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SCANNING ELECTRON MICROSCOPIC STUDY OF IN VITRO
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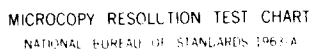
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19) fibroblasts was added to each well. Cell growth and attachment were permitted for 24 hours. Medium was then removed, and solutions for cell fixation, buffer washing and dehydration were added to each well. SEM examination revealed changes in cell growth with increasing periods of aeration suggestive of increasing cell vitality. Cells growing on discs having no aeration were small, round and lobulated; whereas those of seven to fourteen days of aeration were more numerous, and flattened with many microvilli, pseudopodia, and dendritic processes, features consistent with normal cell morphology. These results suggest that EO sterilized polymer implants should be aerated at least seven to fourteen days prior to surgical use. (RW) 

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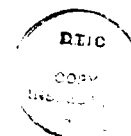
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ABSTRACT

Polylactic acid (PLA) and polyglycolic acid (PGA) have been under investigation for use in the management of hard and soft tissue wounds. Current research has included the incorporation of osteoinductive substances into a PLA-PGA copolymer alloplastic implant material for enhancing the healing of osseous defects. Conventional methods of sterilization such as dry heat, steam heat or ^{60}Co , tend to either destroy or attenuate osteoinductive activity and alter polymer biodegradation. Ethylene oxide (EO) gas sterilization is currently being tested as an alternate method. This study examined the relationship of EO-induced cytotoxicity to the length of time of polymer aeration following EO sterilization. Three groups of copolymer implant disks were studied: 1) 50:50 PLA-PGA copolymer, 2) PLA-PGA polymer with hydroxyapatite (HA) and 3) PLA-PGA with autolyzed, antigen-extracted (AA) bone particles. Polymer discs, as well as particulate HA and AA bone controls, were sterilized with EO for 12 hours. Following periods of two weeks, one week, one day or no subsequent vacuum aeration, samples were placed into 24 well culture plates. A suspension of human fibroblasts was added to each well. Cell growth and attachment were permitted for 24 hours. Medium was then removed, and solutions for cell fixation, buffer washing and dehydration were added to each well. SEM examination revealed changes in cell growth with increasing periods of aeration suggestive of increasing cell vitality. Cells growing

SEM of EO Cytotoxicity

on discs having no aeration were small, round and lobulated; whereas those of seven to fourteen days of aeration were more numerous, and flattened with many microvilli, pseudopodia and dendritic processes, features consistent with normal cell morphology. These results suggest that EO sterilized polymer implants should be aerated at least 7 to 14 days prior to surgical use.

Keywords: Ethylene oxide, sterilization, scanning electron microscopy, cell culture, cytotoxicity

INTRODUCTION

In the past 20 years there has been an increasing interest in the use of absorbable biomaterials for reconstructive surgery, dental and orthopedic applications, and drug delivery systems. The alpha-hydroxypolyesters, polyglycolic acid (PGA) and polylactic acid (PLA), both alone and in combination, have been shown to fulfill many of the requirements of biocompatibility and biodegradation for a variety of biomedical uses (1-3).

The U.S. Army Institute of Dental Research has been investigating the use of a 50:50 copolymer mixture of PLA-PGA as an alloplastic implantable bone repair material (4-6). The incorporation of

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osteoconductive materials, e.g. hydroxyapatite (HA) and osteoinductive substances such as bone morphogenetic protein (BMP), osteogenin and autolyzed, antigen-extracted (AA) bone have all been tested in the copolymer carrier. The inability to aseptically prepare either the alloplastic polymers or the alloimplant bone derivatives requires a sterilization technique which neither diminishes osteoinduction nor alters polymer biodegradation. Boiling, dry heat, conventional steam autoclaving and radiation have all been shown to interfere with both osteoinduction and polymer degradation (7-10). The use of ethylene oxide (EO) gas sterilization therefore, is being investigated as an alternate method.

Ethylene oxide is a cyclic ether widely employed for the sterilization of plastics and rubber products used in medical applications. The mechanism of its action involves replacing available hydrogen atoms with hydroxyl-ether radicals, thereby inactivating amino, sulfhydryl, and carboxyl side-chains on protein molecules of micro-organisms (11). Toxicity from both ethylene oxide and its chemical derivatives, ethylene chlorohydrin and ethylene glycol include vesicular burns of skin and mucosa from prolonged direct contact, respiratory tract dysfunction, hemolytic effects, gastrointestinal disturbances, central nervous system damage and mutagenic effects similar to radiation (12-14). The extent of toxicity however, is related to the amount of EO absorbed and retained by the exposed material. Absorption of EO is dependent on the diffusion coefficient of the exposed material, the time and temperature of sterilization and the concentration of the EO gas mixture (12). However, toxicity of EO can be minimized by allowing

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an adequate period of aeration after gas sterilization to reduce the residual EO. This study examines, by scanning electron microscopy (SEM), the effect on cell viability of varying periods of vacuum aeration following EO sterilization of particulate HA and demineralized bone particles both alone and incorporated into PLA-PGA copolymer.

MATERIALS and METHODS

Biodegradable copolymer discs were fabricated by first solubilizing 50:50 poly (DL,lactide-co-glycolide) (Southern Research Institute, Birmingham, AL.) in acetone. Either autolyzed, antigen-extracted (AA) bone particles prepared (according to a modification of Urist's method) from *Macaca fascicularis* (cynomolgus) non-human primate or repleminiform HA particles (Interpore, Irving, CA.) were added to the copolymer in a 1:1 weight ratio. Polymer was poured into petri plates, cured at room temperature and pressure for 1 day and then vacuum cured at room temperature for 7-10 days. Plain 50:50 poly (DL, lactide-co-glycolide) copolymer without AA bone or HA was similarly prepared. Polymer discs of 4mm diameter and 1.5mm thickness were cut and sterilized for 12 hours at ambient pressure and temperature, in an Anprolene ethylene oxide gas sterilization chamber (H.W. Andersen Products, Inc. Oyster Bay, NY). One ampule containing 84% EO and 16% inert stabilizers was used for the procedure. Discs were then aerated under vacuum for 14, 7, or 1 day or not aerated post-sterilization prior to cell culture. Granular HA and AA bone was also EO sterilized and aerated in a

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fashion similar to the copolymer disks. Discs and granules were prepared in triplicate for each post-EO aeration time period.

Test discs, as well as non-EO sterilized polystyrene placebo discs were placed into the wells (1 disc/well) of 24 well tissue culture plates. Human skin fibroblasts and mouse connective tissue cells (929) obtained from the American Type Culture Collection (Rockville, MD), were grown in Dulbecco's Modified Eagle Medium (DMEM). DMEM was supplemented with 4% l-glutamine, 1.5% sodium pyruvate, 1.5% non-essential amino acids, 0.5% penicillin/streptomycin/ fungizone and either 20% horse serum (for 929 cells) or fetal bovine serum (for human fibroblasts). Each cell type was suspended in DMEM to a final concentration of 5×10^5 cells/ml and added (400 μ l/well) to each well of the appropriate plates. Wells without discs received either particulate AA bone or HA. Cell growth control wells received neither discs nor particles. The plates were incubated at 37°C in a 100% humidity, 5% CO₂ incubator for 24 hours.

Cells were then processed within the culture plate wells for SEM. Culture medium was replaced with Karnowsky fixative (4 % paraform- aldehyde: 1% glutaraldehyde in 0.2M sodium cacodylate buffer, pH 7.4) for 2 hours. Cells were then washed in 0.1M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide for one hour, washed, stabilized in 0.5% tannic acid for 30 minutes, en bloc stained with 1% uranyl acetate for 12 hours at 4 °C, dehydrated through graded ethanol to hexamethyldisilazane for 5 minutes and air dried. Samples were finally gold-palladium sputter coated and photographed on an AMRAY 1645 scanning electron microscope.

RESULTS

Both cell types in control wells, i.e. with no EO sterilized material, grew to confluence. Human fibroblasts appeared as flat, amoeboid-like cells with numerous surface microvilli, elongated dendritic processes and pseudopodia (Fig. 1). Mouse 929 cells appeared as rounded cells uniformly covered with closely packed rounded surface nodules (Fig. 2). Cells grown on placebo discs were similar in appearance.

Cells grown both in the presence of copolymer discs of 7 and 14 days aeration post-sterilization and in the presence of granular AA bone and HA of 7 and 14 days aeration, were cytologically similar to the control cell groups (Fig. 3,4). However, cell density of 7 day aeration samples was not as great as control cell groups, suggesting some retardation of cell replication.

Human fibroblast cells grown in the presence of discs and granules of only one day or no aeration, were both sparse in number and either fragmented or morphologically small, round and lobulated (Fig. 5,6). In addition, 929 cells were not only less numerous and smaller than control cells, but also had a non-nodular surface and an irregular collapsed shape (Fig. 7).

Cells grown in the presence of sterilized copolymer discs showed more profound cytotoxic effects than cells grown in the presence of sterilized particulate AA bone or HA. No remarkable difference was noted in the cell growth which occurred on the polymer discs whether or not AA bone or particulate HA had been incorporated.

DISCUSSION:

Because of the recognized toxicity of ethylene oxide and its reaction products, the American National Institute for Occupational Safety and Health (NIOSH) has established maximum acceptable levels for the content of EO in medical devices. (15) In the United States, these limits vary from 25 to 250 ppm, depending on the application of the device. Although the tendency is to attempt to lower these limits, usually to the 10 to 25 ppm range, the maximum limit in France is 2 ppm and in Germany is 1 ppm (12, 14). Determinations of residual EO can be made by gas chromatography head space analysis (12). The potential usefulness of cell culture cytotoxicity testing lies in the ability to provide a sensitive, low cost assay system of short duration, from which data may be correlated with *in vivo* animal model testing. Discrepancies must be anticipated between animal data and cell culture data however. Wilsnack et al. found, for example, that there are significant differences in the sensitivity of cell culture versus animal model testing (16). Using known concentrations (based on dilution) of EO and EO reaction products, they found that EO was the most toxic, causing degeneration of cells in culture at 25 ppm. Ethylene glycol and 2-chloroethanol were toxic at 100,000 and 1000 ppm respectively. However, EO was found to be 80-fold more toxic in cell culture than in animal testing which showed toxicity at 2000 ppm (16).

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Although it has not yet been possible to determine residual EO concentrations in our samples, the cytotoxic effects observed may be a relative indicator. Toxic effects observed by SEM in this study have been further corroborated by ^{51}Cr assay studies using the same cell culture system (17). Observations made in this SEM study suggest that morphologically, cells appear essentially normal after 7 to 14 days of vacuum aeration. However, the decreased cell density noted, even after the 14 day period, indicate that mechanisms controlling cell replication may still be affected. Functionally, these cells may also be altered. The effect of these levels of EO on osteoprogenitor cells required for bone repair should be determined. Preliminary studies suggest that even after two weeks of vacuum aeration, the osteoinductive capacity of EO sterilized, particulate demineralized bone matrix is reduced (18). Subjectively, it appeared that cells grown on copolymer disks demonstrated a more profound cytotoxic effect than cells grown with granular AA bone or HA. This is probably attributable to a greater residual EO level in the polymer disks than in the granular materials. Whether or not these effects might be significant *in vivo*, has not been determined. It has been the general practice in our laboratory, to allow four weeks of vacuum aeration of EO sterilized implant materials prior to animal implantation. Consequently, significant signs of toxicity have not been observed in our animal experiments.

CONCLUSIONS

Residual ethylene oxide contained in implantable materials recently sterilized, is cytotoxic to cultured human fibroblasts.

The use of a biodegradable alloimplant material such as the PLA-PGA copolymer seems to contain more residual EO for a longer period of time than either particulate HA or AA prepared bone.

A vacuum aeration period of greater than two weeks is recommended to minimize the potential cytotoxicity of EO-sterilized implant materials. The necessary period of aeration should be definitively determined for all implant materials being used or studied. Residual EO appears to be dependent on the configuration and size of the material being sterilized. That is, a large block of copolymer implant might be expected to retain more EO than a thin disk implant after equal periods of aeration.

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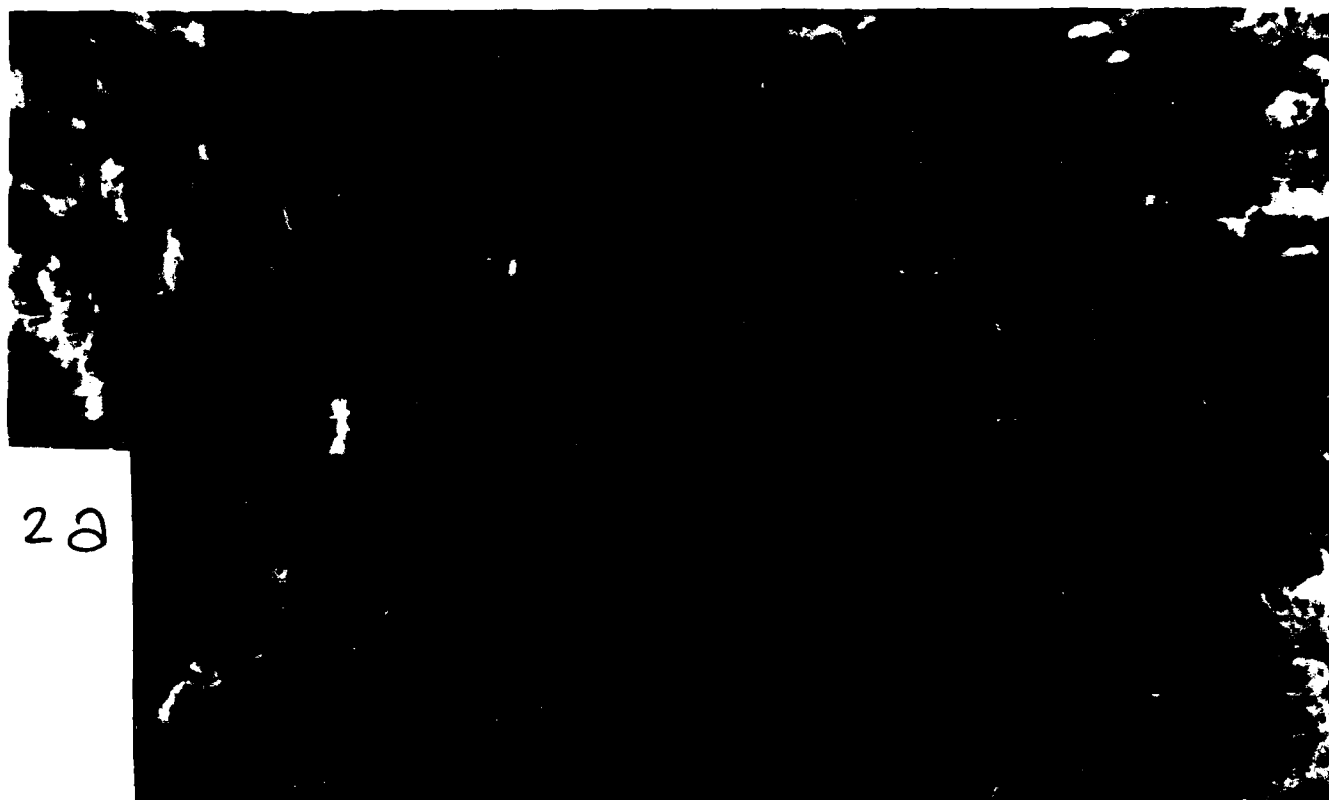
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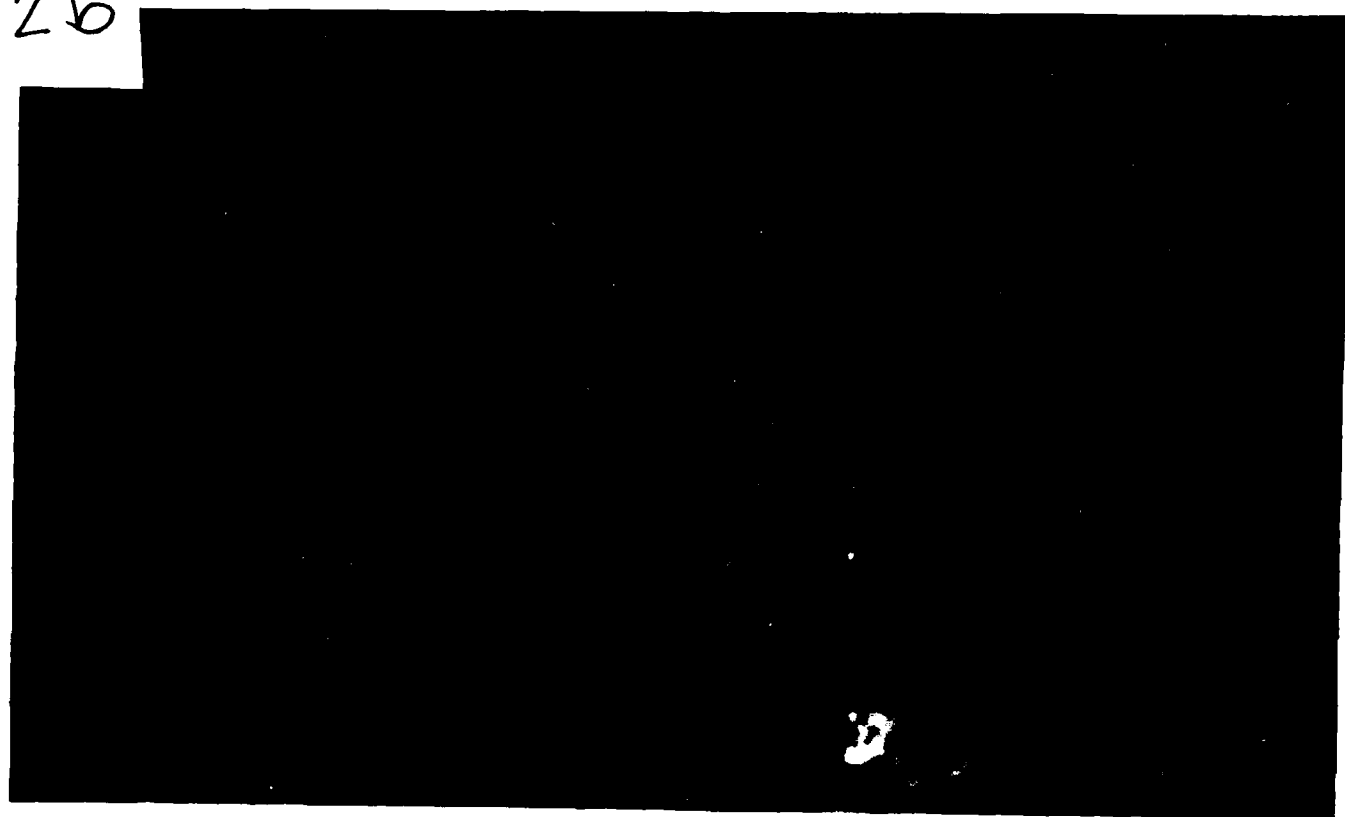
LEGEND

1. Normal human fibroblasts grown in control wells without copolymer or granular AA bone or hydroxyapatite. Cells are confluent with surface microvilli and spread flat with amoeboid pseudopodia. Original magnification X 1000.
2. Normal 929 cells grown in control wells with no copolymer or particulate granules. Cells are confluent, growing in tight clusters of round cells having a dense covering of nodular surface blebs.
a) Original magnification X 300; b) magnification X 1500.
3. a) Human fibroblast after 7 days of EtO aeration grown on plain copolymer disc. Magnification X2000; b) Cells after 14 days aeration grown on copolymer with AA bone disc. Magnification X2000. Cells appear similar to normal control cells, however, lack normal cell population density.
4. 929 cells grown in well with AA bone particles after 14 days of aeration. Like normal cells, most are plump and covered with surface blebs. Some appear flat and spreading due to the decreased density of growth. Magnification X250.
5. Human fibroblast cells grown on polymer with HA disk after one day of EtO aeration. a) Magnification X250; b) magnification X 2000.
6. Human fibroblast cells grown on polymer disc with no aeration after EO sterilization; cells are sparse, fragmented, small, round and nodular.
a) Original magnification X250; b) magnification X 1500.
7. 929 cells grown on polymer disk with no aeration after EO sterilization. Cells are sparse, fragmented, shrivelled, or smooth surfaced, lacking normal morphology. a) Magnification X 250; b) magnification X 1000.

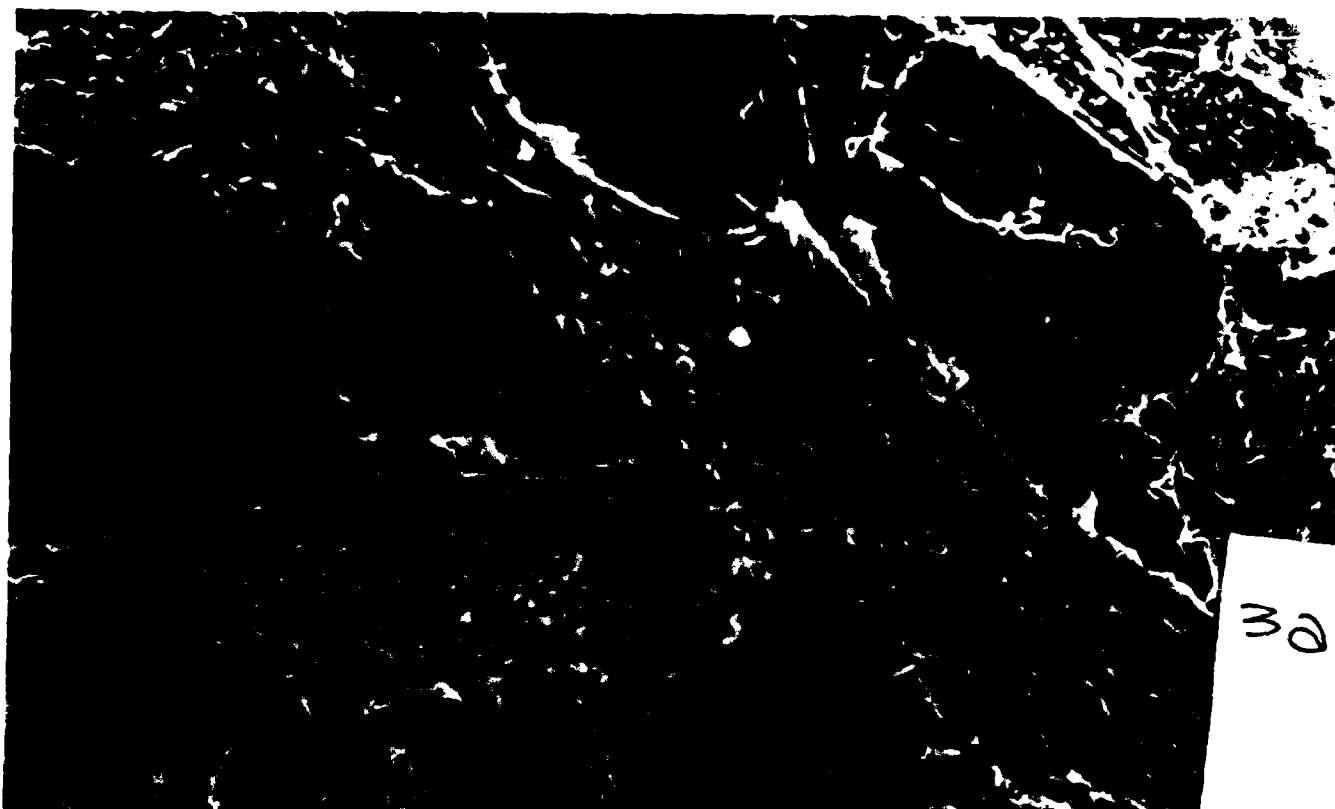




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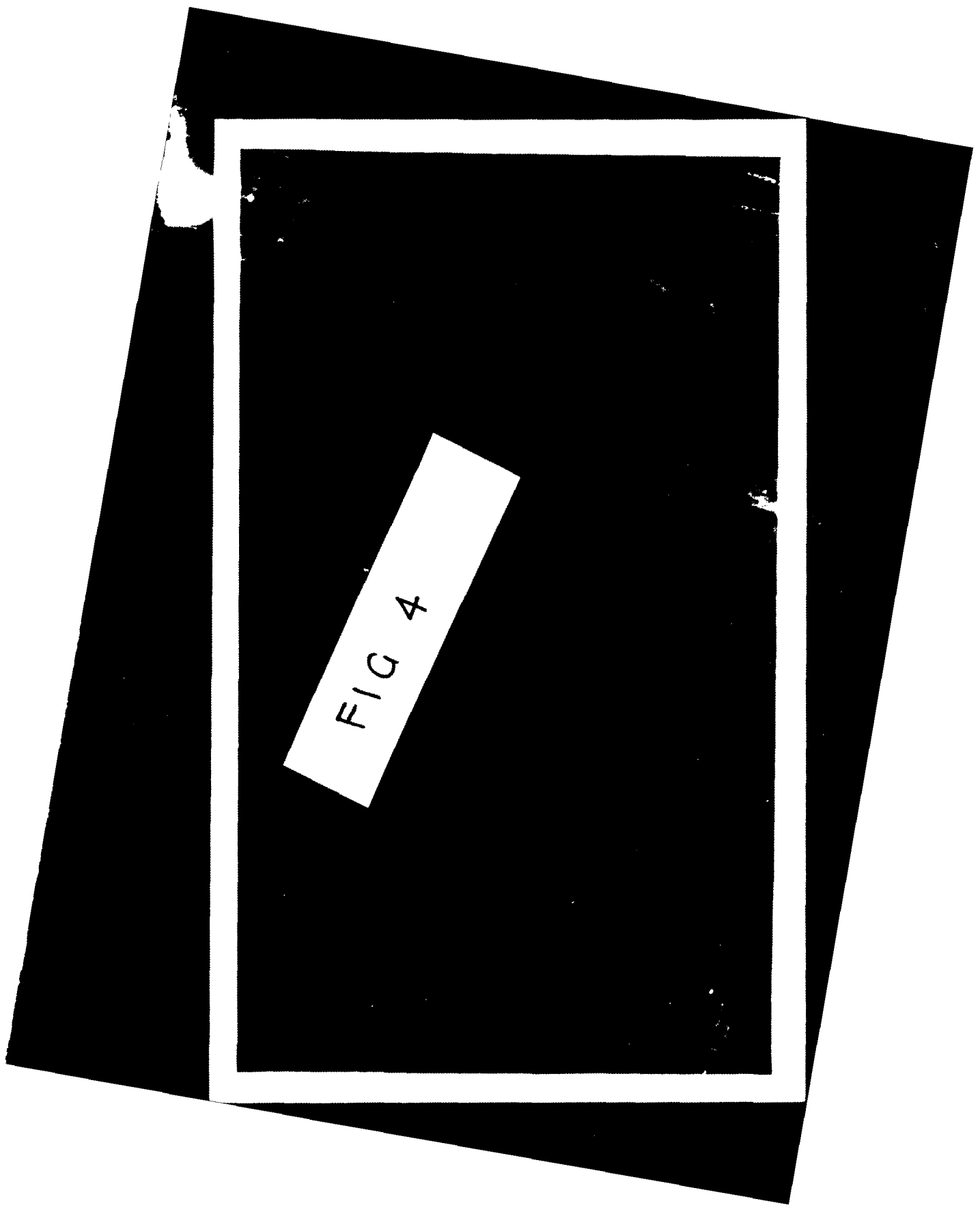
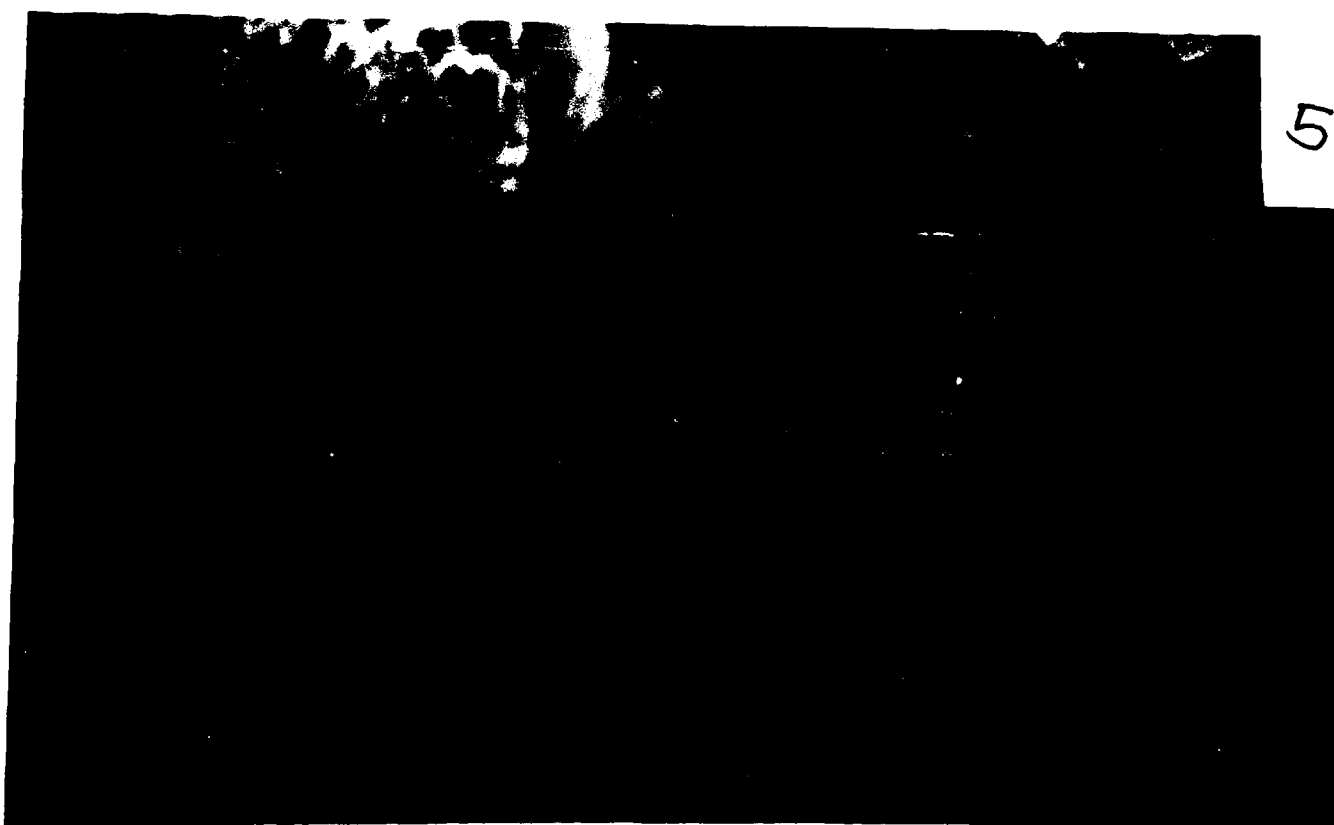


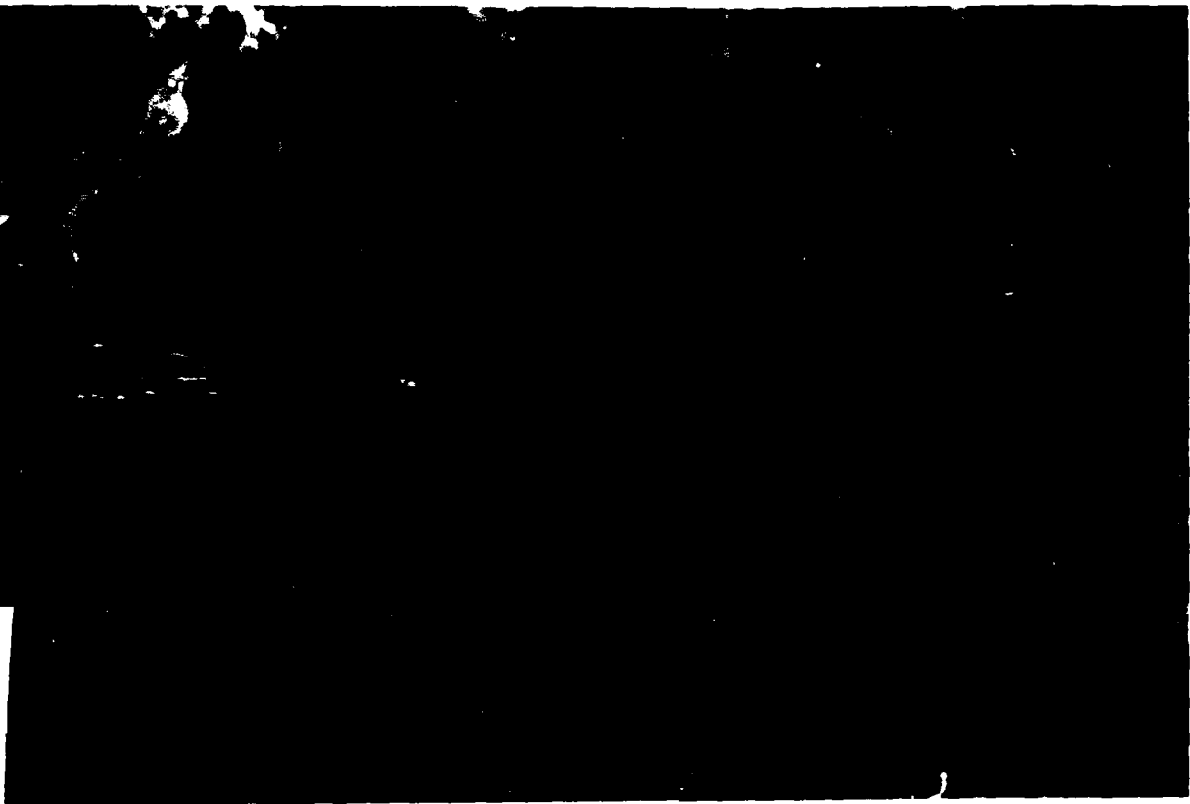
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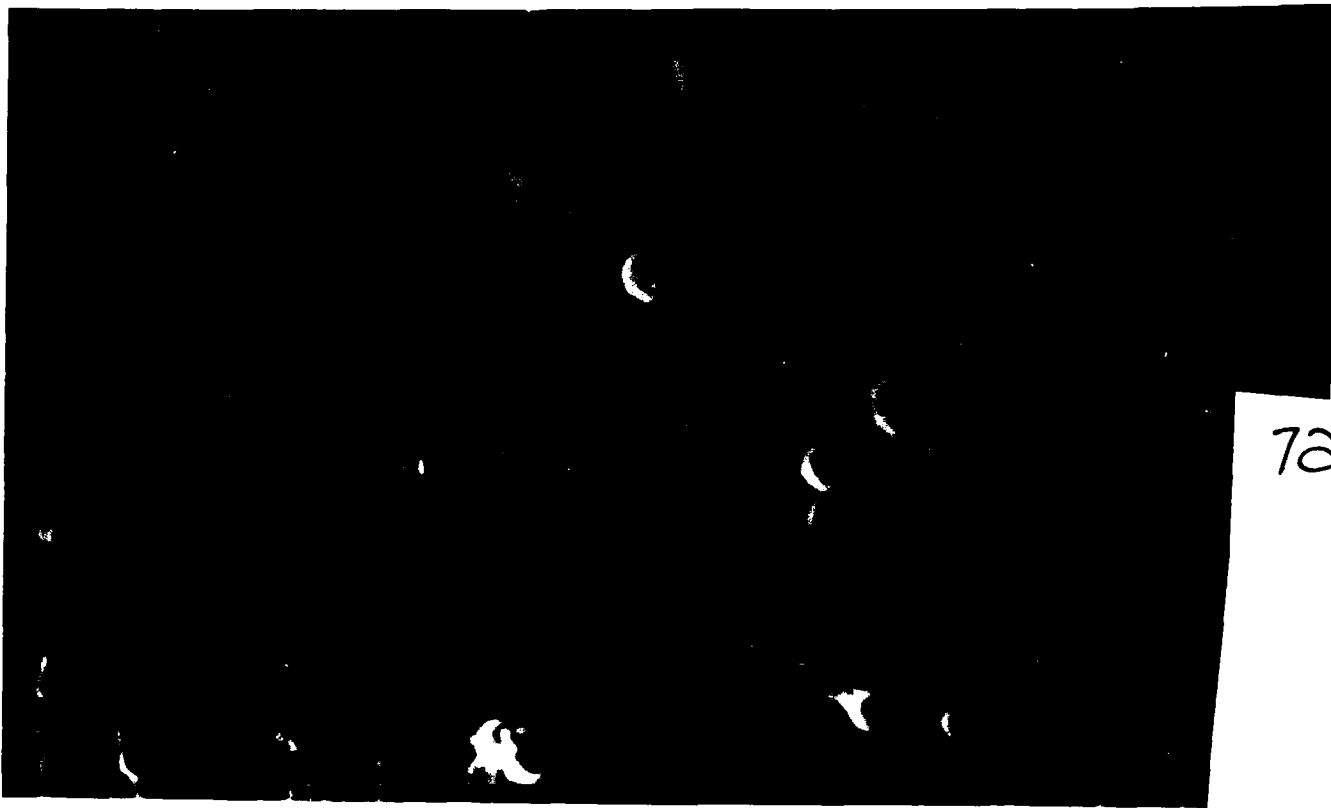
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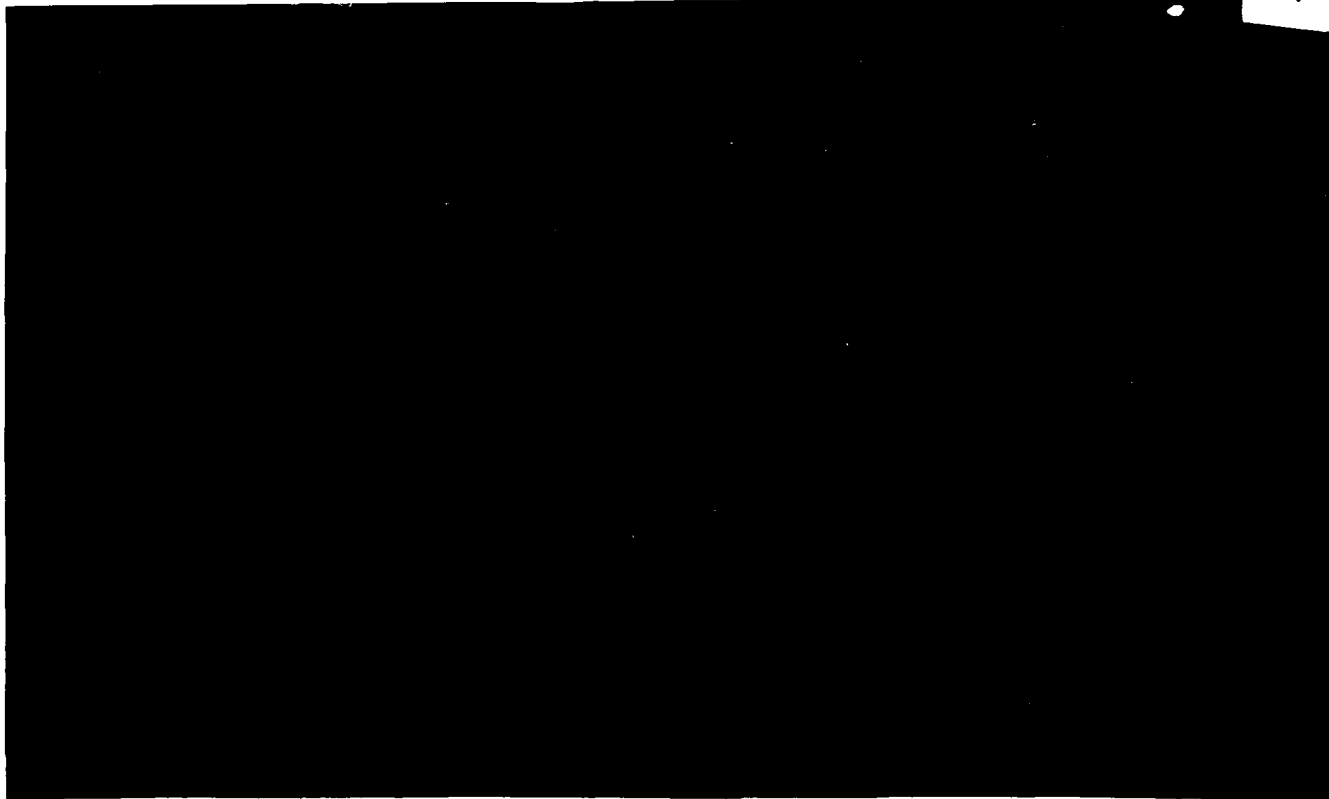
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