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A scanning electron microscopic examination of radiation-induced structural changes in potato tubers

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A SCANNING ELECTRON MICROSCOPIC EXAMINATION OF RADIATION-INDUCED STRUCTURAL CHANGES IN POTATO TUBERS

by

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A SCANNING ELECTRON MICROSCOPIC EXAMINATION OF RADIATION-INDUCED STRUCTURAL CHANGES IN POTATO TUBERS

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ABSTRACT

A SCANNING ELECTRON MICROSCOPIC EXAMINATION OF RADIATION-INDUCED STRUCTURAL CHANGES IN POTATO TUBERS

By Villa M. Mitchell

Both chemical and scanning electron microscopic (SEM) analyses were done on potato tubers ("Superior" variety) before and after a sprout inhibiting dose (10 Krad) of radiation. The chemical analyses were run to detect any changes in the reducing and nonreducing sugar content as well as any changes in the starch concentration. A colorimetric method using 3,5-dinitrosalicylic acid was chosen to estimate the change in sugar and starch content. The results obtained indicate that radiation caused an increase in the reducing and nonreducing sugar (sucrose) content and a decrease in the amount of starch.

SEM analyses of the number and size of the starch granules within tuber cells showed that radiation caused a significant decrease in both values. There did not appear to be a significant difference in either value when comparing the samples 3 days and 1 week after irradiation. Observation of isolated starch granules from a blended tuber did not show any obvious change in granule surface or size.

Irradiated sprout samples did not show a significant decrease in the number or size of the starch granules.

Microscopic examination of the wound periderm did show a decrease in formed cells as a probable result of irradiation. The surface of the irradiated periderm appeared to be smooth and flattened compared to the unirradiated periderm.

SEM analysis of the membrane surrounding the starch granules did not prove to be worthwhile. Transmitting electron microscopic examination of this fraction would be a more useful technique since it will show more detail about the internal structure.

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INTRODUCTION

Research on food irradiation has been going on for many years in the United States and other countries. Irradiation is used to sterilize and preserve foods. As of May 1990, the Food and Drug Administration (FDA) had approved irradiation of such products as pork, spices, fresh fruits and vegetables. Inhibition of growth, maturation and sprouting may be achieved by brief exposure to radiation (12,17,24,26,30).

Foods are irradiated by exposure to high-energy electrons from X-rays or electron beams or by exposure to gamma rays from cobalt-60 or cesium-137. In 1981, the Joint Expert Committee on Wholesomeness of Irradiated Foods, representing the World Health Organization, the International Atomic Energy Agency and the Food and Agriculture Organization of the United Nations, concluded that foods irradiated with an average dose of 1 Mrad or less should be approved without further testing. As of May 1990, the FDA had approved the following foods and dosages: pork, 100,000 RAD; fresh and frozen poultry products, 300,000 RAD; fresh fruits and vegetables, 100,000 RAD; enzyme preparations, 1,000,000 RAD; and dried and dehydrated vegetable substances, 3,000,000 RAD. The units used to measure the radiation energy absorbed are: 1,000 rads = 1 killorad

(Krad) = 10 Grays (Gy); 1,000,000 rads = 1Mrad = 10 kilo-Grays (KGy) (17,26).

Several studies have indicated that complex carbohydrates, such as starch, are depolymerized and degraded to simple sugars as a result of irradiation (1,3,7,8,10,14,16,18,19,27,28). Ananthaswamy et al. showed that irradiated wheat starch, amylose and amylopectin are more susceptible to enzyme actions than unirradiated controls. The products produced resemble those produced from α-amylolysis of starch (1). Rao and Vakil found that irradiation of legumes (green gram) reduced the total content of flatulence-causing oligosaccharides and caused significant production of reducing sugars by glucosidic cleavage of the higher sugar molecules (17). Nene et al. studied the effect of gamma radiation on red gram starch and found alterations of the physicochemical properties. The degradation products have been identified as low molecular weight dextrins (16). While nutritional value is not affected, the physical and rheological properties are altered. Irradiated starch is more water soluble and exhibits reduced swelling power and viscosity (16,19). MacArthur and D'Appolonia found that irradiation caused a reduction in starch pasting properties in three hard red spring wheat cultivars. Swelling power decreased, whereas solubility in water increased. No significant changes were observed when irradiated and unirradiated wheat starch

granules were compared by scanning electron microscopy (SEM) (14).

Gamma irradiation of potatoes at levels of 7-10 Krad causes irreversible sprout inhibition. For best results, it is necessary to allow wounds incurred during harvesting to heal before subjecting potatoes to irradiation. Several changes have been observed in irradiated potatoes as early as 2 hours following irradiation and for as long as 4 weeks. Such changes include: (1) increase in reducing and non-reducing sugars, (2) increase in respiration, (3) increase in activity of different enzymes, (4) decomposition of starch, (5) reduced synthesis of phytoalixins and phenolics and (6) reduced wound periderm formation (7,8,28).

An increase in respiration occurs immediately after irradiation (10 Krad). A maximum respiration rate is reached within 24 hours and returns to normal within 1 to 2 weeks. The increased respiration may be due to mobilization of reducing sugars from stored starch. This is supported by the 25% increase in starch phosphorylase activity within 2 hours after irradiation. Phospho-glucomutase activity was also observed to increase. Maximum sucrose content occurred in 5 to 10 days after irradiation and was thought to be due to an increase in phosphorylase activity (11,27). Invertase activity was found to be about 50% of that of control tubers (27).

It is widely known that the sucrose content increases in potato tubers stored at low temperatures (10,18,27,29). Two explanations have been offered to account for this observation. Workman et al. proposed that a change in the membranes surrounding the starch granules accounts for this increase in sucrose. Isherwood reported that electron micrographs (TEM) of cold storage potato tubers showed the starch granules to still be surrounded by a double membrane (amyloplast membrane). They did find, however, that potatoes that had sweetened due to senescence contained starch granules with disintegrating amyloplast membranes. They concluded that if the membranes remain during storage, that at some stage in the interconversion the carbohydrate intermediates must be transported across this membrane. They studied changes in phosphate esters and properties of key enzymes such as ADP glucose pyrophosphorylase, phosphorylase and sucrose phosphate synthetase. No evidence can be found to indicate that any one of these enzymes is responsible for the increase in sucrose concentration. Comparison of the hexose phosphates; glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate showed that the relative proportions remained largely unchanged. All of their studies on the glycolytic enzymes indicated that the controlling factor in the sucrose synthesis must be outside the glycolytic sequence. Sucrose synthesis probably occurs outside the amyloplasts since starch granules do not contain

any appreciable sucrose inside the amyloplasts. Because sucrose seems to be formed from starch during storage at low temperatures, they concluded that transport across the amyloplast membrane is necessary (11).

Isherwood suggests that movement of metabolites across the amyloplast membranes represents a balance between electron transport activated influx and passive efflux. He suggests that the lower the temperature the greater the efflux. This conclusion is supported by the fact that poisons, such as cyanide, act on the electron transport system and cause immediate sweetening (10).

Ohad et al. studied the effect of cold storage on the starch and sucrose content and on the amyloplast membranes. They found that storage at 4 °C caused the sucrose concentration to increase after 4 days reaching a maximum at 8 days and decreasing thereafter. The starch content decreased markedly at 4 °C. These changes were not observed at 25 °C. The amyloplast membranes began to disintegrate during cold storage. This disintegration could possibly allow enzymes involved in starch degradation to come in contact with their substrate (18).

Hayashi and Kawashima suggest that the degree of sucrose accumulation in irradiated potato tubers is 2 to 3 times larger than that caused by cold storage. They investigated the pathway of sucrose accumulation in irradiated potato tubers. The activities of phosphorylase,

UDP-glucose pyrophosphorylase, phosphoglucomutase, phosphoglucoisomerase, sucrose synthase and sucrose phosphate synthase and the respiratory rate were monitored. They reported the following results: (1) the sucrose content and the respiration rate increased, (2) the phosphorylase activity showed a rapid increase for 2 days and then returned to normal, (3) the UDP-glucose pyrophosphorylase activity increased slightly for 2 days but then dropped to a lower level than was observed for the unirradiated controls, (4) the phosphoglucomutase activity showed no significant increase, (5) the phosphoglucoisomerase showed no increased activity, (6) the sucrose synthase showed no immediate increase in activity but later showed a rapid increase. A maximum activity was achieved in 3 days followed by a decrease but not to the level of the unirradiated controls. The sucrose synthase activity had not increased on the first day following irradiation but the sucrose content had already started to increase. Sucrose synthase activity was high while the sucrose content increased, (7) the sucrose phosphate synthase showed an immediate increase in activity reaching a maximum in 3 days. It later decreased to the level found in unirradiated tubers. Sucrose phosphate synthase activity was at a high level when the sucrose concentration was increasing. This enzyme catalyzes the synthesis of sucrose-6-phosphate which is converted to sucrose by sucrose phosphatase. The above

observations suggest that the UDP-glucose pyrophosphorylase may be involved in formation and breakdown of UDP-glucose. The high phosphorylase activity may account for the increase in respiration and increase in sucrose content. Sucrose synthase may be involved in the increase in sucrose synthesis as well as an increase in sucrose degradation (7,8).

From their studies no repression of glycolytic reactions or respiration is indicated. The authors concluded that the changes in enzyme activities contribute to the increase in sucrose, but they could not explain the later decrease in sucrose concentration while the enzyme activities were still high. Rao and Vakil proposed that in green gram the enzymes are not stimulated or inactivated by radiation. They proposed that radiation forms weak points in the chain molecules of sugars and that the hydrolytic enzymes preferentially attack these points (19). The concentration of radiation-induced breakdown products increase as the water content increases until an equilibrium in water content is reached (16). Nene et al. found that α-amylolysis liberated more maltose from irradiated (1 Mrad) red gram flour and starch samples than from unirradiated controls (16). Potato and wheat starch exhibited rapid breakdown releasing more reducing sugars.

Also reported to be affected by irradiation was the formation of the wound periderm and synthesis of chlorophyll

and solanine. When under stress, potatoes form a periderm. In unirradiated potato tubers this periderm is dry and loosely bound to the cortex. In irradiated (10 Krad) tubers the periderm is moist and tightly bound to the cortex. Ghanekar et al. found that levels of phenolics, free lipids and suberin were lower in irradiated potatoes than in unirradiated controls. The irradiated tubers were observed to be more susceptible to rot than were controls. The lowered resistance to rot may be due to reduced synthesis of resistance compounds such as phytoalexins and phenolics (5).

The use of SEM to study structural changes brought about by different types of food processing is finding increased applications (2,3). One limitation of SEM analysis of different foods appears to be sample preparation. Many foods are of low water content and are processed by air drying. These samples present very little problem in SEM studies. Foods that are largely water must be prepared by different techniques if their structure is to be preserved. Air drying causes significant damage to the sample due to pressure differences between the gas and liquid sides of the meniscus. A typical procedure for highly hydrated samples includes: (1) chemical fixation, (2) chemical dehydration, (3) critical point drying and (4) sputter coating (4,6,9,25).

The purpose of chemical fixation is: (1) to preserve the structure of the cells, (2) cessation of all cellular

activities and (3) to prepare the sample for further treatments such as exposure to the electron beam. Many researchers follow a two-step sequence in chemical fixation. The first step is fixation of proteins with an aldehyde fixative. This is often followed by a post-fixation of unsaturated lipids in osmium tetroxide. Glutaraldehyde is useful for preserving the fine structure of a cell by forming both intra and intermolecular bonds with several amino acid residues in the side chains of proteins. Osmium tetroxide oxidizes unsaturated lipids forming a diester product that is stable enough to resist dehydration. Saturated lipids are changed somewhat but are still susceptible to extraction by the dehydrating solvent. Carbohydrates, such as glycogen and starch, are only partially extracted after glutaraldehyde fixation. The relative insolubility of these two compounds in the dehydrating solvents also prevents further extraction. Many researchers feel that this post-fixation with osmium tetroxide is unnecessary for plant tissue that is to be studied by SEM (4,9,15,19,23).

Other factors that need consideration are: (1) pH, (2) temperature, (3) concentration and osmolality of the fixative and (4) duration of fixation. The pH is maintained as close as possible to the physiological value of the specimen. For plant tissue this is usually a pH range of 6.8 - 7.1. Phosphate buffers are often used to carry the

fixative molecules into the cells. Phosphate buffers have the advantage of being inexpensive, nontoxic, similar to natural tissue fluids and compatible with both primary and secondary fixatives (4,9).

A fixation temperature of 0-4 °C has been widely used. At these low temperatures autolytic enzymes are less active and extraction of cellular components is reduced. Other researchers report that a temperature of 25-35 °C is better than lower temperatures. Regardless of the temperature used, there seems to be general agreement that the sample and fixative should be near the same temperature. Both penetration and fixation rates are lower at the low temperature range (4,9,15).

A concentration range of 1.5-4% glutaraldehyde is considered suitable for most plant and animal tissues. An osmolality of 230-685 milliosmoles is commonly used with the range of 400-450 milliosmoles being preferred. The optimum duration of fixation is difficult to the determine. The fixative chosen, size of the sample and temperature range are all factors that need to be considered. Generally with 1.5-4% glutaraldehyde a fixation time of 2-4 hours is acceptable for small tissue samples (4,9,15).

Chemical dehydration involves replacement of all free water in a sample with an organic solvent that is miscible with the transitional fluid that is to be used in critical-point drying (CPD). This dehydration process inevitably

causes shrinkage and extraction of cellular components. Dehydration is commonly carried out with a graded series (e.g., 30,50,70,85,95,100,100%) of either ethanol or acetone in water. The degree of shrinkage is controlled by both the use of a graded series of solvent and limiting the exposure time. Exposure times of 5-10 minutes are considered adequate for each solvent concentration. The exposure time chosen is based on sample size. The time should be as short as possible to minimize shrinkage and extraction. Acetone is not generally recommended for plant tissue due to extreme extraction of lipids. Lipid extraction is reported to be far less with ethanol (4,15).

The goal of critical point drying is to dry the sample preserving its size and shape without subjecting it to interfacial tension. Evaporation of water from a specimen subjects it to high surface tension which distorts the surface detail. Once the sample has been chemically dehydrated with acetone or ethanol it can be infiltrated with an appropriate transitional fluid such as CO_2. The temperature is then raised above the critical temperature of the transitional fluid which causes the fluid to expand and vaporize. As this occurs the liquid becomes less dense and the gas becomes increasingly dense until the densities are identical. At this point the miniscus disappears and the surface tension is zero. If the temperature is maintained above the critical temperature the gas can be released from

the critical-point dryer. In essence the sample is dried without being damaged by a change in surface tension (4,15).

Biological samples have a composition of low atomic weight and absorb electron beams. To overcome this problem the sample can either be impregnated with heavy metal salts or coated with a thin metal film. A typical procedure is to sputter coat the sample with a film of gold or palladium. In this process metal ions are ejected from a target metal, such as gold, by plasmas of argon. A low vacuum is used which ensures that a continuous film is deposited on the sample (4,15).

Because only one reference was found dealing with SEM analysis of starch after irradiation (14), we decided that examination of potato starch as well as tuber sections would be worthwhile. Several authors have reported an increase in reducing sugars and a decrease in starch concentration following irradiation (7,8,11,27). Our goal in this study was to correlate the change in carbohydrate content with structural changes in the starch granules, membranes or tuber sections. In addition structural changes in the wound periderm, from irradiated potato tubers, were anticipated. Throughout this study, results obtained from irradiated samples were compared to those from the nonirradiated controls.

The procedures used to determine reducing sugar and starch content were adaptations of those used by Lindsay

(13), Ohad (18) and Robyt et al. (21). The procedures used to prepare samples for SEM analysis were taken from general recommendations by several authors (2,3,4,6,20,23,25).

CHAPTER 2

MATERIALS AND METHODS

This discussion is divided into two major categories: (1) chemical analysis of carbohydrates and (2) SEM examination of starch and potato tuber sections. Both irradiated and nonirradiated tubers ("Superior" variety) were used in each study. The potatoes were stored at 25 °C in air.

Irradiation

Potato tubers were exposed to a ^{137}Cs source of gamma radiation in a Mark I cesium irradiation (J. L. Shepherd and Associates) having an influx of 3.3 Gy/min. The exposure time was 33 min which gave a total exposure of 10 Krad.

Sample Preparation for Chemical Analysis

Both nonirradiated and irradiated potato tubers were sliced and oven dried at 50 °C for 4 days. The average weight of the nonirradiated potatoes was 156 g and that of the irradiated potatoes was 141 g. The irradiated tubers were divided into 2 portions. The first portion was dried 3 days after irradiation while the remaining portion was dried 1 week after irradiation. The dried tubers were ground to a fine powder and stored in sample vials until

needed. In the following discussions the irradiated samples will be referred to as 3 day and 1 week samples.

3,5-Dinitrosalicylic Acid Reagent

The 3,5-dinitrosalicylic acid reagent was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid in 20 mL of 2 M NaOH and 50 mL of water. Thirty grams of Rochelle salt (Na-K-tartrate) was added, and the solution was made to a final volume of 100 mL. This reagent was protected from CO_2 and light (13).

Calibration Curve - 3,5-Dinitrosalicylic Acid Method

Standard glucose solutions (0.2% w/v) were used to prepare solutions for the calibration curve. Dilutions were made, and the solutions were mixed with 2 mL of the 3,5-dinitrosalicylic acid reagent. The samples were heated for 4 min in a boiling water bath, cooled to room temperature and mixed with 2 mL of water. The absorbance of each solution was read at 570 nm, on a Perkin-Elmer Lambda 3B UV/Vis Spectrophotometer, and was used to prepare the calibration curve (Figure 1).

Glucose Oxidase Reagent

The glucose oxidase reagent was prepared by dissolving 30 mg of glucose oxidase (Sigma, Type VII-5), 3 mg of horseradish peroxidase (Sigma, Type VI-A) and 10 mg of ortho-dianisidine hydrochloride in 100 mL of tris/phosphate/glycerol buffer, pH 7.0. The buffer consists of 36.3 g of

tris and 56.5 g of sodium dihydrogen phosphate dihydrate dissolved in 400 mL of water and 400 mL of glycerol. The solution was diluted to a final volume of 1 L and refrigerated in a brown bottle (21).

Calibration Curve - Glucose Oxidase Method

A D-glucose solution (1×10^{-3} g/mL) was used to prepare solutions for the calibration curve (Figure 2). The solutions ranged in concentration from 10 mg/mL to 100 mg/mL. One milliliter of these solutions was added to 2.0 mL of the glucose oxidase reagent. The solution was incubated at 37 °C for 30 min and then 4.0 mL of 5 N HCl was added. The absorbance was measured at 525 nm.

Phosphate Buffer (0.1 M)

A 0.1 M phosphate buffer, pH 7.0, was prepared by mixing 202.5 mL of a 0.2 m sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) buffer and 47.5 mL of a 0.2 M sodium monohydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) buffer and diluting to a total volume of 500 mL.

The 0.2 M sodium phosphate dibasic buffer was prepared by dissolving 13.8 g of $NaH_2PO_4 \cdot H_2O$ in 500 mL of water. The 0.2 M sodium phosphate monobasic buffer was prepared by dissolving 35.0 g of $Na_2HPO_4 \cdot 12H_2O$ in 500 mL of water.

Extraction of Total Reducing Sugars and Analysis with 3,5-Dinitrosalicylic Acid

Three samples weighing 25.0 mg each were extracted

4 times with hot ethanol (80% v/v). A volume of 10 mL was used for each extraction. Each time the suspensions were centrifuged (3,000 g, 5 min). The supernatant liquids were collected for each sample, and the ethanol was evaporated. One milliliter of water and 2 mL of the 3,5-dinitrosalicylic acid reagent were added to each sample. The samples were heated in a boiling water bath for 10 min and were then cooled to room temperature. Two milliliters of water was added, and the samples were centrifuged to clarify the solutions. The solutions were then decanted into colorimeter cuvettes with a path length of 1 cm, and the absorbance was read at 570 nm.

Analysis of Total Reducing Sugars with 3,5-Dinitrosalicylic Acid (Dry Powder Method)

An alternate procedure was used in which the powdered potato sample (60 mg) was mixed with 6 mL of the 3,5-dinitrosalicylic acid reagent and 3 mL of water. The mixture was heated for 10 min in a boiling water bath and then cooled to room temperature. Six milliliters of water was added, and the samples were centrifuged (3,000 g, 5 min). The clear supernatant solutions were used to measure absorbance at 570 nm. In order to obtain enough clear supernatant liquid, it was necessary to triple the volumes used for the calibration solutions.

3,5-Dinitrosalicylic Acid Analysis of Total Reducing Sugars After Sucrose Inversion

Dried potato powder (0.6 g) samples were mixed with 3 mL of water and 0.2 mL of invertase (Candida Utilis, Grade X, Sigma) solution. This gave a final invertase concentration of 0.33 mg/mL. The samples were incubated at 37 °C for 2 hr. The solutions were then mixed with 6 mL of the 3,5-dinitrosalicylic acid reagent. After being heated for 10 min in a boiling water bath the solutions were cooled to room temperature and mixed with 5.8 mL of water. The samples were centrifuged (3,000 g, 5 min), and the absorbance of the supernatant liquids was read at 570 nm.

3,5-Dinitrosalicylic Acid Analysis of Starch

Dried potato powder (0.1 g) from nonirradiated, 3 day and 1 week irradiated samples was extracted 4 times with hot ethanol (10mL) and centrifuged (3,000 g, 5 min) after each extraction. The residues were mixed with 10 mL of water and were heated in a boiling water bath for 15 min to solubilize the starch. An α-amylase preparation (porcine pancreas, amylase, Sigma) was added to a final concentration of 15 mg/mL. The samples were incubated at 37 °C for 1 hr and were then tested for undigested starch with I_2-KI. The samples were centrifuged (3,000 g, 5 min) and 1 mL of the supernatant liquid from each sample was diluted to 10 mL with water. One milliliter of these solutions was added to 2 mL of the 3,5-dinitrosalicylic acid reagent. These

solutions were heated in a boiling water bath for 10 min and were then cooled to room temperature. Two milliliters of water was added, and the absorbance of each solution was read at 570 nm.

Glucose Oxidase Analysis of Starch

Dried potato powder (0.1 g) from nonirradiated, 3 day and 1 week irradiated samples was extracted 4 times with hot ethanol (10 mL) and centrifuged (3,000 g, 5 min) after each extraction. The residues were mixed with 10 mL of water and were heated in a boiling water bath to solubilize the starch. An α-amylase preparation was added to final concentration of 15 mg/mL. The samples were incubated at 37 °C for 1 hr and then were tested for undigested starch with I_2-KI. Aliquots (0.25 mL) of these samples were diluted to 10 mL total volume. One milliliter of these solutions was added to 2 mL of the glucose oxidase reagent (Aspergillus Niger, Type VII-5, Sigma). The samples were incubated for 30 min at 37 °C. Four milliliters of 5 N HCl was added, and the absorbance was read at 525 nm. A blank was used to zero the instrument. The blank was prepared the same way as the sample solutions except that 1 mL of water was used instead of the glucose solution.

SEM ANALYSIS

All samples, with the exception of the starch granules, were dried in a Polaron E3000 critical point dryer and sputter coated in an ISI 5400 high resolution sputter coater. All samples were examined in an International Scientific Instrument Co. electron microscope (ISI-SX-30) operating at 15 KV.

Potato Tuber Sections

Sections of tubers (irradiated and nonirradiated) were taken prior to oven drying. The sections from irradiated tubers were taken 3 days and 1 week after irradiation. The samples were prepared as follows: (1) fixed in glutaraldehyde (3% in 0.1 M phosphate buffer) for 2 hr, (2) washed 2 times with 0.1 M phosphate buffer, (3) washed 2 times with water, (4) dehydrated in a graded series of ethanol (30, 50, 70, 85, 90, 95, 100, 100%) for 10 min, (5) critical point dried and (6) sputter coated for 30 sec with gold.

Periderm

Both irradiated and nonirradiated tubers were bruised and left for 4 days for the wound periderm to form. The periderms were isolated, fixed, dehydrated, dried and coated as outlined above.

Starch

Irradiated (3 days and 1 week after irradiation) and

nonirradiated tubers were ground in a stainless steel Waring blender. Sodium dithionite (4 g/Kg mesh) was added, and the mesh was filtered through four layers of gauze. The mesh was resuspended in a large volume of 2.5 mM tris-chloride, pH 7.4, containing 7.5 mM NaCl and 0.5 mM B-mercaptoethanol. The mixture was refiltered, and the filtrates were combined and allowed to settle for several hours. The starch granules were washed 3 times with the buffer and were then broken in the blender by 50 strokes of 15 sec each. The starch mixture was centrifuged at 200 g, and the sediment was washed 3 times with the buffer. The supernatant fluids were combined and saved for isolation of the membrane fraction. The starch sediment was air dryed and then was dusted on a microscope stub that had been painted with silver paint. The starch samples were then sputter coated for 30 sec with gold.

Membrane Fraction

The membrane fraction connected with the starch granules was isolated from the supernatant fluids collected in the above procedure. After centrifugation (3,000 g, 5 min) to remove broken starch granules and cellular dibris, the membrane fraction was sedimented by centrifugation at 45,000 g in an International Equipment Co. model B-20 centrifuge.

The membrane fraction was: (1) fixed in 3% glutaraldehyde for 2 hr, (2) washed 2 times with 0.1 M

phosphate buffer, (3) washed 2 times with water, (4) dehydrated in a graded series of ethanol (30 - 100%), and (5) critical point dried in an aluminum foil holder that had pin-hole perforations. The dried sample was then dusted onto a stub that had been coated with epoxy. The membrane samples were then sputter coated for 30 sec with gold.

Sprout

Sprout sections from nonirradiated and irradiated (3 days after irradiation) tubers were taken at the same time after harvest. The irradiated sprouts had stopped growing, but the nonirradiated tuber sprouts were still growing. The sprout sections were fixed in glutaraldehyde (3%), washed with buffer, washed with water, dehydrated in ethanol (30 - 100%), critical point dried and sputter coated for 30 sec with gold.

SEM Analysis of Starch in Potato Tuber and Sprout Sections

At a magnification of 156X, sections from nonirradiated and irradiated (3 day and 1 week) tubers were examined for the following information: (1) the number of starch granules found in four separate 0.52mm^2 areas, (2) the area (μm^2) of the granules found within these regions, (3) the size (μm^2) of individual cells, (4), the number of cells with and without starch granules, and (5) the shape of the granules. The sprout sections, from before and after

irradiation, were examined at a magnification of 730X for the number, size and shape of starch granules found in nine areas 0.52mm^2. The data was examined by basic statistical analysis to see if there was any difference in the samples after irradiation.

CHAPTER 3

RESULTS AND DISCUSSION

Calibration Curve - 3,5-Dinitrosalicylic Acid Method

A calibration curve (Figure 1) for the range of 0.08 - 0.72 mg/mL was constructed from readings on a Perkin-Elmer Lambda 3B UV/Vis Spectrophotometer, at a wavelength of 570 nm. This concentration range was chosen to permit estimation of total reducing sugars before and after starch hydrolysis.

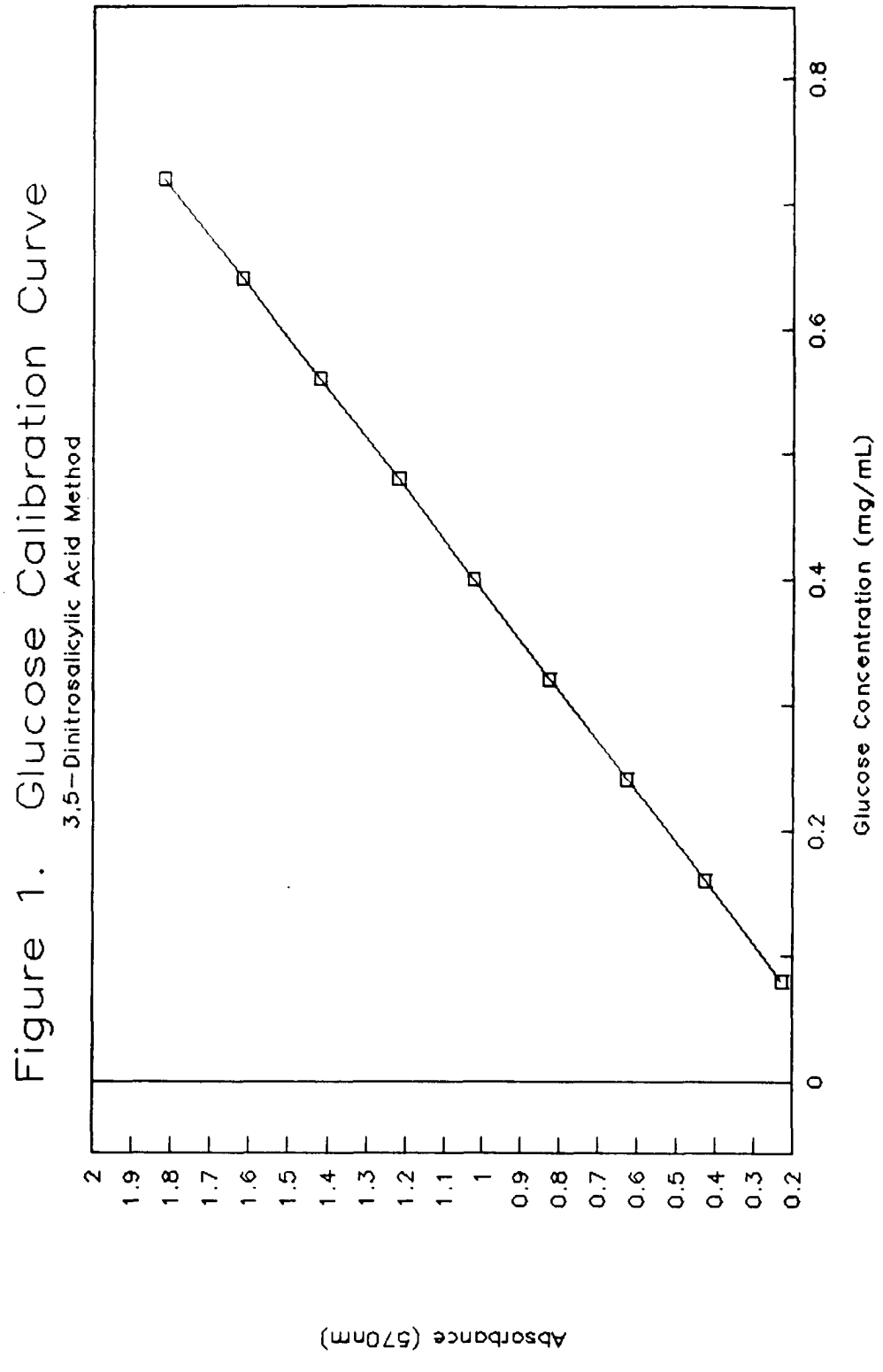
Extraction of Total Reducing Sugars and Analysis with 3,5-Dinitrosalicylic Acid

Determination of the total reducing sugar content in the ethanolic extracts from nonirradiated and irradiated (3 day and 1 week) tuber samples showed that the reducing sugar content remained unchanged after irradiation. The nonirradiated tuber was 0.472% dry wt., and the irradiated tuber (1 week) was 0.477% dry wt. (Table I).

Analysis of Total Reducing Sugars with 3,5-Dinitrosalicylic Acid (Dry Powder Method)

A comparison of the results from the ethanolic extraction method (Table I) with those from the dry powder method (Table II) indicated that there was no need to use the more lengthy extraction procedure. The two methods gave

Figure 1. Glucose Calibration Curve -
3,5-Dinitrosalicylic Acid Method



similar results. Solutions made using the dry powder gave higher percentages than did the extraction method. The average % dry wt. from the nonirradiated tubers was 0.544% compared to 0.540% for the 3 day tubers and 0.473% for the 1 week samples (Table II). There was a 12.9% decrease in total reducing sugar content during the week following irradiation.

Total Reducing Sugar Analysis with 3,5-Dinitrosalicylic Acid Following Sucrose Inversion

The nonreducing sugar (sucrose) content was determined by comparing the results before sucrose inversion (Table II) to those following sucrose inversion (Table III). After inversion the average total reducing sugar content (% dry wt.) was 1.18% for the nonirradiated tubers compared to 1.32% for the 3 day tubers and 1.46% for the 1 week sample. The total nonreducing sugar content was 0.325% for the nonirradiated tubers compared to 0.390% for the 3 day sample and 0.493% for the 1 week tubers. This was a 20% increase in total sucrose content during the 3 days following irradiation and a 52% increase over the 1 week period.

Analysis of Total Reducing Sugars with 3,5-Dinitrosalicylic Acid Following Starch Hydrolysis

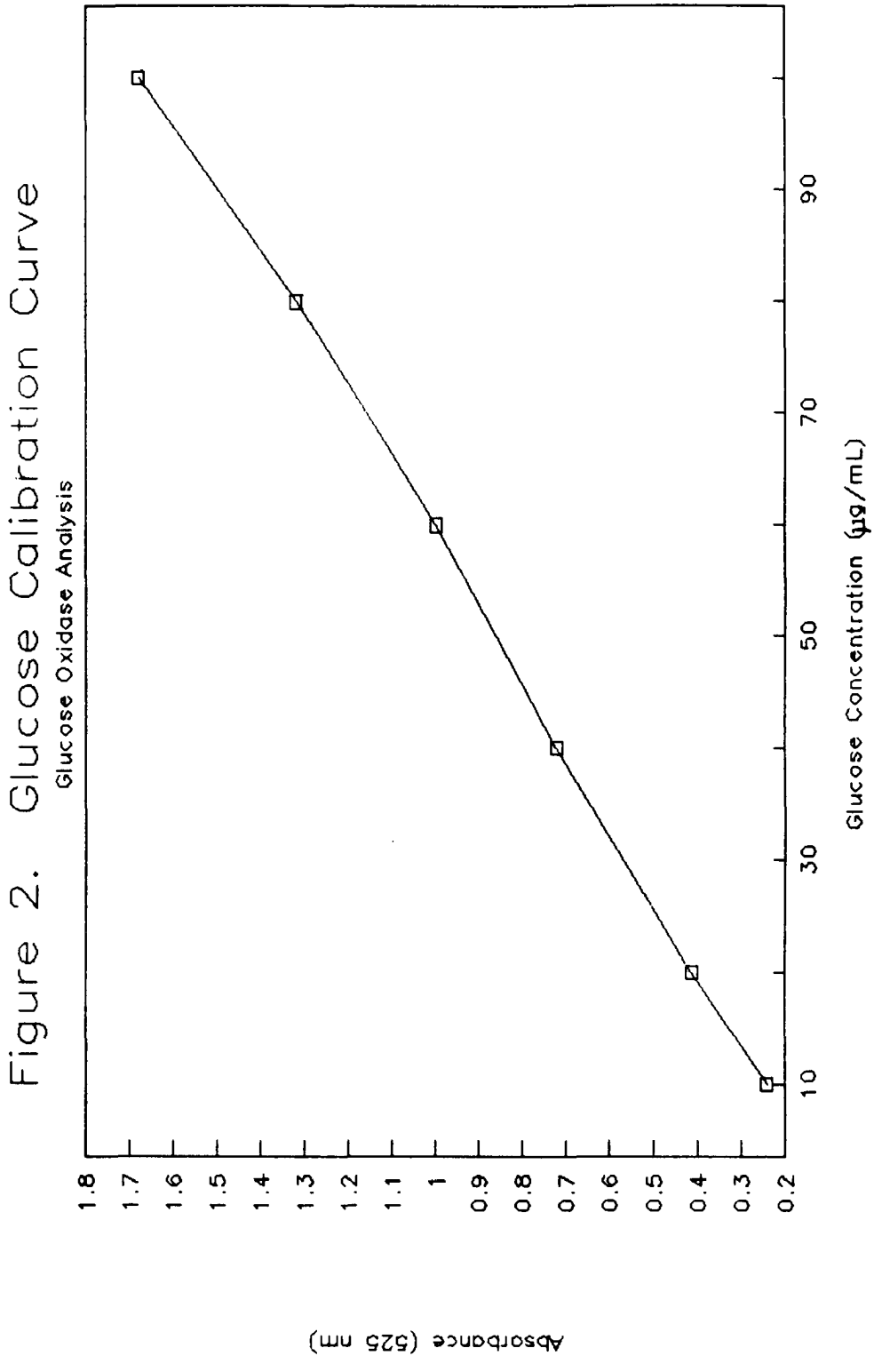
The total reducing sugar (glucose) content was determined after digestion of the starch with α-amylase.

The average glucose content (% dry wt.) decreased from 71.2% to 54.2% during the 3 days following irradiation and was at 48.5% after 1 week (Table IV). This was a 24% decrease for the 3 day period and a 46.8% decrease during the 1 week following irradiation.

Glucose Oxidase Analysis of Starch

This alternate method was run on one tuber from each of the three categories (nonirradiated, 3 day, 1 week) just for comparison. From a glucose calibration curve (Figure 2), the total glucose content was determined. The starch content (% dry wt.) decreased from 85.8% to 62.0% during the 3 days following irradiation and was at 45.0% after 1 week (Table V). When compared to the literature value of 76% starch (average value), the results appear to be high for the nonirradiated tuber. The 3,5-dinitrosalicylic acid was the method chosen for the remaining analyses.

Figure 2. Glucose Calibration Curve
Glucose Oxidase Method



SEM Analysis of Potato Tuber Sections

As can be seen from a comparison of the micrographs (Figures 3A,B,C) and from the statistical analysis (Table VI), there was a significant decrease in the number of starch granules during the week following irradiation. A comparison of the nonirradiated and the three day sample showed a significant decrease as did a comparison of the nonirradiated and the 1 week sample. There was not a significant decrease in the number of granules during the period from 3 days to 1 week. The area of the starch granules decreased significantly when the nonirradiated sample was compared to both the 3 day sample and the 1 week samples. There was, however, no significant change in the granule size from 3 days to 1 week. This decrease in granule size is not obvious from a comparison of the micrographs (Figures 3A,B,C), but the statistical analysis is indicative of this. The cell size did not significantly change after irradiation. The cells were irregularly shaped and therefore, the areas were not easily calculated. The values reported (Table VI) represent the product of the length times the width. The lengths and widths were measured at the longest and widest points for each cell.

The number of empty cells differed significantly when a comparison was made between the nonirradiated and 1 week sample. There was not a significant difference from 3 days to 1 week (Table VI).

Figure 3A. Nonirradiated Potato Tuber Section

magnification = 593X

58.9 mm = 100 μ

KV = 15

Figure 3B. Irradiated Potato Tuber Section (3 day sample)

magnification = 445X

44.7 mm = 100 μ

KV = 15

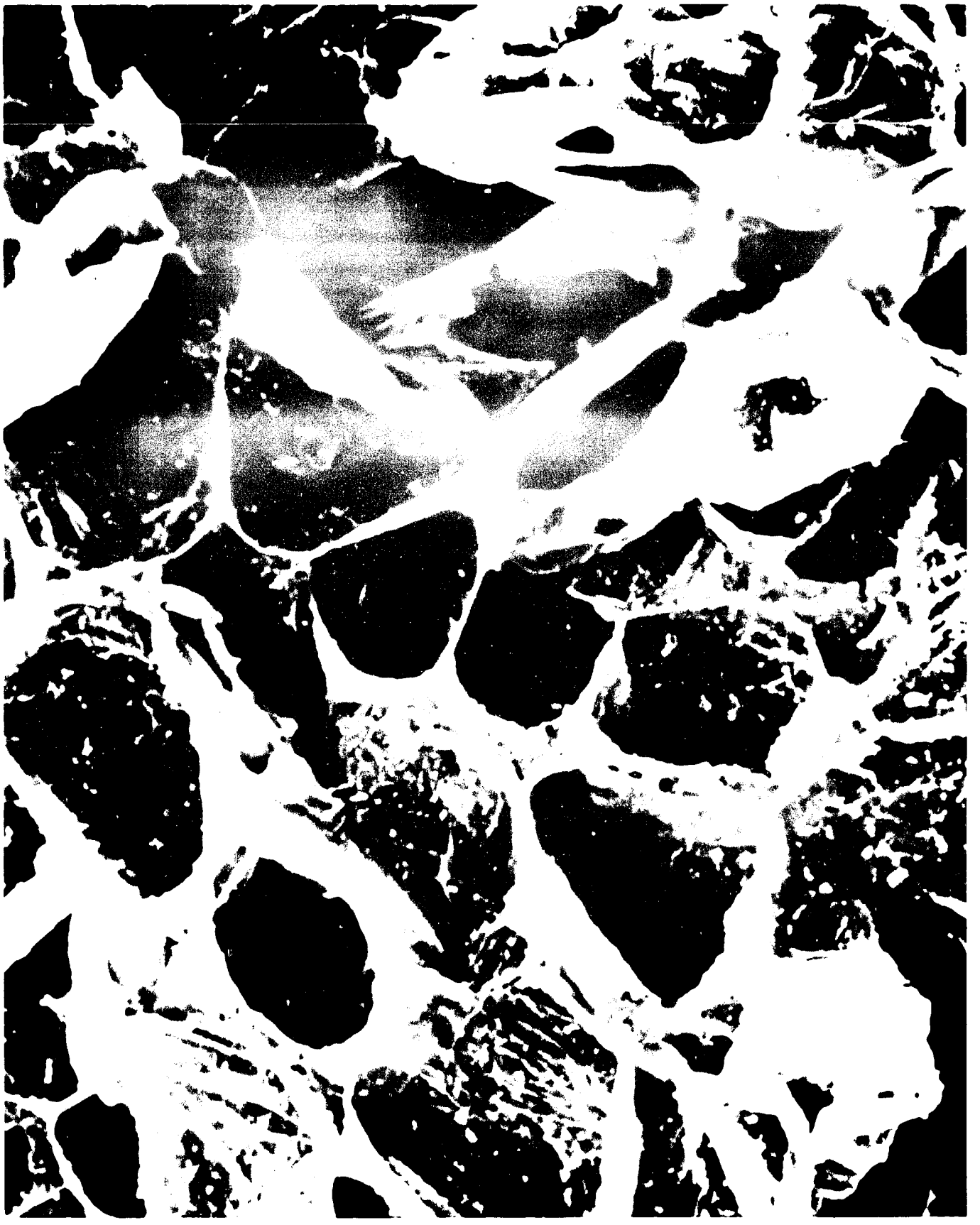


Figure 3C. Irradiated Potato Tuber Section
(1 week sample)

magnification = 556X

6.01 mm = 10 μ

KV = 15

The starch granules appear to have different shapes. A comparison of the granule shapes from the various samples showed that for the 1 week sample 16.7% were round, 50% were elliptical and 33.3% were irregularly shaped. The three day and nonirradiated samples gave the following results: 67.7% round, 16.1% elliptical, 12.9% irregular; 45.2% round, 41.1% elliptical and 13.7% irregular respectively. There was no obvious trend as far as a change from round to elliptical or irregular.

SEM Analysis of Starch Granules

A comparison of the micrographs (Figures 4A,B,C) indicates that exposure to radiation did not cause a large difference in the appearance of the starch granules. This was also the observation made by MacArthur and D'Appolonia when they compared starch granules from irradiated and nonirradiated wheat (14). A visual comparison of the samples did not reveal any obvious difference in size as was reported for the granules within the tuber cells. This may be due to the fact that the tuber samples were taken close to the surface where the radiation may have had a greater effect. The isolated granules were randomly taken from a whole potato that had been blended. An analysis of isolated starch grains and tuber sections from various regions within the potato tuber will be examined in a later study.

Figure 4A. Starch Granules (nonirradiated sample)

magnification = 1920X

10.0 mm = 14 μ

KV = 15

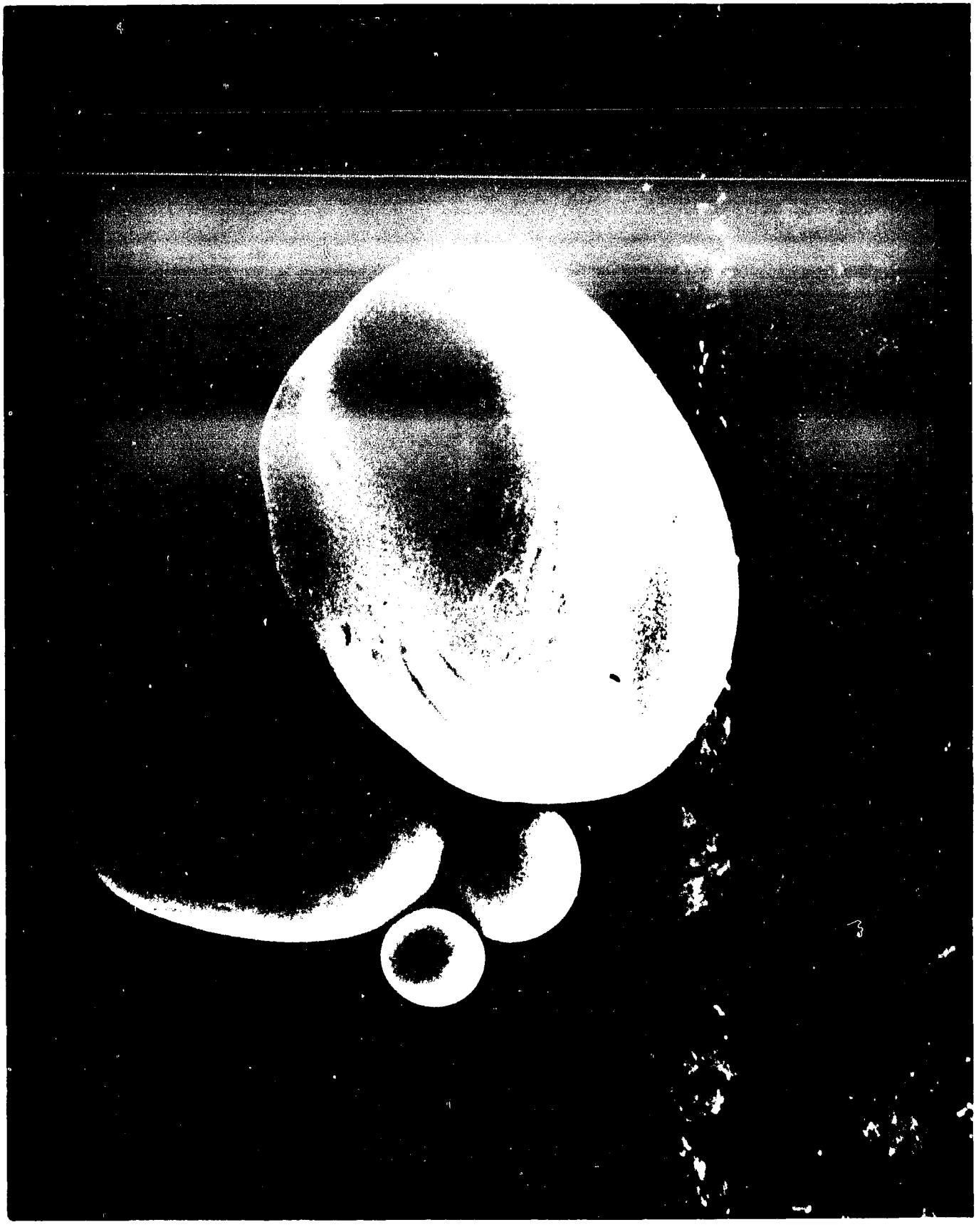


Figure 4B. Starch Granules (3 day sample)

magnification = 1930X

10.0 mm = 14 μ

KV = 15

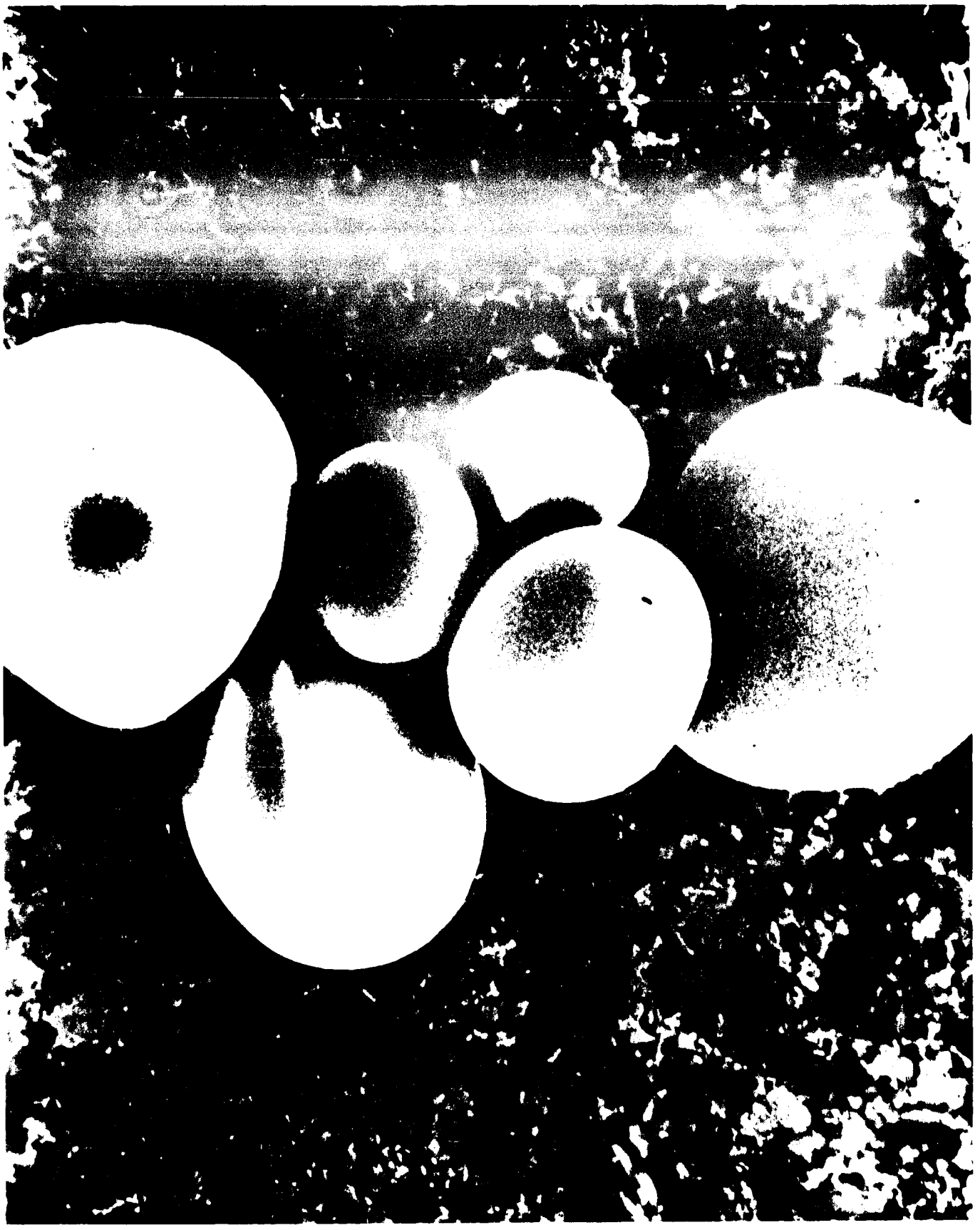
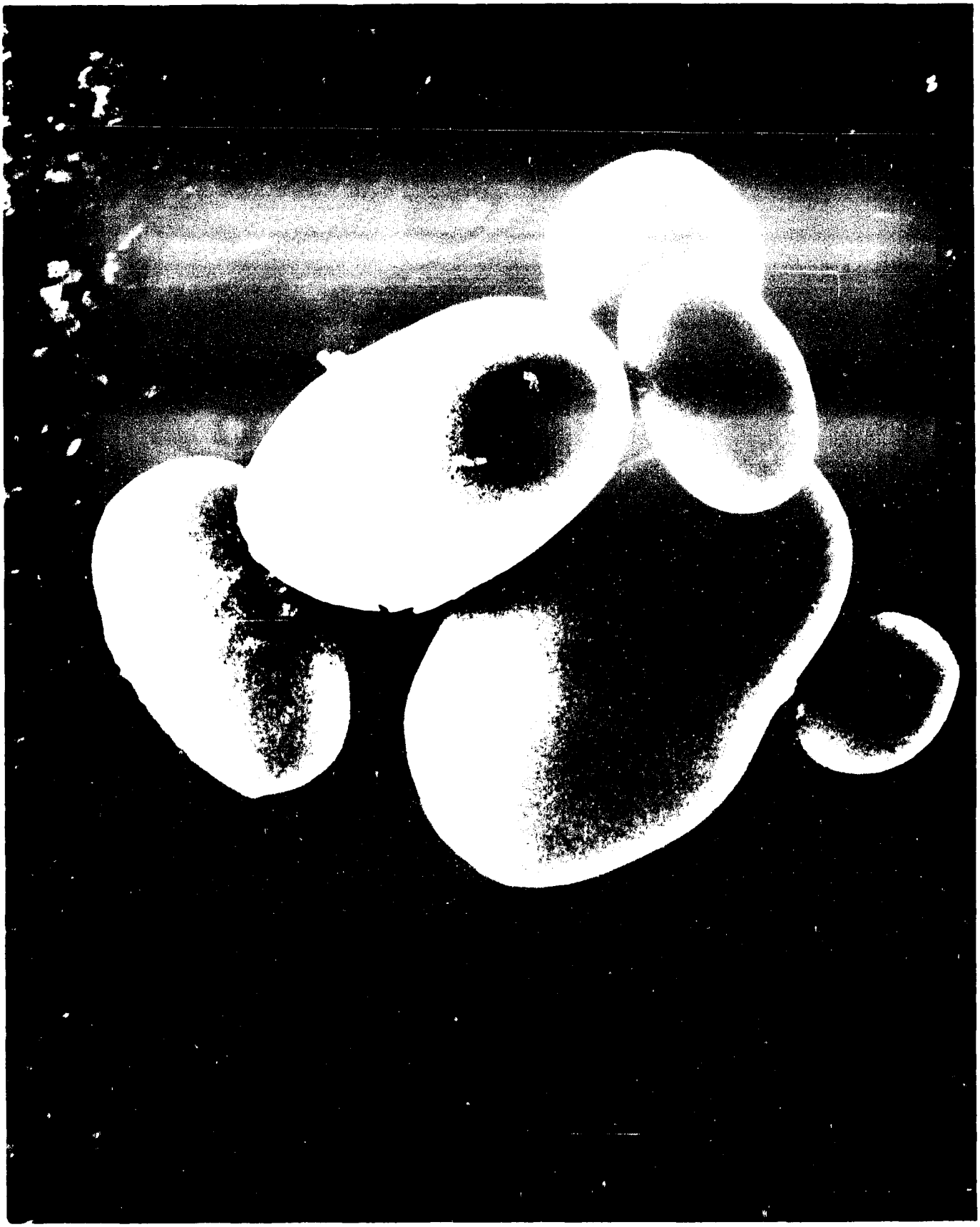


Figure 4C. Starch Granules (1 week sample)

magnification = 1930X

10.0 mm = 14 μ

KV = 15



SEM Analysis of Sprout Sections

From an examination of the micrographs, there appeared to be a difference in the sprout section after irradiation (Figures 5A,B). There seemed to be a decrease in the number but not in the size of the starch granules. This decrease in the number of starch granules was not supported by the statistical analysis. The statistical analysis did show that the areas of the starch granules remained unchanged after irradiation (Table VII).

SEM Analysis of the Wound Periderm

There was a difference in the moisture content and tightness of binding of the periderm to cortex tissue as reported by Ghanekar et al. (5). SEM analysis of the periderm sections did show a smoother surface and fewer distinct cells for the irradiated sample. The cell walls were not as obvious and appeared to be irregularly shaped in the irradiated sample (Figures 6A,B).

SEM Analysis of Membrane Fractions

An attempt was made to isolate and examine the membrane surrounding the starch granules. This fraction was isolated by sedimentation in an ultracentrifuge at 45,000 g. The micrographs of the three membrane fractions (Figures 7A,B,C) are very similar. They do not show a distinct difference in these fractions.

Figure 5A. Nonirradiated Sprout Section

magnification = 2060X

19.3 mm = 10 μ

KV = 15

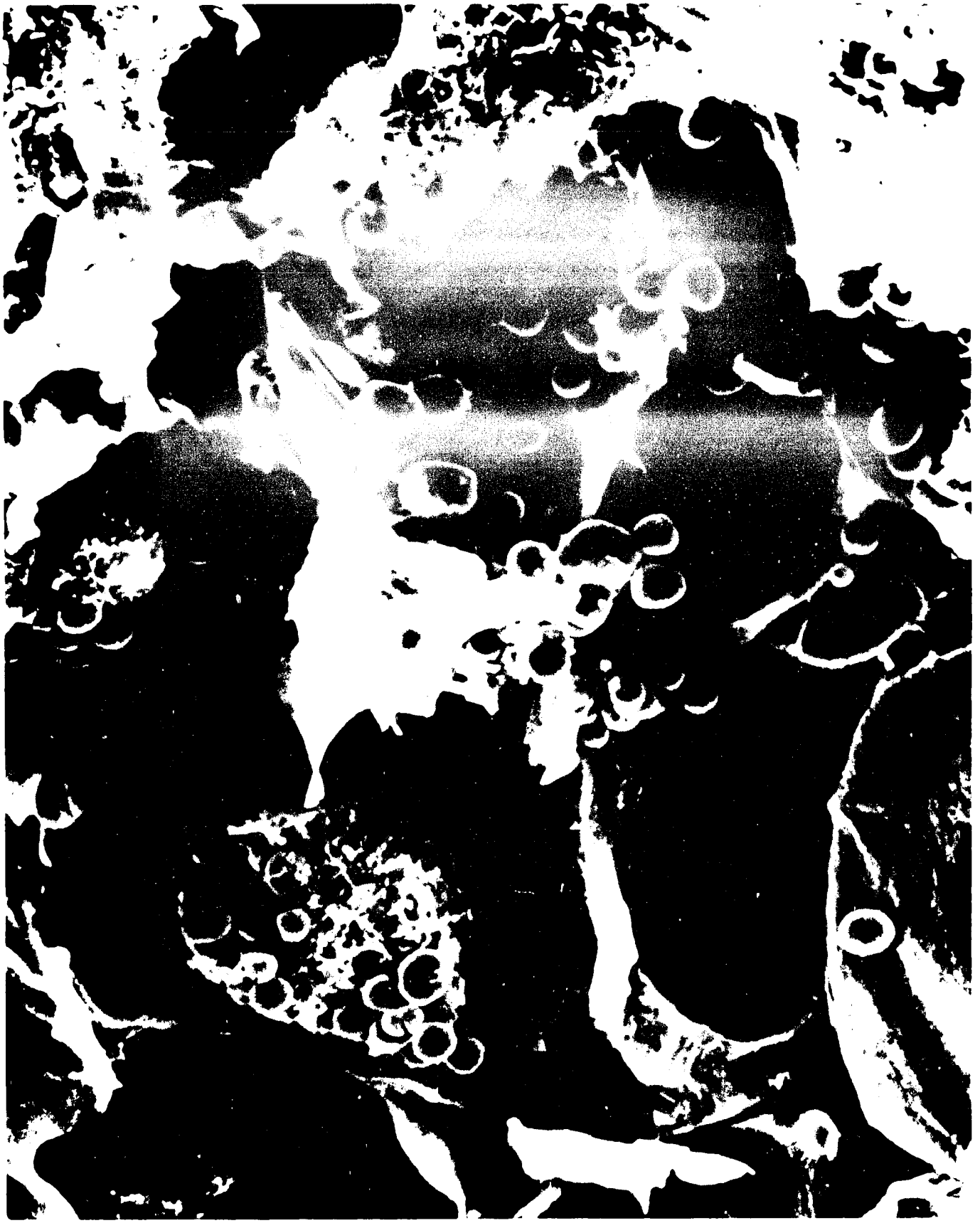


Figure 5B. Irradiated Sprout Section

magnification = 1910X

10.0 mm = 14 μ

KV = 15

Figure 6A. Nonirradiated Wound Periderm

magnification = 1780X

16.9 mm = 10 μ

KV = 15

Figure 6B. Irradiated Wound Periderm

magnification = 2080X

10.0 mm = 13.5 μ

KV = 15

Figure 7A. Nonirradiated Starch Granule Membrane

magnification = 14,580X

15.4 mm = 1 μ

KV = 15

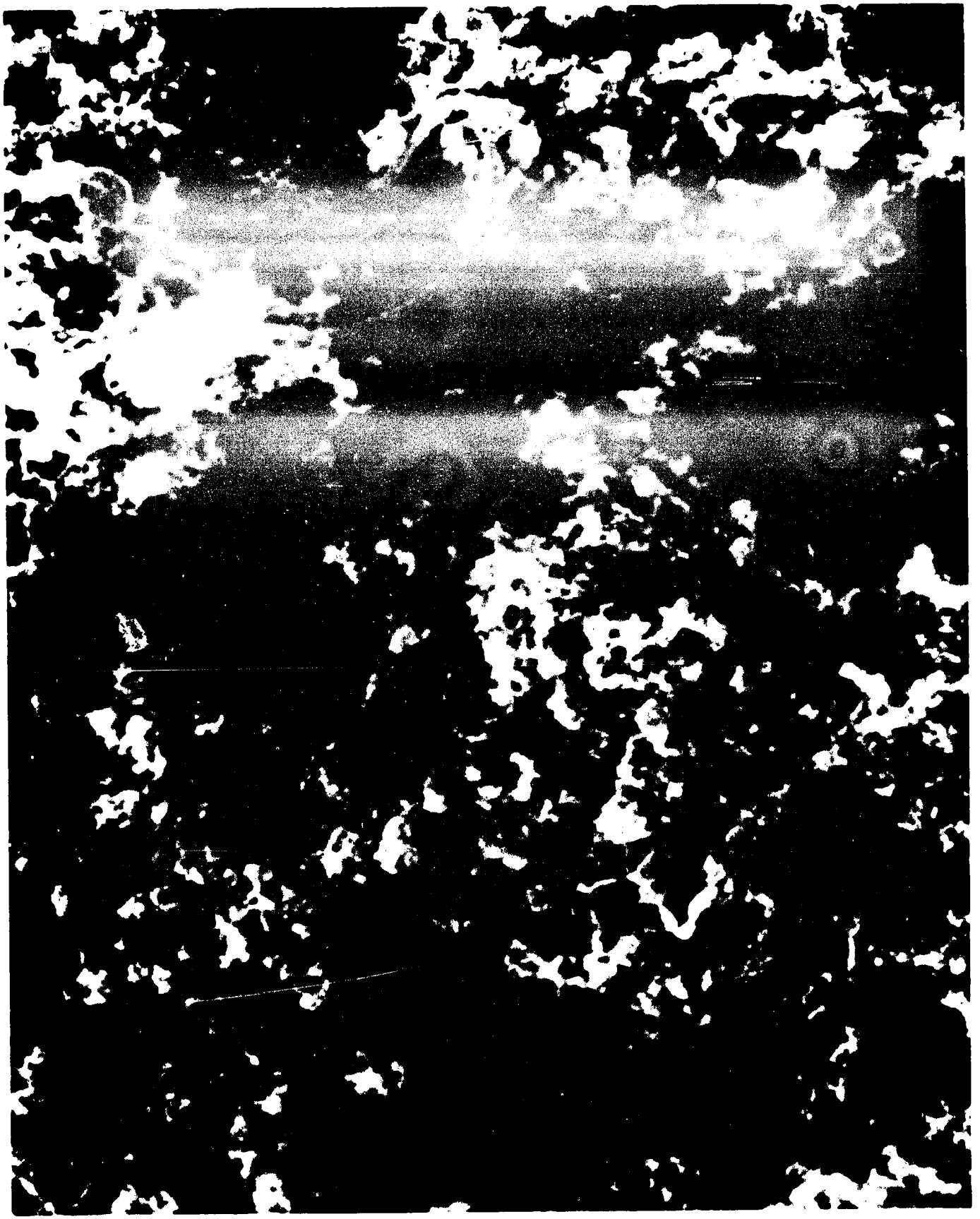


Figure 7B. Irradiated Starch Granule Membrane (3 day sample)

magnification = 16,540X

15.0 mm = 1.0 μ

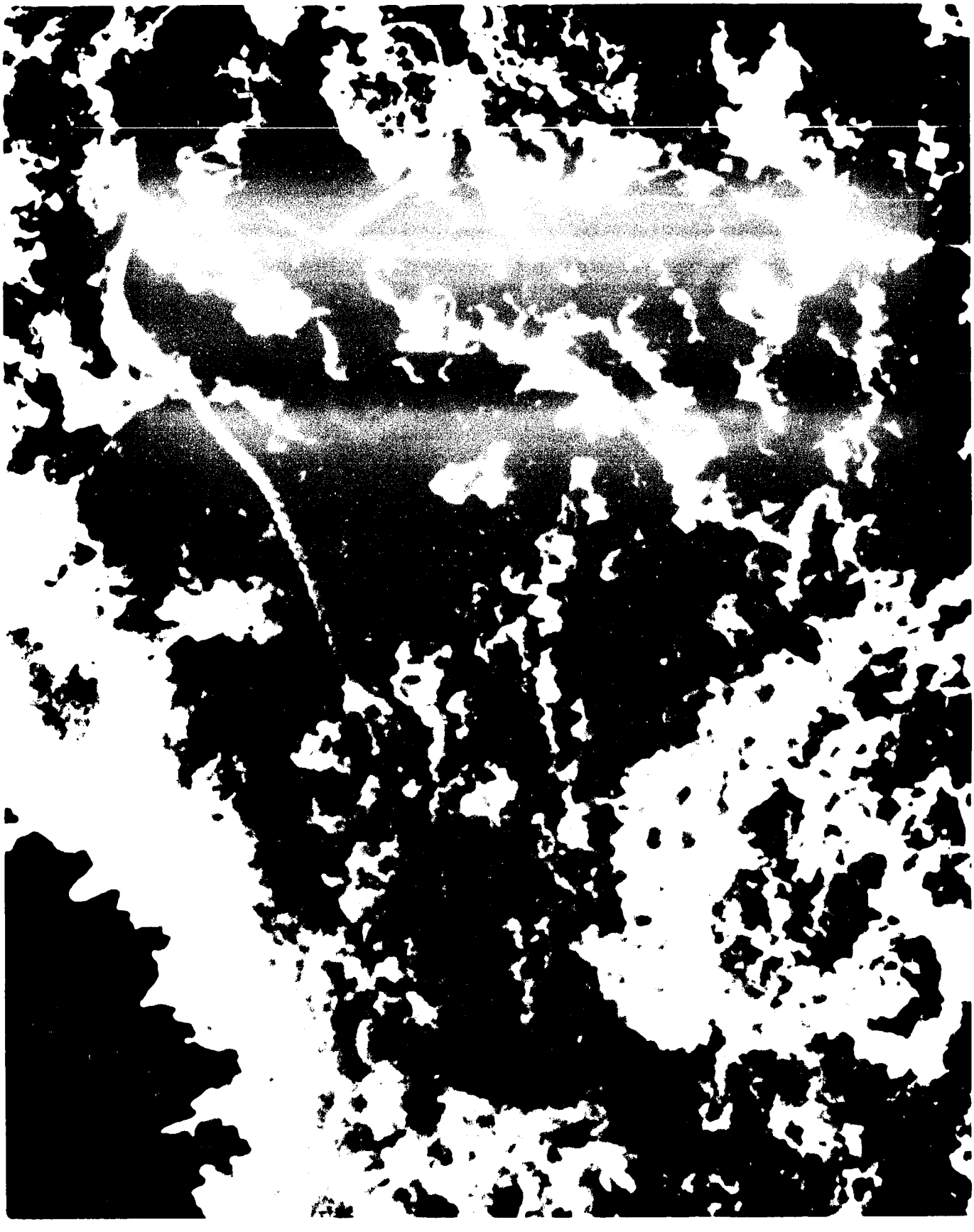
KV = 15

Figure 7C. Irradiated Starch Granule Membrane (1 week sample)

magnification = 15,240X

17.2 mm = 1.0 μ

KV = 15



Transmitting electron microscopy (TEM) would perhaps be a better method to use when examining membrane fractions. More detail can be seen with TEM than with SEM.

CHAPTER 4

CONCLUSIONS

Many researchers have analyzed irradiated potato tubers (10 Krad) for the reducing sugar, nonreducing sugar and starch content. Most reported an increase in both reducing and nonreducing sugars and a decrease in starch. No reference was found in the current literature for a scanning electron microscopic analysis of these tubers.

A colorimetric estimation of reducing sugars with 3,5-dinitrosalicylic acid was run on irradiated and nonirradiated tubers. The reducing sugar content was determined on the nonirradiated, 3 day and 1 week samples before and after sucrose inversion and after starch hydrolysis. The results from these analyses indicate that the reducing sugar content did decrease by 12.9% during the week following irradiation. The sucrose content increased by 52% during the week after irradiation. The starch content was decreased by 46.8% in the week that followed irradiation. All factors, such as storage conditions, were kept the same for all samples. The tubers were stored at 25 ^{o}C since it has been reported that low storage temperatures make a considerable difference in the reducing sugar content. According to the literature, storage at 25 ^{o}C does

not promote starch degradation nor an increase in reducing or nonreducing sugars.

SEM comparison of both irradiated and nonirradiated tuber and sprout sections showed a significant decrease in the number and size of starch granules found within the tuber cells after irradiation. No change was observed for the sprout sections. From this observation it would appear that starch is most likely being degraded. This finding agrees with the chemical analysis which showed a decrease in starch content and an increase in sucrose after irradiation.

SEM observation of the isolated starch granules does not show any difference in the appearance of their surface or size. As stated before this seems to contradict the results from the tuber analysis. It should be kept in mind that the tuber sections were close to the surface and the starch granules may have been changed more than those near the center of the tuber.

Since it has been proposed that radiation may cause a change in the membrane associated with starch granules, micrographs of the membrane fractions before and after irradiation were examined. There did not appear to be a difference in these fractions. As stated previously, the resolution of the scanning electron microscope may be too low to reveal sufficient detail. This study should be done using transmitting electron microscopy.

SEM analysis of the wound periderm revealed that there is a change in the microstructure. This is supported by literature reports of a change in the chemical analysis and tightness of binding to the cortex tissue. It appears from the micrographs that the individual cells are more pronounced before irradiation. After irradiation there is a smooth flat appearance to the periderm.

From these initial studies, the results on sugar analysis and structural changes seem to agree well with observations reported in the literature. It should be noted, however, that additional data is needed to make more conclusive statements about the sugar and starch content as well as SEM observations. This data was not collected before the time of this writing because the "Superior" variety of potato used was unavailable. Additional samples will be run for all analyses in the fall of this year after more potatoes are harvested.

APPENDIX

Table I. Total Reducing Sugar Analysis with 3,5-Dinitrosalicylic Acid (Extraction Method)

Tuber Sample	Sample Wt (g)	Absorbance	Average % Dry Wt
Nonirradiated	0.250	0.630	
	0.250	0.582	0.472
	0.250	0.631	
3 day - Irradiated	0.250	0.625	
	0.250	0.632	0.485
	0.250	0.638	
1 week - Irradiated	0.250	0.625	0.477
	0.250	0.622	
	0.250	0.620	

Table II. Total Reducing Sugar Analysis with 3,5-Dinitrosalicylic Acid (Dry Powder Method)

Tuber Sample	Sample Wt (g)	Absorbance	Average % Dry Wt
Nonirradiated wt(g)			
148.1	0.625	0.560	
	0.613	0.650	0.560
	0.639	0.623	
145.2	0.570	0.658	
	0.575	0.711	0.487
	0.570	0.655	
175.8	0.630	0.640	
	0.622	0.635	0.586
	0.631	0.638	
3 day - Irradiated wt(g)			
140.2	0.639	0.548	
	0.615	0.579	0.548
	0.632	0.675	
138.9	0.620	0.560	
	0.618	0.579	0.532
	0.618	0.577	
1 week - Irradiated wt(g)			
144.5	0.649	0.590	
	0.616	0.412	0.434
	0.601	0.407	
140.6	0.630	0.566	
	0.632	0.571	0.513
	0.635	0.563	

Table III. Total Reducing Sugar Analysis with 3,5-Dinitrosalicylic Acid (After Sucrose Inversion)

Tuber Sample	Sample Wt (g)	Absorbance	Average % Dry Wt
Nonirradiated wt(g)			
148.1	0.610	1.210	
	0.613	1.250	1.19
	0.620	1.238	
145.2	0.619	1.254	
	0.613	1.230	1.18
	0.615	1.228	
175.8	0.611	1.215	
	0.598	1.203	1.18
	0.605	1.214	
3 day - Irradiated wt(g)			
140.9	0.615	1.368	
	0.616	1.372	1.32
	0.615	1.375	
138.9	0.595	1.352	
	0.630	1.385	1.31
	0.621	1.369	
1 week - Irradiated wt(g)			
144.5	0.607	1.499	
	0.599	1.486	1.46
	0.602	1.483	
140.6	0.611	1.498	
	0.607	1.495	1.45
	0.620	1.521	

Table IV. Starch Analysis with 3,5-Dinitrosalicylic Acid

Tuber Sample	Sample Wt (g)	Absorbance	Average % Dry Wt
Nonirradiated wt(g)			
148.1	0.101	0.384	
	0.101	0.382	72.8
	0.101	0.384	
145.2	0.103	0.388	
	0.100	0.380	72.1
	0.102	0.389	
175.8	0.108	0.380	
	0.110	0.385	68.7
	0.103	0.388	
3 day - Irradiated wt(g)			
140.9	0.110	0.305	
	0.110	0.306	53.2
	0.110	0.304	
138.9	0.107	0.308	
	0.101	0.301	55.2
	0.110	0.305	
1 week - Irradiated wt(g)			
144.5	0.116	0.294	
	0.116	0.290	48.1
	0.116	0.289	
140.6	0.108	0.288	
	0.111	0.285	49.0
	0.120	0.295	

Table V. Starch Analysis - Glucose Oxidase Method

Tuber Sample	Sample Wt (g)	Absorbance	Average % Dry Wt
Nonirradiated	0.651	0.0995	
	0.476	0.1049	85.8
3 day - Irradiated	0.446	0.1057	
	0.388	0.1020	62.0
1 week - Irradiated	0.292	0.1011	
	0.293	0.1011	45.0

Table VI. Statistical Analysis of Data from Potato Tubers

Measurement	Nonirradiated		3 Day Sample		1 Week Sample	
	Mean	Confidence Limits	Mean	Confidence Limits	Mean	Confidence Limits
# of Starch Grains (a)	67.5±6.18	47.83-87.17 (95%)	13*±5.58	0-30.75 (95%)	2.0*±0.82	0-4.6 (95%)
Starch Granule Area (b)	467±106	253-681 (95%)	104.25*± 10.29	83.13-125.36 (95%)	86.6*±15.5	49.9-123.4 (95%)
Cell Size (b)	161.38± 17.48	123-199 (95%)	190.90± 15.24	159-222 (95%)	144.52± 12.9	117-171.3 (95%)
Empty Cells	3.25±1.38	0-7.63 (95%)	12.75± 1.93	6.6-18.9 (95%)	18*±1.73	12.5-23.5 (95%)
Cells with Granules	16.5±0.96	13.46-19.54 (95%)	5.5*±1.55	0.56-10.4 (95%)	1.75*±0.63	0-3.75 (95%)

(a) in 0.52 mm^2 area at 156X; (b) (μm^2)

* significant difference at 0.05 level of confidence

Table VII. Statistical Analysis of Data from Potato Sprouts

Measurement	Nonirradiated		Irradiated	
	Mean	Confidence Limits	Mean	Confidence Limits
# of Starch Grains (a)	16.67±4.81	5.58-27.76 (95%)	7.44±1.44	4.11-10.78 (95%)
Starch Granule Area (b)	6.66±0.86	4.81-8.51 (95%)	7.64±1.07	5.34-9.94 (95%)

(a) in a 0.52mm^2 area at 730X

(b) (μm^2)

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