produce the maximum understanding of the actual three-dimensional shape of the object while preserving as much as possible the actual detail of the micrograph. For this reason our contour representations are shown together with the actual micrographs. And again, we chose stereo representations for their unique ability to represent three-dimensional shapes from flat two-dimensional printed pages.

Figures V-5 through V-8 are stereo pair presentations of actual micrographs obtained from serial thick sections, presented together with contours abstracted from each serial section reconstruction. Figure V-9 is a stereo pair computer-generated reconstruction of the entire object. Note that a number of details have been removed in Figure V-9. This is because even with the stereo pair presentation, it is extremely difficult to look through a "shell" at detail within. Also, the rear surface of the outer shell has been drawn with lighter lines for easier understanding. All these operations on the data were performed using the editor described in the appendix, and our general computer algorithms for generating stereo pairs was capable of accepting all this data, as well as instructions for drawing different aspects of the reconstruction with different line thicknesses.



LEFT

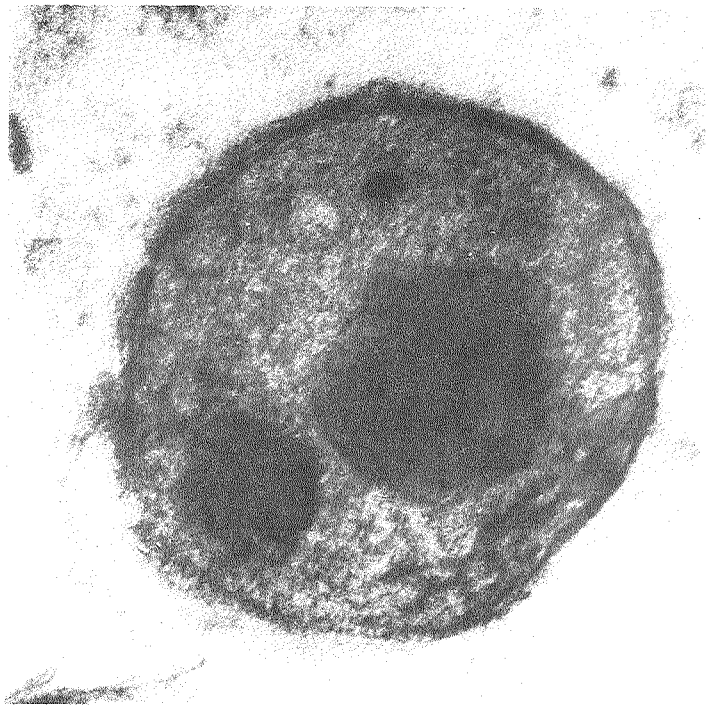


FIGURE V-5A

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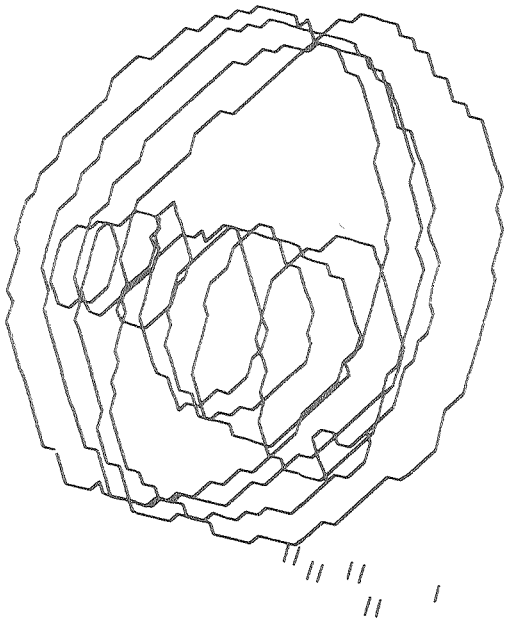

RIGHT

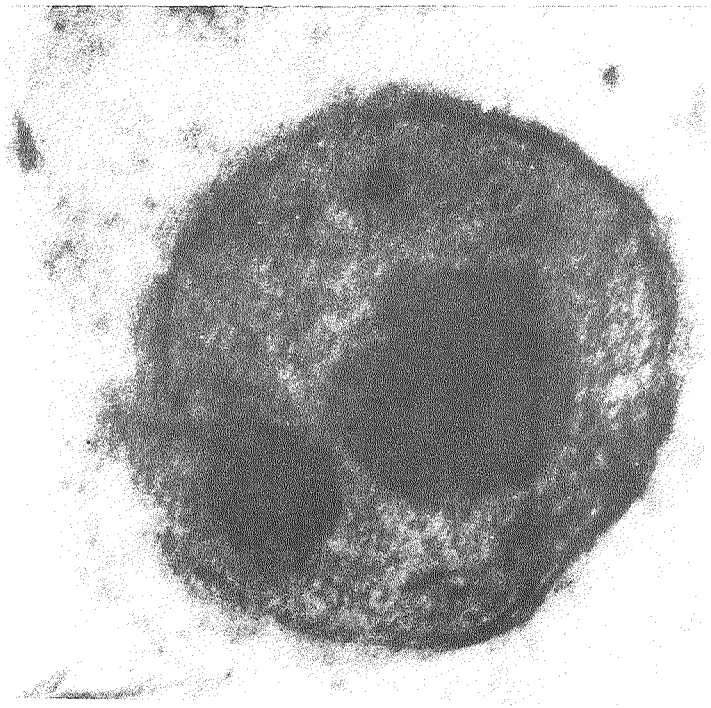

FIGURE V-5B

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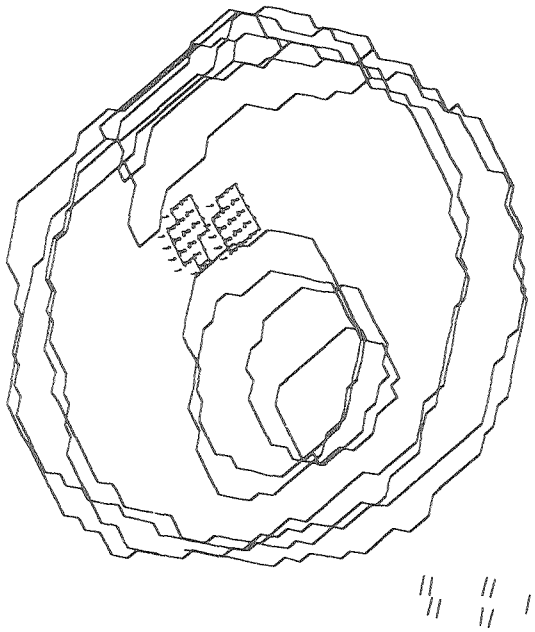


LEFT

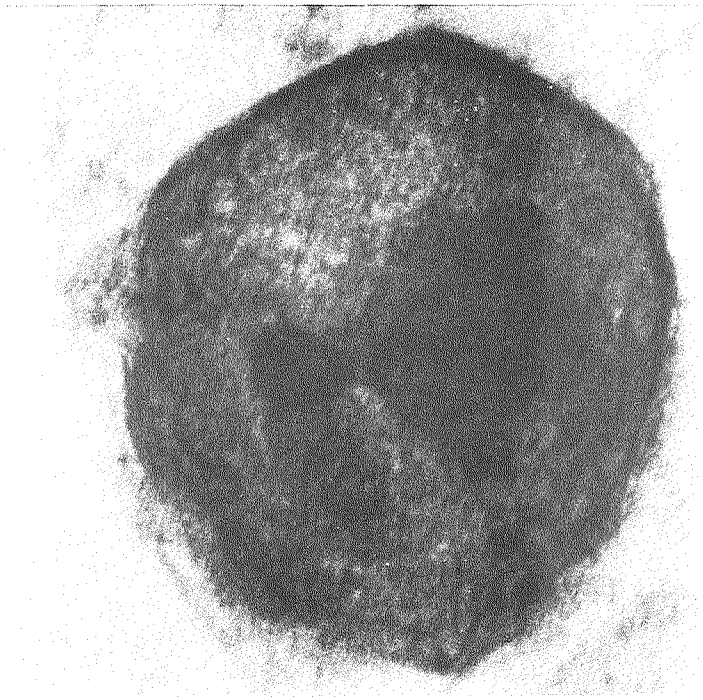
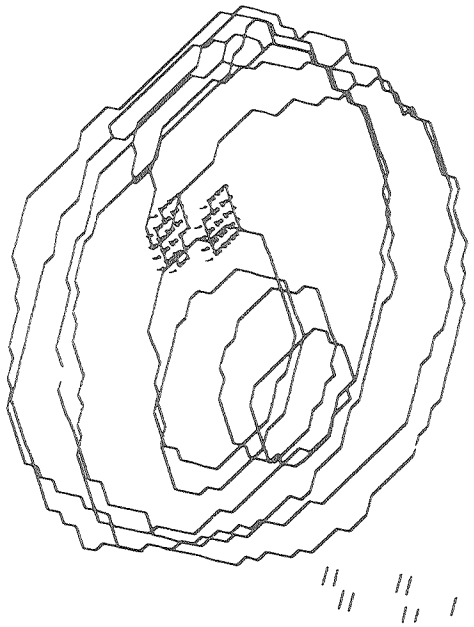


FIGURE V-6A

XBB 778-7925



RIGHT

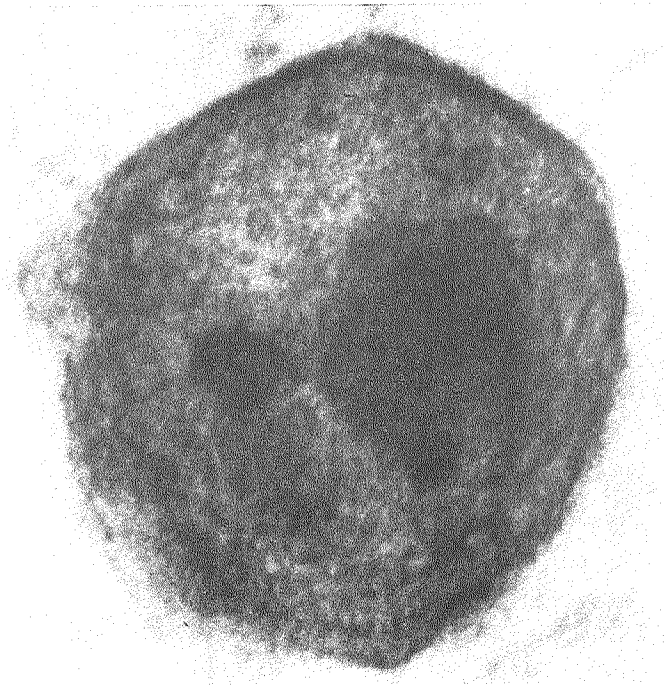
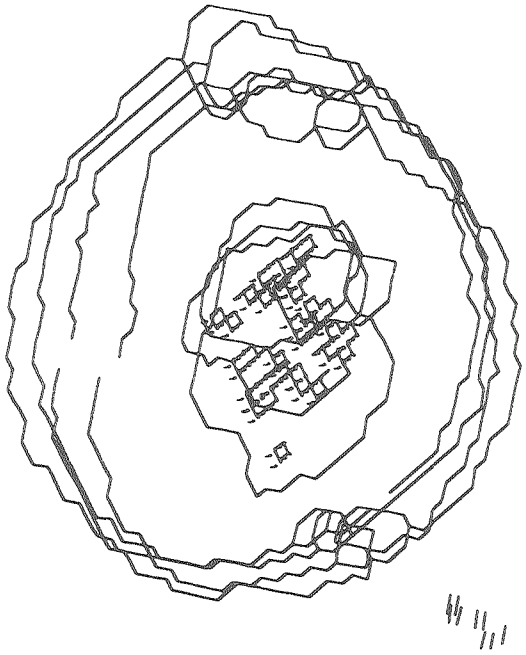


FIGURE V-6B

XBB 778-7923



LEFT

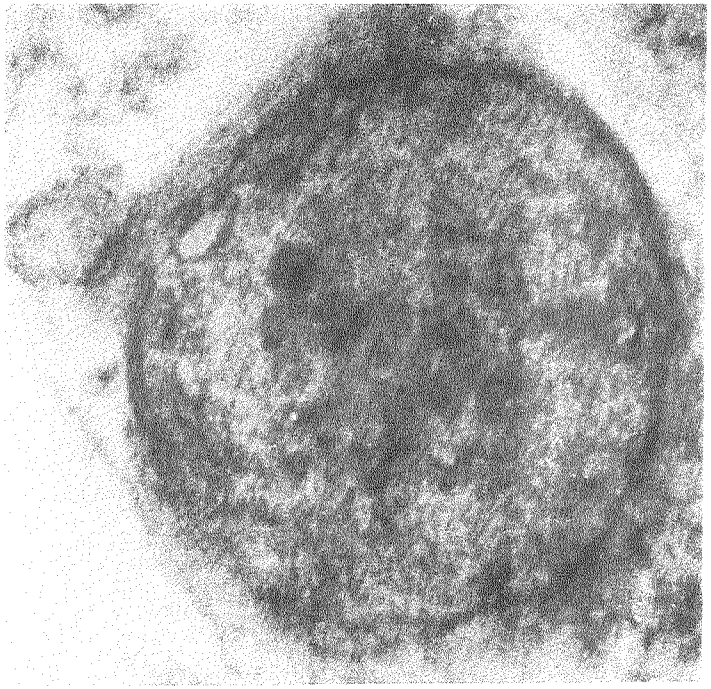
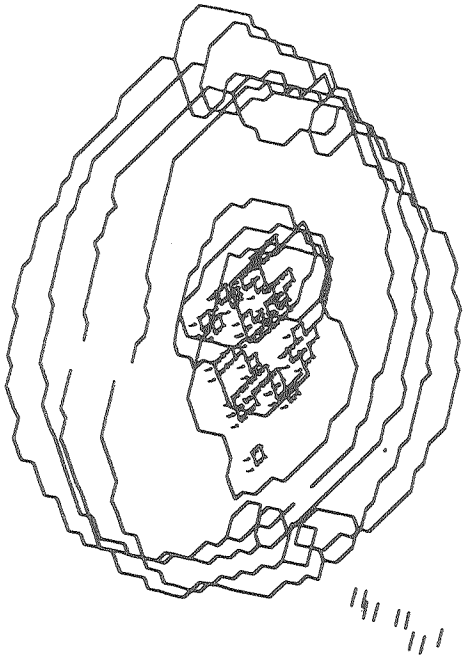


FIGURE V-7A

XBB 778-7920



RIGHT

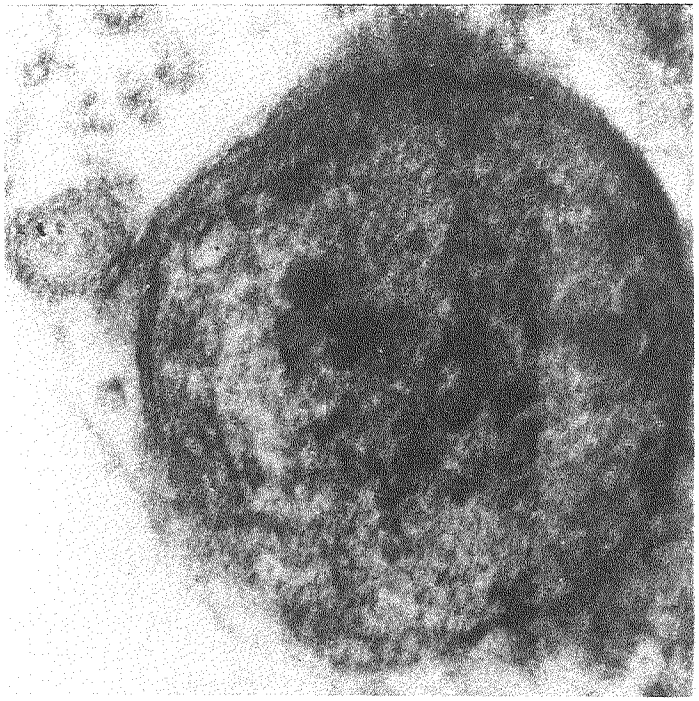
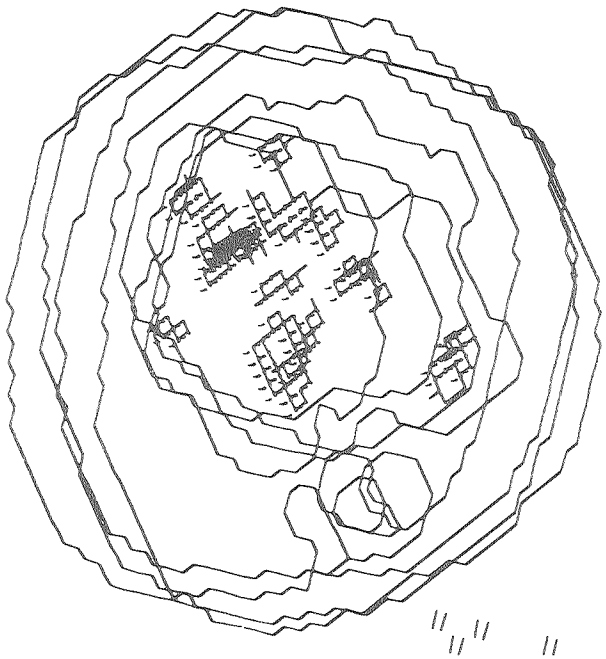


FIGURE V-7B

XBB 778-7921



LEFT

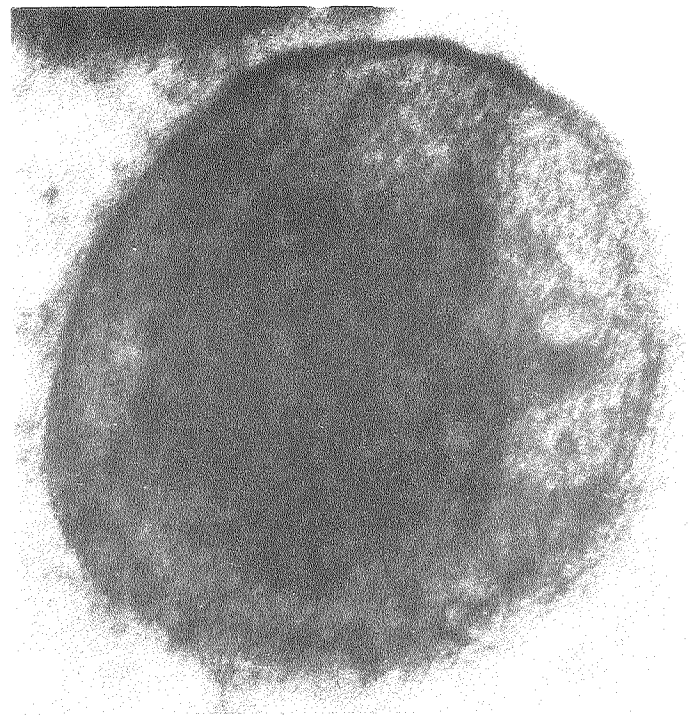
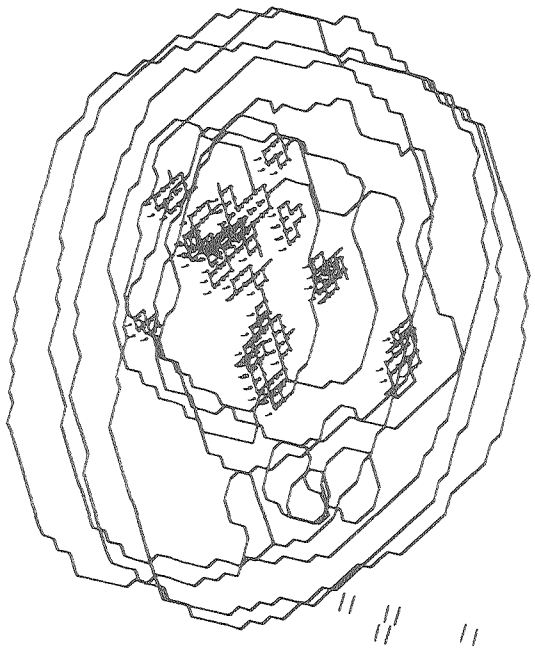


FIGURE V-8A

XBB 778-7919



RIGHT

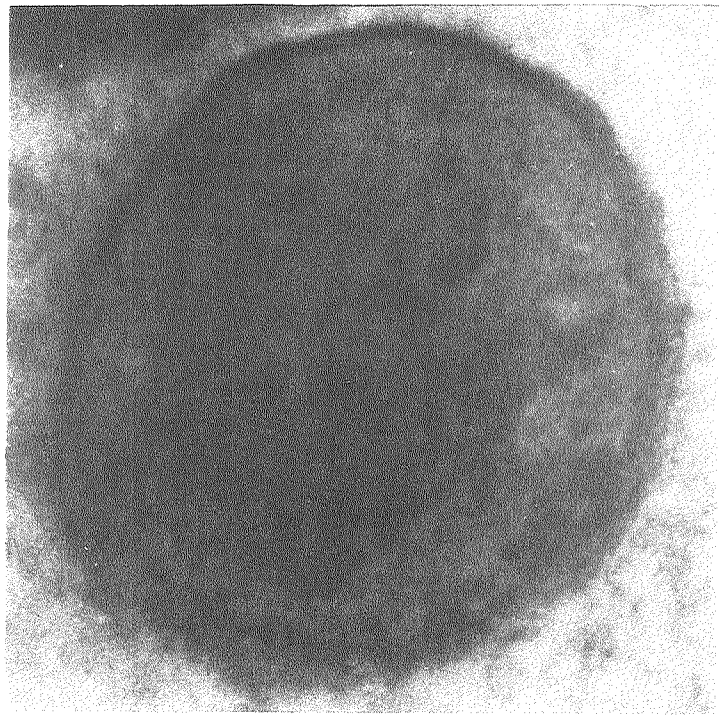
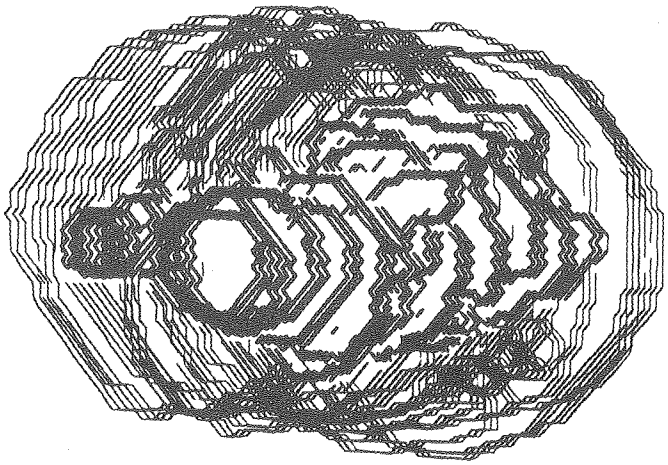


FIGURE V-8B

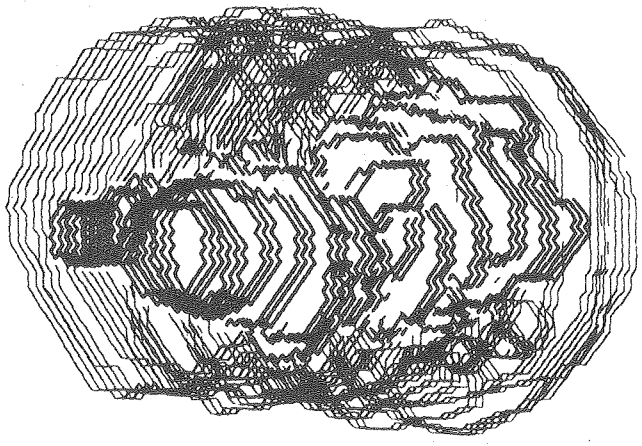
XBB 778-7918



LEFT

FIGURE V-9A

XBL 7711-10513



RIGHT

FIGURE V-9B

XBL 7711-10512

VI. CONCLUSIONS AND DISCUSSION

By bringing the power of the electron microscope to bear on the problem of hydrocarbon production in Guayule, we have made three contributions. The first of these is the first examination of the structure of Guayule, a copious hydrocarbon producer, at the electron microscope level. The second is our success at following the biosynthesis of hydrocarbons in Guayule using mevalonic acid as a tracer. The final contribution derives from our development of a system to reconstruct the three-dimensional shape of organelles (suspected of involvement in hydrocarbon synthesis) using stereo micrographs of serial thick sections (.3 microns) viewed in the high-voltage electron microscope.

In the study of the morphology of Guayule, particular attention was given to structures associated with hydrocarbon production in the plant. Consequently there was emphasis on the structure of the resin canals and the subcellular components (organelles) of the cells which surround the canals. For the reasons discussed in Section III-E of this thesis, these organelles are strongly suspected of involvement in hydrocarbon biosynthesis in Guayule.

The morphological material presented also emphasizes the structure of the chloroplasts of Guayule leaves. This was done in recognition of the role of chloroplasts as the primary producers of all materials used in later steps of

biosynthesis in the plant, and also because of the results of our tracer experiments (Section IV) which implicate the chloroplasts in later steps of the biosynthesis of hydrocarbons than was previously thought.

Micrographs of Guayule chloroplasts show dark staining inclusions (as do the chloroplasts of many other species), called plastoglobuli, which may be storage sites from which the chloroplasts derive carbon dioxide in times of high light intensity and low carbon dioxide availability--that is, when the leaf stomates close to avoid moisture loss. This speculation derives from the hypothesis developed in Section IV which relates hydrocarbon production in Guayule to a mechanism for providing carbon dioxide to Guayule chloroplasts in times of stress. A further basis for this speculation is provided by the study of the bark chloroplasts of the deciduous desert tree Cercidium performed by Adams and Strain (1969). The results of experiments with isotopically labeled carbon dioxide led the authors to suggest that the many plastoglobuli found in the bark chloroplasts serve as a reservoir of photosynthetically fixed carbon (Adams & Strain, 1969). Since plastoglobuli are not starch inclusions but rather osmiophilic and generally terpenoid (Greenwood et al., 1963; Bailey & Whyborn, 1963), there is the implication that plastoglobuli serve as a reserve of necessary materials which are not carbohydrates. Given the evidence developed in the mevalonic acid experiments described previously, the conjecture

that the plastoglobuli of Guayule serve as reserves of carbon dioxide seems not unreasonable, though far from proven.

The morphological studies are regarded as only a beginning for a thorough investigation of Guayule. For example, a comparative study of high and low yielding varieties of Guayule and comparisons with other larger but non-rubber-producing relatives might prove very valuable to the development of Guayule's rubber-producing potential.

For our second contribution, we used mevalonic acid, the precursor for the biosynthesis of alkenes, in two different ways to explore hydrocarbon synthesis in Guayule. First, we found that by administering a high concentration of mevalonic acid to the roots of seedlings, densely staining inclusions developed in the chloroplasts of the plant. This indicates that the mevalonic acid is transported to the chloroplasts for some biosynthetic step.

We then administered tritium-labeled mevalonic acid to seedling roots in timed doses to follow the metabolism using autoradiographic techniques. As described in the section dealing with our autoradiographic results, this points to the fact that rubber production in Guayule is linked with a carbon dioxide reserve system in the Guayule plant. It further allows the speculation that the plastoglobuli found in the chloroplasts of the plants may be storage sites of chemicals similar to mevalonic acid which release their carbon dioxide during times of high carbon dioxide stress in high sunlight.

These results indicate that we may be able to tell a great deal about the hydrocarbon-producing potential by examining chloroplasts. We may be able to use the high resolving power of the electron microscope to significantly aid in the breeding and selection of higher producing varieties. We are also left with the general success of the autoradiographic method with the implication that we may be able to follow the biosynthetic steps even further and determine what biochemical steps are occurring in the chloroplasts as well as in other areas of the plant.

Our final contribution to the understanding of hydrocarbon production in Guayule is the development of our system to produce the three-dimensional shape of subcellular structures strongly suspected of involvement in hydrocarbon biosynthesis. This system makes use of the advantages offered by the high-voltage electron microscope. By making three-dimensional reconstructions from stereo views of thick sections--thick sections that can only be successfully viewed in the high-voltage electron microscope--we have found both another application for this powerful tool and a way of making reconstructions of three-dimensional objects from considerably fewer serial sections.

Our method of obtaining contours using stereo projections of the thick slices increases the resolution of the reconstruction. Also, the method we developed to present both the contours derived from these thick sections as well

as stereo views of the thick sections themselves enhance the understanding of the three-dimensional structures.

Finally the reconstruction of part of the structure of an organelle from the root of Guayule makes a small step toward the full understanding of the hydrocarbon-producing mechanism in Guayule. Such understanding of the three-dimensional structure of elements associated with hydrocarbon production hopefully will assist in the improvement of yields in this and possibly other hydrocarbon-producing plants.

It is the firm conviction of the author that biological conversion of solar energy in any of the many forms described in this work is an exciting prospect for supplying the energy needs of our nation. The prospects for developing better breeds of plants for the production of energy and materials is an exciting prospect for science in the next 20 years.

0 0 0 0 4 6 0 7 3 6 1

APPENDIX

Appendix A

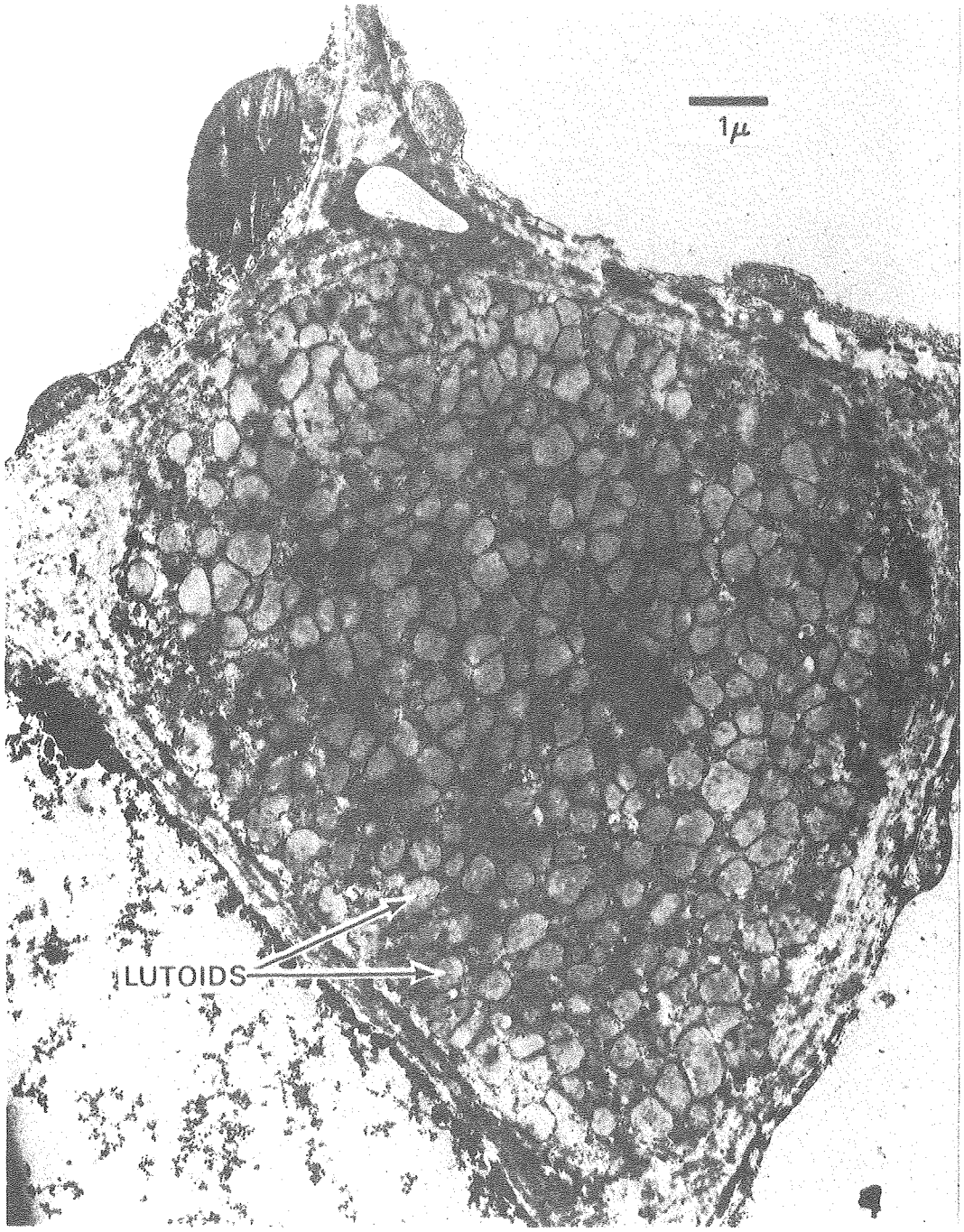
MORPHOLOGY OF OTHER RUBBER PLANTS

Probably the best known hydrocarbon-producing plant is the Hevea tree (Hevea brasiliensis). Hevea contains its rubber within structures called latex vessels. These latex vessels are tube-like linear arrangements of specialized living cells, in contrast to the extracellular spaces which form the resin canals in Guayule. Like Guayule's resin canals, the vessels form ring-like configurations interspaced with the annual rings of phloem bundles.

Latex vessels are common in the plant families Euphorbiceae (Hevea, for example), Asclepiadaceae (milkweed), and Compositae (sunflower) (Bauer & Glaeser, 1975). Latex vessels are also found within other plant families and not all members of the above families have them. Guayule is a member of the Compositae family for example. The latex vessels are so named because of the milky white latex which they usually contain, but this latex does not ordinarily contain more than a trace of rubber if any. The contents are usually lower molecular wright terpenoids (Bauer & Glaeser, 1975).

Dickenson has made an extensive study of the structure and ultrastructure of Hevea latex vessels (Dickenson, 1964). Micrographs are presented in this section of the latex vessels of Hevea and of a South African member of the milkweed family, Asclepias fruticosa.

0 0 0 0 4 6 0 7 3 6 2

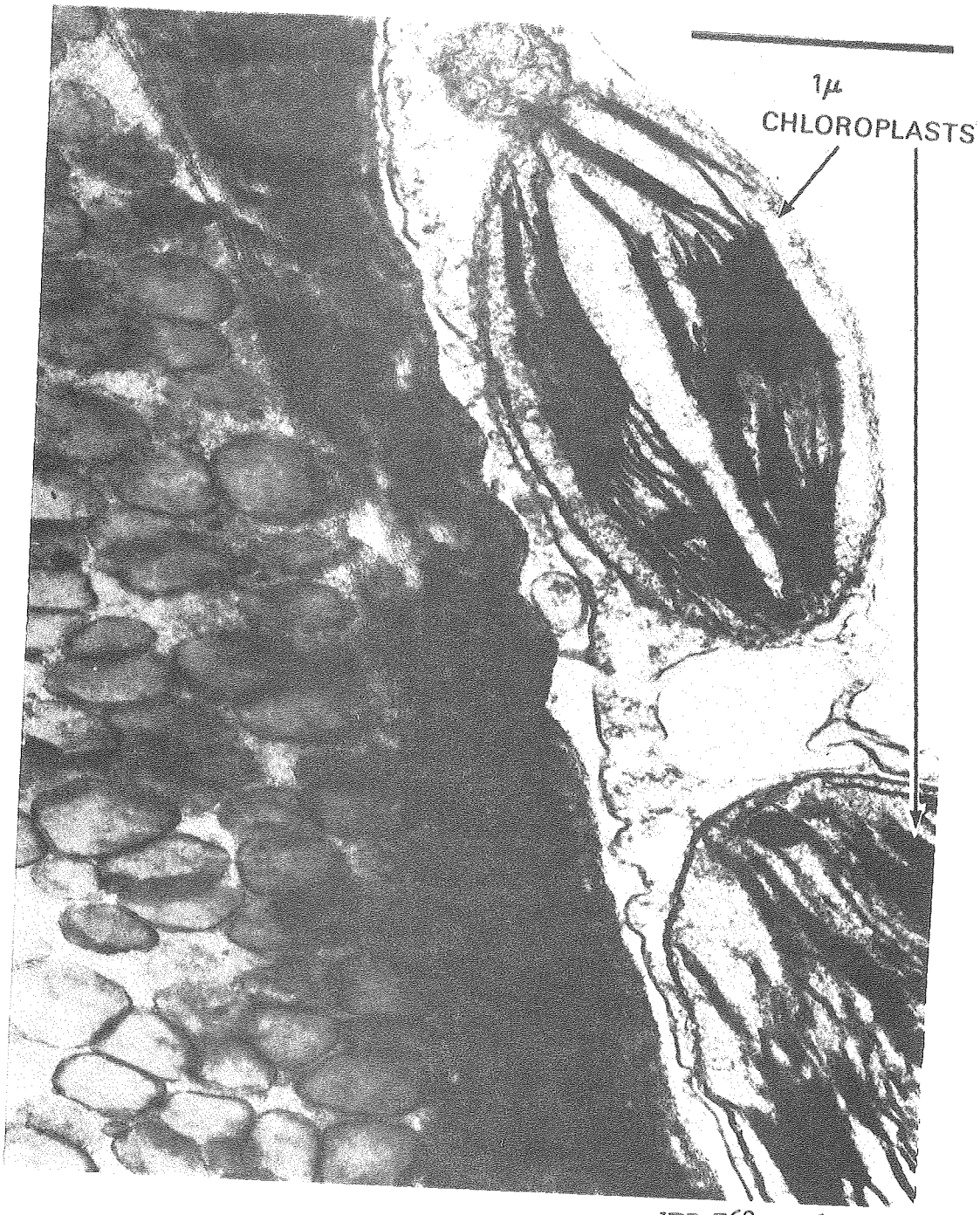


XBB 768-7597

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.

FIGURE A-1

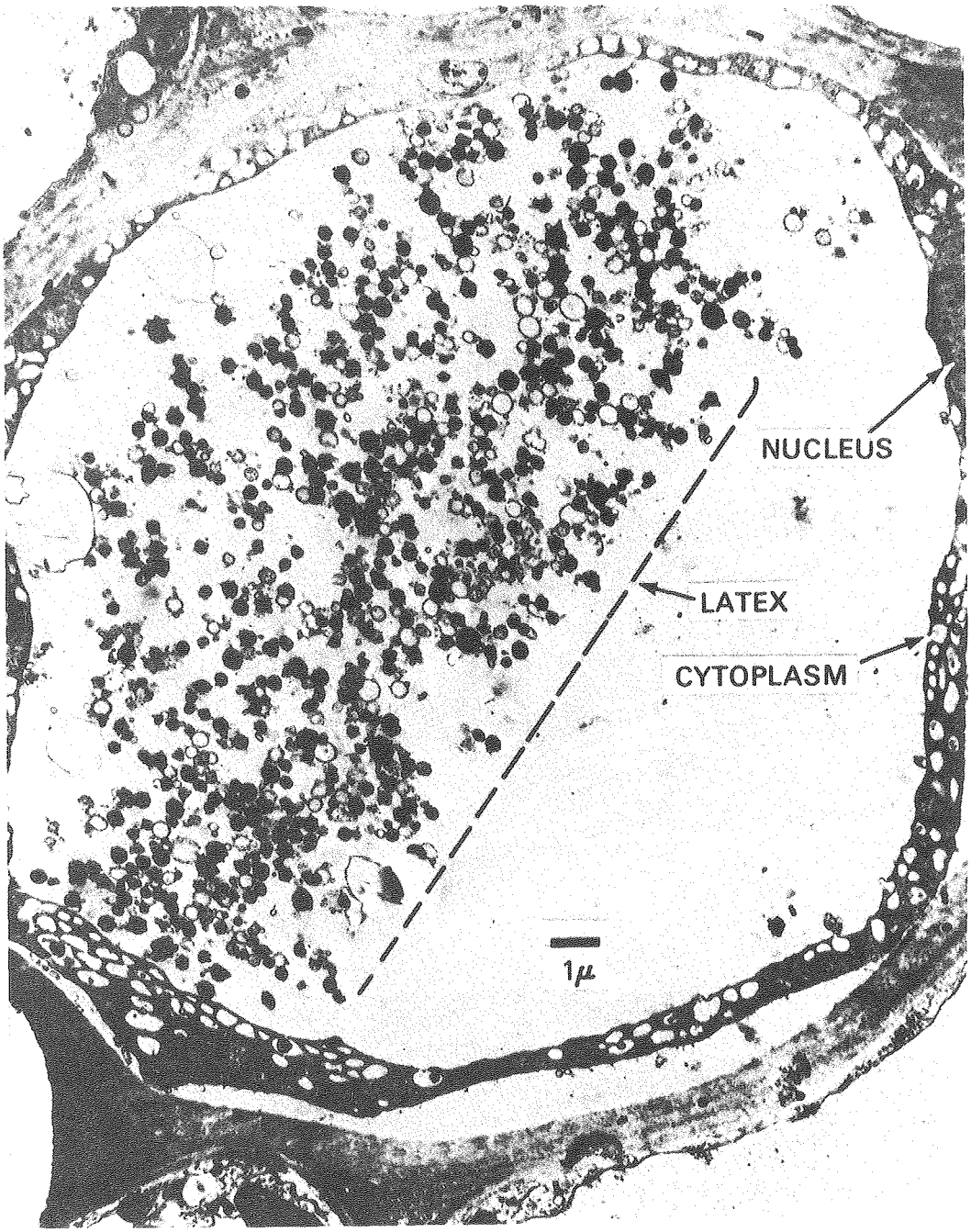


XBB 768-7596

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.

FIGURE A-2

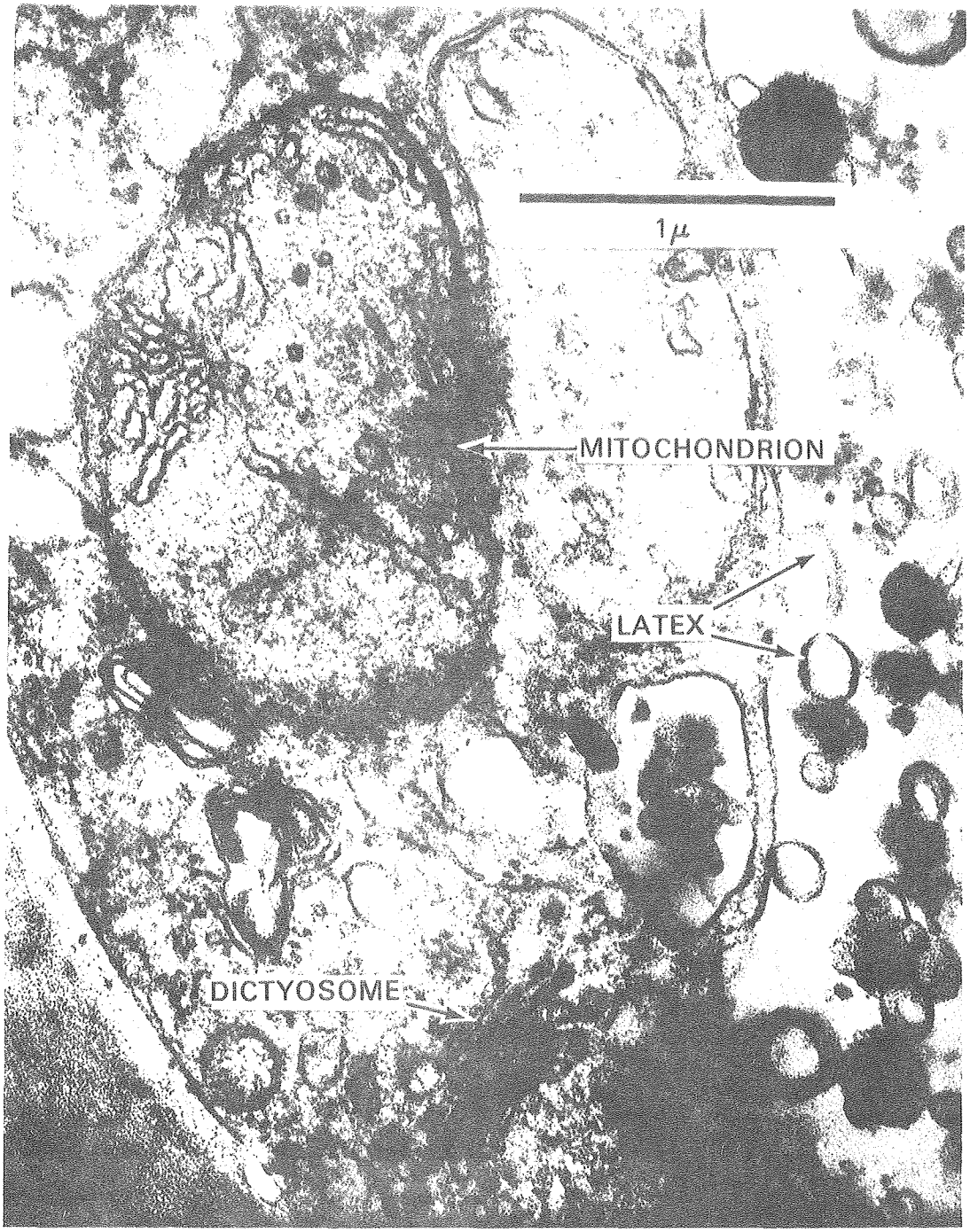


XBB 768-7595

Micrograph 3. Asclepias fruticosa

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.

FIGURE A-3



XBB 768-7594

Micrograph 4. Asclepias fruticosa

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

FIGURE A-4

0 0 0 4 6 0 / 3 6 4

The tissue from which the micrographs were made was provided by Dr. Bruce Bartholomew of the U.C. Botanical Graden. The material was fixed in Gluteraldehyde and osmium, dehydrated in ethanol and propalene oxide, and embedded in epon. Sections were stained in uranyl acetate and Reynolds lead stain.

Figure A-1 is of a latex vessel of a very young stem of *Hevea*. Figure A-2 is a higher magnification view of the edge of the latex vessel and the contents of a neighboring cell. Note the plastoglobuli in the chloroplasts of the neighboring cell.

Figure A-3 is of the latex vessel of *Asclepias fruticosa*. Figure A-4 is a higher magnification view which shows details of the cytoplasm.

Appendix B

STEREO MICROGRAPH ORIENTATION

Proper stereo imaging requires the proper alignment of two planar projections. If the two plane images are not aligned correctly with respect to each other, several sorts of distortions can result. The most common distortion results from failure to align the planar images correctly with respect to the axis of rotation. A slight misalignment will result in difficulty in perceiving stereo, while a greater misalignment will make stereo viewing impossible.

However, even if the two planar images are rotationally aligned correctly with respect to the stereo axis, there are a number of errors still possible. Figures A through H on the following pages illustrate these possibilities.

The upper right-hand corner of Figure A is a perspective drawing of a rectangular box. The front of the box is labeled "F" and the back labeled "B." Drawn in the box is a figure T, and in front of this a figure 1. The characters T and 1 are taken as abstract representations of any general shape which might be embedded in the box, such as an organelle embedded in a plastic section.

If the contents of the box (T and 1) were photographed from the directions $\pm\theta$ in the drawing, the resultant photographs would resemble these depicted at the bottom of Figure A. The depicted photographs are labeled "L" and "R," and

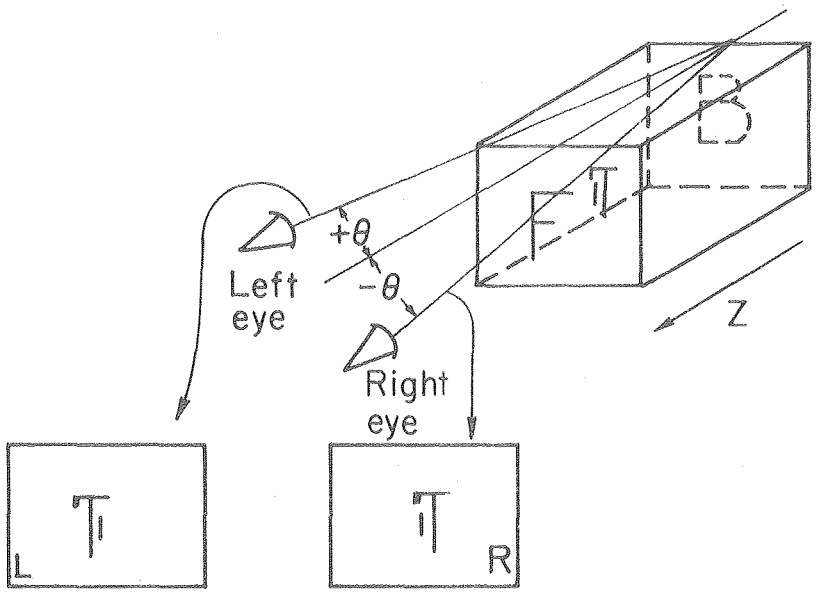


Fig. A

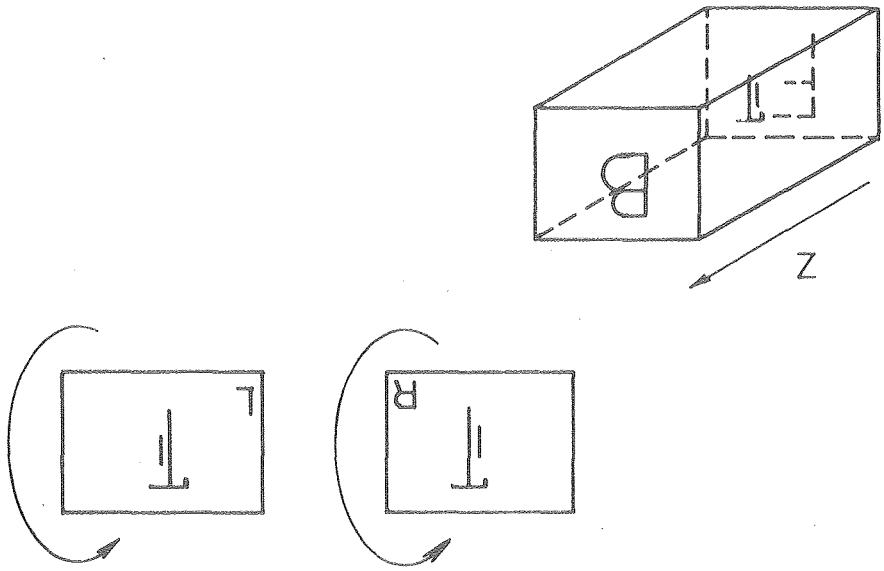


Fig. B

$$\alpha \Rightarrow \left\{ \begin{array}{l} Z \rightarrow -Z \\ \pi \text{ rotation about } Z \end{array} \right\}$$

XBL779-3788

if these photographs were viewed by the left and right eyes, respectively, in a stereo viewer, the stereo image created would properly reconstruct the three-dimensional relationships of T and 1. The same is true of stereo pairs obtained of any general three-dimensional structure.

Figure B depicts the two planar images (photographs) rotated by 180°. If the planar images were so rotated and then viewed with a stereo viewer, image "L" seen with the left, "R" by the right, the resultant stereo image would be as shown by the perspective box drawn in the upper right-hand corner of the figure. The contents of the box (T and 1) would now appear upside down, and 1 in back of T. The same transformation would occur for any structure embedded in the box, and this is indicated by the rotating and switching of the labels "F" and "B" on the front and back surfaces of the perspective box. This transformation has been labeled alpha.

The perspective box in Figure C demonstrates the resultant stereo image perceived if the two planar images were shifted left for right in the stereo viewer. Again, front and back are switched in the stereo image, but there is no rotation. This transformation has been labeled beta.

The perspective box in Figure D represents the stereo image perceived if the planar images are rotated by 180° and then shifted left for right in the stereo viewer. This transformation is labeled gamma, and is a combination of alpha and beta. In the resultant stereo image, the positions of front

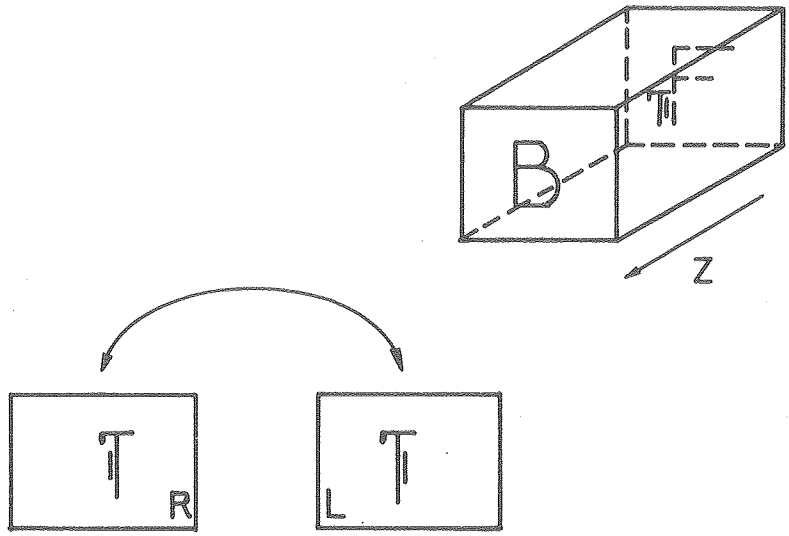
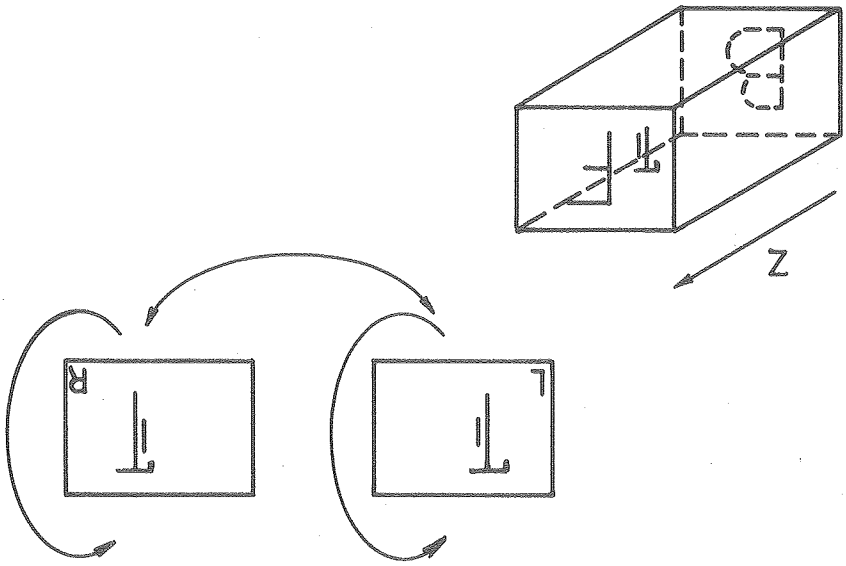


Fig. C

$\beta \Rightarrow \{Z \rightarrow -Z\}$



$\gamma = \alpha\beta = \beta\alpha \Rightarrow \{\pi \text{ rotation about } Z\}$

Fig. D

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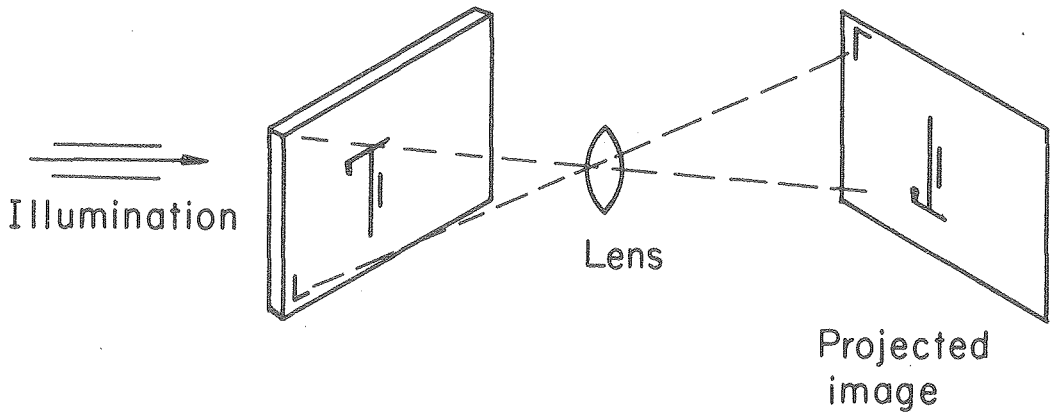
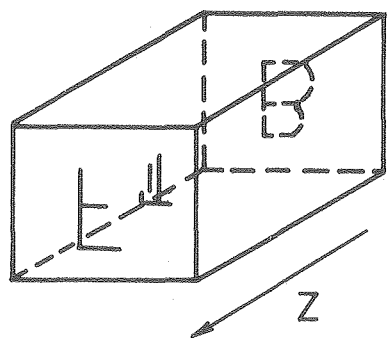
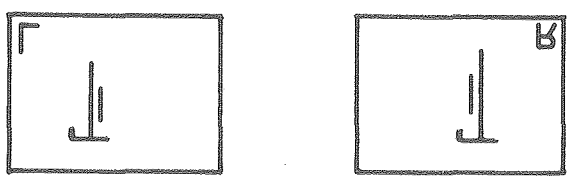


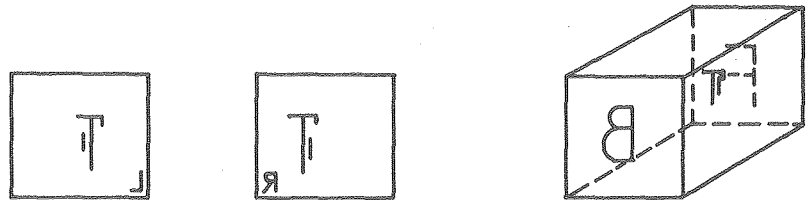




$\delta \Rightarrow \left\{ \begin{array}{l} \text{Left-right exchange (mirror)} \\ \pi \text{ rotation about } Z \end{array} \right\}$

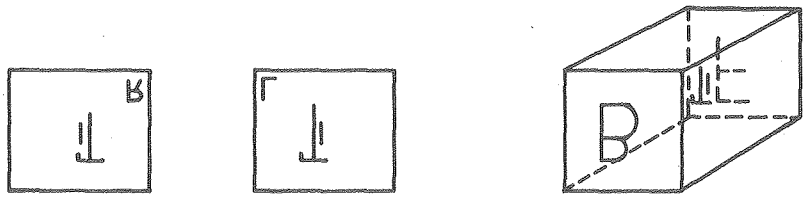
Fig. E

XBL779-3789



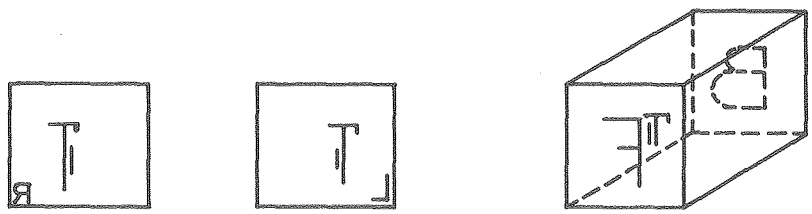
$\theta = \alpha\delta = \delta\alpha \Rightarrow \left\{ \begin{array}{l} \text{Left-right exchange (mirror)} \\ Z \longrightarrow -Z \end{array} \right\}$

Fig. F



$\lambda = \beta\delta = \delta\beta \Rightarrow \left\{ \begin{array}{l} \text{Left-right exchange (mirror)} \\ \pi \text{ rotation about } Z \\ Z \longrightarrow -Z \end{array} \right\}$

Fig. G



$\mu = \gamma\delta = \delta\gamma \Rightarrow \left\{ \text{Left-right exchange (mirror)} \right\}$

Fig. H

	1	α	β	γ	δ	θ	λ	μ
1	1	α	β	γ	δ	θ	λ	μ
α	α	1	γ	β	θ	δ	μ	λ
β	β	γ	1	α	λ	μ	δ	θ
γ	γ	β	α	1	μ	λ	θ	δ
δ	δ	θ	λ	μ	1	α	β	γ
θ	θ	δ	μ	λ	α	1	γ	β
λ	λ	μ	δ	θ	β	γ	1	α
μ	μ	λ	θ	δ	γ	β	α	1

Fig. I

XBL779-3791

and back are maintained and the contents of the box appear to be upside down.

Figure E demonstrates how a different set of distortions can arise. This set of conditions occurs when a negative is printed using an enlarger, or when the negatives are projected in our stereoscopic projection machine for the deriving of contours. Please note that the relative orientations of negative, lens and projected image are distorted in the top of Figure E. In practice, the planes of the negative and projected image are parallel and lie perpendicular to the axis of the lens. The "exploded" view presented at the top of Figure E distort these relationships in order to avoid a depiction which, though "correct," would require the reader to view the figure as if from "behind" the plane of the projected image or of the negative. The representation presented in Figure E is as if the arrangement of negative, lens, and projected image were opened, like the pages of a book, toward the viewer.

When a negative is projected with the emulsion side toward the lens (the standard orientation), the projected image undergoes a mirror inversion, as depicted in Figure E. If the so projected images of our stereo pairs are now viewed with a stereo viewer in the orientation depicted at the bottom of Figure E, the stereo image perceived will be as depicted in the perspective box. Front and back will remain invariant, but the image will appear upside down. More importantly, the

0 0 0 4 6 0 7 3 6 7

contents of the box will undergo a mirror inversion with left switching for right. Note that the figure R has become R. This will be true of any structure imbedded in the box, and this is represented by the "backwards" "R" and "B" on the front and back surfaces of the box. This transformation has been labeled delta.

Figures F, G, and H illustrate the results of combining the operation delta with the operations alpha, beta, and gamma (that is, rotating and/or switching the projected left and right images before placing them in the stereo viewer).

Finally, Figure I shows the multiplication table of the group of operations described above. The operations do form a mathematical group, and multiplication of one operation by another is defined as applying the first transformation to the stereo pair and then the second. Actually, the order of application is immaterial since the group is abelian.

To use the multiplication table, one looks down the left side of the table to find the first operation and then reads across that row to the column headed by the second operation. The number "1" represents the unity or null transformation. All of the operations are self inverses (applying any transformation twice restores the original relationships) and so the diagonal of the multiplication table is composed of "1"s. Since the group is abelian, the multiplication table is symmetric about the diagonal.

This section has been included because the author has

never seen this information presented in any other place. Since almost all the rearrangements are likely to occur when working with stereo pairs, the systematic classification of the information should prove valuable. For example, it is convenient to know which operations produce an exchange of back for front since sometimes it is easier to see relationships within a structure if the structure can be viewed from "front" and "back." Also, when developing reconstructions using serial sections, it is very important to keep front and back sorted out. Finally, the multiplication table of operations will be of great assistance when multiple transformations are applied to stereo pairs. This can occur when electron microscope stereo pairs of negatives are printed, the prints photographed to make slides, and the slides projected, perhaps upside down or backwards in the slide projector.

There is an additional caveat regarding the magnetic lenses of the electron microscope. These lenses can and do produce rotations and inversions of the images. Before we could begin reconstructions using stereo pairs of serial sections we had to determine what effect the microscope's lenses were producing.

In order to guarantee proper designation of the tops and bottoms (front and back) of serial sections, stereo pairs were obtained of an autoradiography sample. The photographic grain has a known relationship (on top of) to the biological section. Alternatively, a formvar film with gold colloid

0 0 0 0 4 6 0 7 3 6 8

particles or polystyrene spheres could be used as well.

No effort was made to determine whether the microscope lenses produced a mirror inversion since "handedness" is irrelevant to our reconstruction problem. Obviously, investigations of helical structures would require a determination of inversion. Electron microscope lens inversion could also be a source of confusion if stereo pairs of the same region were taken at different magnifications.

Appendix C

DESCRIPTION OF COMPUTER PROGRAMS

The computer programs written to produce stereo perspective views of our reconstructions are stored on PSS (Program Storage System) Library Bauert, subset TRYFH. The two main routines used in the program, INIT3D and DANDR (Wright, 1973) are both derived from the NCAR (National Center for Atmospheric Research) routines stored on PSS Library IGSNCAR, subset IGNCULB. The NCAR package was adapted to the Lawrence Berkeley Laboratory computer center operating system by William Johnston (1975). The routine INIT3D was used essentially as it was, while the routine DANDR was extensively rewritten to meet our particular needs. What follows is a general description of the functions of each of the routines in the package and more detailed explanations of the working of routine DANDR which actually draws the pictures.

Program REDT

This program is the main calling routine in the program. In it, one specifies a title for the graphics produced, specifies the number of contour planes to be drawn, and specifies also the size of the arrays in which the contours are embedded. These arrays may be up to 200 × 200, though commonly we have used arrays of the order of 60 × 60 to 80 × 80. In this routine too are specifications for the hidden line remover

which can be called in. Finally this routine states the viewpoints for the stereo pairs and then calls the routines to perform their functions.

Subroutine INIT3D

This routine is from the IGSNCAR package and is left as it was found on the library with only a few changes to allow large core storage for the contour data. Its function is to produce a two-dimensional projection, from a viewpoint specified in program REDT of a rectangular volume with maximum coordinates (n,n,n). This routine is called once for each of the two stereo views produced.

Subroutine DANDR

Once the previous routine has established the two-dimensional coordinates of the projection of the three-dimensional box, DANDR uses the information to draw the features of the embedded structure. The logic of this routine has been rewritten to conform to our particular needs, and Figure A demonstrates the workings of this routine.

DANDR looks at an input array, such as the 5 × 5 array presented in Figure A-1, four elements at a time. That is, it will look at the subarray composed of elements 1, 2, 3, and 4 in the figure. Since there is nothing in these elements, the routine then goes to look at the next four, which are array elements 3, 4, 5, and 6. Here the routine discovers

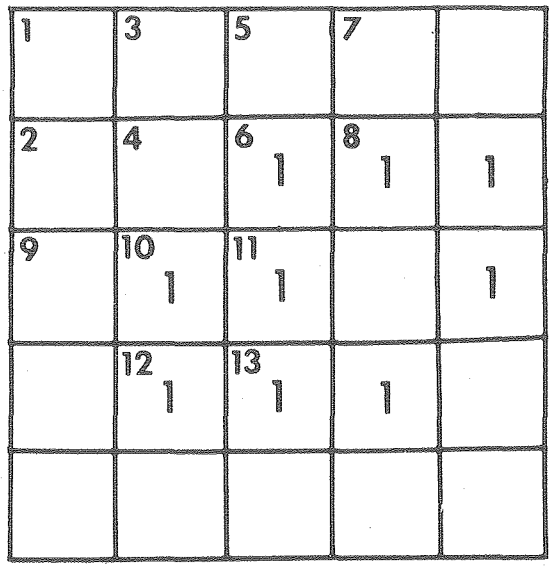


FIG. A-I

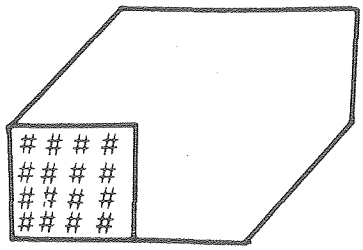


FIG. A-II

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a single element, and again draws nothing. A single element occurring anywhere in the block of four is skipped over by the routine.

The routine then looks at the subarray composed of 5, 6, 7, and 8. Here it finds "1"s in array element 6 and 8 and consequently draws a horizontal line connecting the two points. The program then goes on to finish out the top two rows and returns to look at the block composed of elements 2, 9, 4, and 10. The routine continues to sweep through the inputted array four elements at a time to determine what structures are there and what to draw.

Solid areas, such as that shown in the array block 10, 11, 12, and 13 in the figure, are treated differently than nonsolid elements. The routine does not draw all the possible lines involved here, but rather produces asterisks covering the area of subarrays. Array areas which are solidly packed are stored differently in the routine than lines, and at the option of the investigator they may be drawn or simply used in the hidden line remover to blank out images from subsequent planes without themselves being drawn.

Figure A-II demonstrates the picture that routine DANDR would draw of the array in Figure A-1.

Subroutines SARGE and DRAW

These two routines together are used to draw parts of the reconstruction from information supplied by DANDR. A

hidden line remover can be called into these routines to remove only those lines which are hidden in the part of the reconstruction being drawn at the time.

The other two components of our computer information processing system are two sets of routines stored on the PSS library BAUERT as subsets GPN and GRAF.

Program GPN

This set of routines is used to convert the output of the GRAF-Pen to coordinate data. The GRAF-Pen machine itself produces a string of binary bits which represent the coordinates of the pen on the table, but this string is not in a language the computer understands. Therefore the primary function of this routine is to serve as a translator from the GRAF-Pen code to ASCII.

A second feature of this routine, which makes it very convenient to use, is a scaling operation. One can produce any scale or orientation of coordinate data desired by simply first entering three GRAF-Pen spark inputs and then using the teletype keyboard to input the user coordinates for these points. The routine then solves the three sets of linear equations for the factors which convert further input to the desired coordinate data.

Program GRAF

This set of routines is used to edit data using the

Tectronix 4014 CRT terminal once the data have been digitalized with the GRAF-pen. The operation of this routine is best understood by examining Figure B, which is a copy of the display of the Tectronix screen in operation.

In the center of the screen is a square box in which lies the contour data to be edited. On the right-hand side of the box is a list of commands which can be selected. The routines produce a set of crosshairs on the screen which can be located anywhere on the screen by means of thumb wheels on the console. If the crosshairs are on one of the operations, that operation is selected. If the crosshairs are on a point within the square, it is assumed that that is where a point will either be added or subtracted, depending on the command previously chosen. The operation of each of the commands on the "menu" on the right of the picture is described below.

REWIND. This command causes the input file of contour data to be rewound.

PLUS. This command will cause points to be added to the data set whenever the crosshairs are placed at a location inside the box and an input command is given. Points can continually be added until a new command is specified.

MINUS. This command causes a point to be removed from the data set whenever the crosshairs are placed over it and the input command given. The entire display is redrawn after each removal of a point using this command. There is another command for removing data points which does not cause

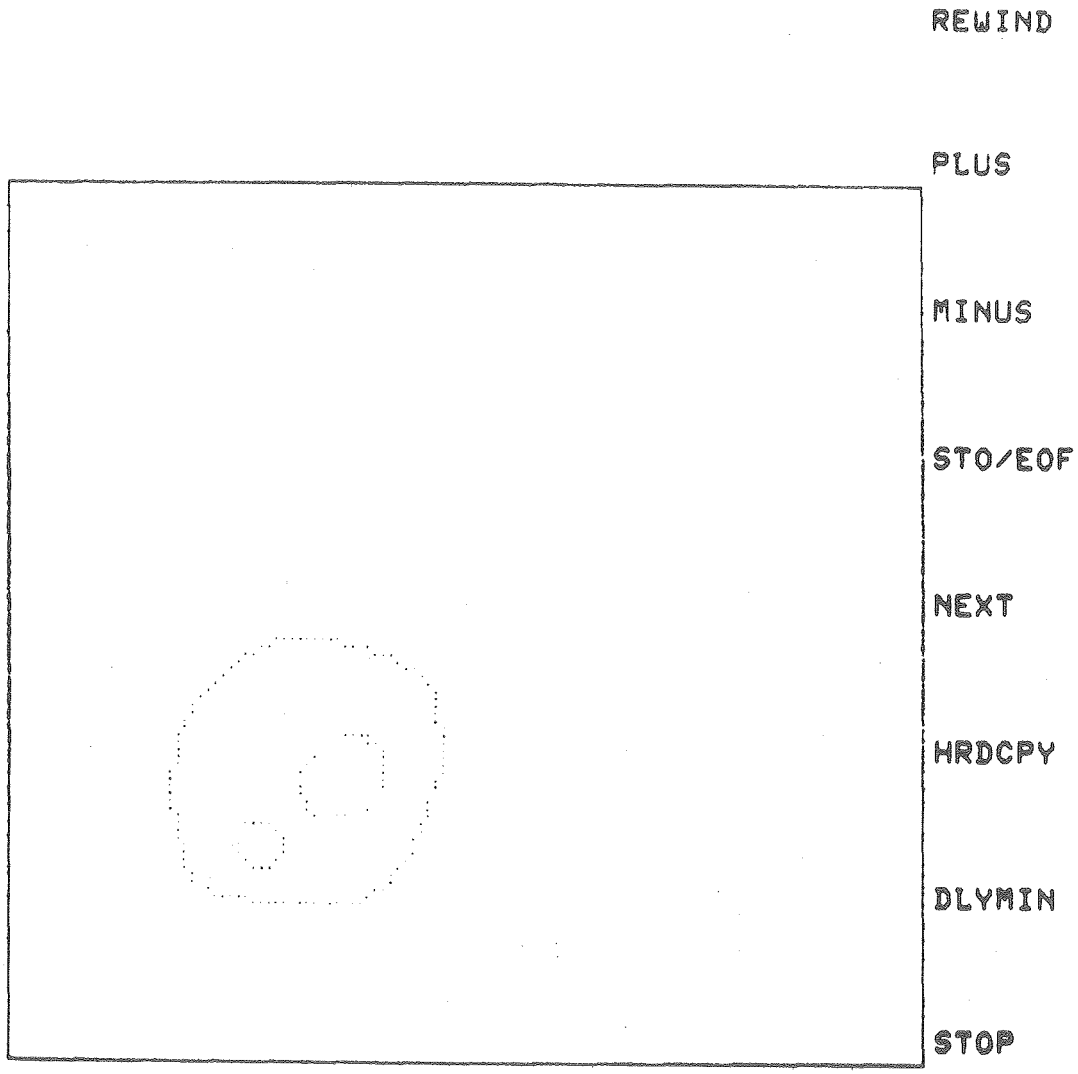

FIGURE B

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the redrawing and it is described below.

STO/EOF. This command causes the contour data displayed on the screen to be stored on the output file and places an "end of file" mark at the end of the data. The stereo reconstruction routine in the computer expects to see a data structure of contour planes separated by end of file marks.

NEXT. This command causes the next file of contour data to be displayed on the screen.

HRDCPY. This command causes the contour present on the screen to be drawn on both microfiche and on 35mm film. This is a very convenient way to keep a record of the edited contour data.

DLYMIN. This command causes points to be removed when the crosshairs are placed over them and the input signal given. However, the contour set is not redrawn with this command, and one does not see the points disappearing. To see which points have actually been removed, one must enter the "MINUS" command. Whenever many points are to be removed DLYMIN is the command of choice.

STOP. This command causes the routine to stop execution and to return control to the operator.

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