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The Effects of an Artificial Substrate upon In Vivo Cell Proliferation: An Autoradiographic Study

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THE EFFECTS OF AN ARTIFICIAL SUBSTRATE

UPON IN VIVO CELL PROLIFERATION: AN

AUTORADIOGRAPHIC STUDY

By

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To My Family--
the many sacrifices
they have made in order to
make my education
possible.

Edward Eugene Black was born in Oklahoma City, Oklahoma on July 25, 1937. He was graduated from Faver High School, Guthrie, Oklahoma, May, 1954.

From 1955 to 1959 he attended San Jose State College, San Jose, California where he obtained the Degree of Bachelor of Science. He was graduated from the Loyola University School of Dentistry, Chicago, Illinois in June, 1964 with the Degree of Doctor of Dental Surgery.

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In 1966 he was accepted on the Residency Staff in Oral Surgery at Long Beach Veterans Administration Hospital, Long Beach, California to complete his Oral Surgery training.

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INTRODUCTION

Included in the many processes responsible for growth and proliferation of cells are synthesis of cellular components and mitosis and development. In order that cells may synthesize, divide and metabolize normally, the proper substrate or building blocks must be made available.

Investigators have used cells in tissue culture in an effort to stimulate cellular proliferation and have determined the effect of various natural and synthetic media on their maintenance and growth. Growth promoting factors have been demonstrated in many tissue homogenates and extracts, but for the most part these attempts were made with unidentifiable components of biological materials.

In this thesis will be discussed the results of an investigation on the influence of an in vivo synthetic substrate upon subcutaneous mesenchymal cell homeostasis.

REVIEW OF LITERATURE

This review deals with some aspects of growth-promoting factors in various tissues. No claim is made of complete coverage of the literature. Rather, the problems are to be focused on a discussion of selected, and, it is hoped, representative investigations. Such selection is of necessity subjective and arbitrary.

Our knowledge of the factors which influence the multiplication and growth of cells in the animal organism is surprisingly limited. As early as 1858, Virchow suggested that in the organism itself an injured tissue produces a formative irritation, manifested as an increase in cell division. It was principally in connection with investigations on wound healing that the question of the presence of growth-promoting factors in the organism was raised.

In tissue cultures of animal tissue, Carrel (1912) succeeded in proving the presence of growth-promoting factors. He observed that tissue cultures grew much more rapidly after application of an extract of embryonic tissue. He assumed that the embryonic extract contained substances like the "proteoses" which he prepared by digestion of protein. The "trephones" which are secreted products of leukocytes were identical with this substance.

Fisher (1929) proved the presence in traumatized animal tissue cultures of growth promoting substances which he assumed were released when the cells were injured.

In 1925, Carrel and Baker found that embryo web porridge was effective in accelerating wound healing. They found that upon fractionation of embryo tissue juice by a 50% alcoholic solution, the protein fraction contained the

activating substance or the essential nutritive substance. Experiments were undertaken to purify the protein by the process of repeated precipitation but it was found that precipitation destroyed the growth-promoting properties.

Harvey and Howes (1930) showed the effect of high protein diet on the velocity of growth of fibroblasts upon stomach wounds of the rat. They found that the period preceeding the initiation of growth is not affected by a high protein diet, however, once growth has started, the velocity of it is distinctly increased by such a diet.

Following the line of investigation set forth by Carrel, Nielsen (1939) gives an account of controlled experiments in the healing of wounds in dogs and shows that the employment of the embryonic extract in dressing these wounds shortened the period of healing by an average of almost 32%. The embryonic material referred to was called "epicutan."

Waugh (1940) used this protein derivative of embryonic tissue, epicutan, and presents eighteen case histories which demonstrate marked acceleration of wound healing under the influence of the extract. The wounds were powdered, epicutan being adsorbed to kaolin powder, and covered with sterile gauze. The dressings were changed every five days. These results added impetus to the claim that epicutan reduces the time of healing by 30%.

Numerous investigations then were performed on the application of embryonic extracts to experimental wounds in laboratory animals and to indolent wounds in humans. In the majority of cases, favorable results were obtained.

It was therefore natural to determine by animal experiments whether cell growth-promoting adult tissue extracts could also accelerate the course of wound healing.

In 1944, Auerbach and Doljanski recorded experiments on rats with parenterally applied cell growth activating extracts. The experiments were performed on 30 white rats, averaging 180 grams in weight. In each experiment two animals of equal size and of the same sex were chosen. One circular wound of 20 mm. diameter was produced on the back, the skin being excised down to the superficial fascia. The wounds were treated by injecting intraperitoneally 1 cc. of saline extract of adult chicken heart on every second day. The control animals remained untreated. The experimental and control wounds were measured at intervals of 48 hours and injections continued until the wounds were completely healed. The results indicated that from these injections the closure time of the wounds was on the average of 21.8% shorter than in the untreated controls. These results were in agreement with earlier experiments which observed stimulation of wound healing following injections of extracts of embryonic tissue.

In 1946, Hoffman, Dingwell and Andrus in a series of experiments and clinical trials used a tissue extract of sterile adult sheep hearts minced in a blender with tyrode's solution to stimulate healing. A group of five human volunteers were obtained in an effort to evaluate the stimulating effect of the extract without relying on clinical impression, secondhand controls or experimental animals.

Under novacaine anesthesia wounds were made on both anterior thighs of nearly equal size and depth. They varied from 3 to 4.5 sq. cm., and from simple complete skin excision, to removal of a block of tissue down to the deep fascia. After 48 hours the larger side was chosen for treatment and the contralateral lesion used as a control. An extract saturated gauze was placed in

contact with the lesion and covered with paraffined gauze while the control le-
sion was treated by leaving a Dankin pack on the wound. At regular intervals
the surface circumferences of the wounds were traced on a transparent plastic
disk, the areas measured with a planimeter and the wound sizes marked.

Four of the five treated wounds were healed before the controls, and in
one case healing was simultaneous. In the wounds in which blocks of tissue
were excised, it was felt that the accelerating effect of the extract was due
in part to the relative rapidity with which granulation tissue filled the de-
fect or gap. Hoffman et. al., concluded that in adult sheep heart extract
there was a substance or substances which exerted a growth-promoting effect
when added to any heterologous tissue either in vitro or in human wounds.
They further felt that such substances acted directly on the metabolic mech-
anism of cellular proliferation.

Negative results however were obtained by Dvorak and Bryan (1933), using
macerated tissue of various organs, and Young et. al., (1946), using adult
sheep heart as the stimulating agent.

Menkin (1941) in an endeavor to find out if there may not be a growth-
promoting factor or factors in inflammatory exudates, injected subcutaneously
the exudate of rabbits repeatedly into the base of the ear of other normal rab-
bits. He found a marked irregular proliferation of new cells. There also was
a marked proliferation and keratinization of the epithelial layer. He con-
cluded that these results suggested the presence of growth-promoting factors
liberated by injured cells which accounted for the proliferative forces in the
region of the area of injury.

From this wound hormone hypothesis, Young, Fisher and Young (1941) mea-
sured the rate of closure of primary and secondary experimental wounds in

rabbits. A series of 657 open wounds in the skin were studied. The secondary wounds healed more rapidly than the primary ones in a statistically significant number of cases. They were of the opinion that some accelerating factor must operate in the healing of a secondary wound which is lacking in the healing of the primary. They regarded the primary as the source of the factor which accelerates or tends to accelerate the secondary. They thought it conceivable that this factor might be a diffusable growth-promoting substance or trephone.

Auerbach (1952) attempted to account for the slowing down of the proliferation of granulation tissue in the last phase of wound healing prior to closure by epithelization. He proposed that this slowing down of the proliferation was caused by the liberation of an inhibiting factor in the organism. To test this hypothesis, two experimental wounds of equal size were produced at different time intervals, so that any inhibiting influence produced in the last phase of the first wound healing process could be made to coincide with the active phase of proliferation of the granulation tissue in the second wound.

The wounds, 20 mm. in diameter, were produced in 16 animals. One wound was placed on one side of the dorsal midline and a second wound of equal size added symmetrically on the other side after an interval of six-seven days. In order to assess the time during which the assumed inhibitory substance was effective, three other sets of experiments under the same conditions were utilized but the time intervals of the second wound were varied in each.

The results showed that an inhibiting effect appears during a limited period of the wound healing process, which has its influence on another healing process in the same organism. Auerbach thought that this effect might indicate the existence of an inhibiting factor appearing during this period.

Sandblom and Muren (1954) repeated the experiments of Young, Fisher and Young in an attempt to verify the wound hormone theory. The method employed was essentially the same as that applied by Young et. al. After the hair on the back of the rabbits was clipped, three circular defects 16 mm. in diameter were made through the skin, but not through the subcutaneous muscle. Five days later, the circumference of the wounds was traced on cellophane and transferred onto paper graduated in sq. mm. for measurement of the size of the wounds. This procedure was repeated every other day until epithelization was complete. In one series ten rabbits were used. A cutaneous defect was made on one side and ten days later on the other side. The secondary wounds here healed faster than the primary thus confirming the reports of Young et. al. However, in the second series of animals the conditions were identical except the animals were shorn on one side at a time, the hair on each side being clipped just before the production of the skin defect. In this series, the secondary wounds healed practically the same as the primary.

The investigation failed to produce any evidence favoring the assumption of the release of a repair-promoting factor in the organism following trauma. The difference found in the rate of healing in recently shorn areas and in less recently shorn areas was explained by the probable differences in the circulation of the skin suddenly deprived of its protective coat and thereby exposed to environmental temperatures. Another possibility was that the trauma sustained by the skin in connection with the clipping or shaving of the hair might in some way temporarily influence the healing capacity by releasing some humoral principle capable of inhibiting tissue repair.

The same negative results were also recorded by Williams, Mason and Brad-

shaw in 1951.

Up to 1950, the literature reports all previous experiments suggesting the presence of wound hormones. However, after that date none did again until 1957 when Menkin obtained a growth-promoting factor from inflammatory plural exudates. This agent stimulated the growth of breast tissue as well as of epithelium of the skin. Menkin states that the tissue changes were possibly "precancerous" in nature, but at the same time considers the growth factor as possibly significant in tissue repair after inflammation. He states that this agent was not a nucleoprotein, but a peptide structure was suggested.

In 1964, Calnan, Fry and Saad pursued further this concept and they again concluded that there was no evidence for a circulating wound hormone or wound healing substance. Their experiments involved the local effect of resuture, the effect of multiple sequential rupture and resuture, and the effect of time on secondary suture in parallel wounds on the back of rats. All of these investigations measured tensile strength of the wounds as an index to healing. The results supported the theory of some local wound-healing factor in the wound edges, but not a circulating hormone.

Lundy and Adams (1944) reported that the presence of blood seemed to be a stimulating effect on the growth of vascular and connective tissue. They thought that glutathione was responsible for the beneficial effect. Their report gave examples of a variety of wounds which appeared to respond to the application of dried erythrocytes. The dried powdered erythrocytes were applied with a sterile spatula or dusted on from a sterile container and the wound then covered with a sterile gauze dressing. The condition of the wound improved gradually but steadily.

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With this concept in mind, Cole (1948) used placental blood and pooled red cells in an endeavor to stimulate cellular proliferation. The placental blood was collected, pooled and then mixed with sterile K-Y jelly so that the consistency was that of a paste. The mass of red cells before being used was mixed with an extract of placenta, the extracts being obtained by squeezing the placentas of the same patients whose blood was used. Of the ten case histories presented, Cole found the placental mass to be more potent in its healing properties than the red cell mass. He thought that this might be due to a greater amount of "growth hormone," steroids, glycogen or vitamin C in placental tissue.

Although during the past decade the importance of protein metabolism in the healing process had been emphasized, the manner in which lack of protein affected healing was still a controversy. In a series of experiments by Kobak et. al. in 1947, male rats were divided into two groups, one of which was maintained on a low protein ration containing 1.9% protein, and the other on a control ration containing 21.1% protein. The diets were isocaloric and contained an adequate quantity of vitamins, minerals and fat.

Following the period of protein depletion, the animals were fasted for approximately eight hours, weighed and anesthetized. A wound was then produced by an upper abdomen incision approximately 2.5 cm. in length. The wound was repaired with stainless steel sutures. On selected post operative days the animals were again fasted, weighed, anesthetized and blood was withdrawn for serum protein determination. Tensile strength determinations were made on both groups. The effect of protein deficiency resulted in a characteristic delay in healing from the third to the fifth post operative days. Fibroplasia occurred

despite the lowered serum proteins, although it was marked by a delay in its early phases.

Following the work of Kobak, in 1948, Localia showed that the parenteral administration of methionine to protein depleted rats shifted the curve of wound healing of abnormal wounds toward normal. In that study the healing of skin was excluded from the experiments. However, in 1949, he recorded the effect of methionine on the healing of surface wounds in protein depleted rats and humans. He found that sulfur containing amino acids restored the healing time toward normal in spite of continued protein depletion, although it did not speed the healing of surface wounds in normal rats.

With the knowledge that methionine would increase the rate of healing of experimental wounds, Williamson (1951) felt that methionine, rather than other essential amino acids, was the limiting factor in the healing process. The concept of a negative nitrogen balance existing shortly after wounding and the fact that injured animals would retain methionine sulfur and incorporate it into protein, even when this negative balance existed, prompted Williamson to try to further elucidate the role of protein metabolism in the healing process.

Four groups of 24 female albino rats were used. Group I animals were fed a basal diet and the remaining groups' diets modified from the basal in containing varying amounts of different proteins and "protein sulfur." All of the diets were isocaloric and contained the same amount of vitamin supplement as in the basal diet. Twenty-four hour urine samples were collected during the course of the experiment and the urine was analyzed for total nitrogen, urea nitrogen, amino acid nitrogen and total sulfur. Williamson showed that the protein sulfur retention appeared to be correlated with the rate of healing. He suggested that the amount of retained sulfur in excess over that utilized

for normal tissue protein synthesis was an important factor in determining the rate of healing of experimental wounds.

In 1952, Williamson again repeated these experiments but this time in an attempt to see if wound healing could be affected by cystine to the same extent as an equivalent amount of methionine, on the basis of sulfur. The experiments were carried out in a similar manner to those previously reported. He found that equivalent amounts of cystine and methionine, on the basis of sulfur, have the same effect on the healing index as calculated from tensile strength data. A correlation between the sulfur retention and the healing index also was observed, for after wounding there appeared to be a greater retention of sulfur than might be expected from the amount of nitrogen which was retained. (Usually N:S is 15:1).

Since the conversion of methionine to cystine is irreversible in vivo, he concluded that the methionine in the diet is first converted to cystine before it becomes available for the healing process. Further work, however, indicated that methionine, per se, is required to some extent during wound healing, over and above that which is converted to cystine. This was proven by using ethionine which blocks the conversion of methionine to cystine as well as the incorporation of methionine into protein.

Tier in 1951 developed a theory of the growth and multiplication of the cells in the animal organism. His theory proposed that growth and multiplication would be governed on one hand by local growth regulating factors in the tissue itself and on the other hand by a superior system, in which the growth hormone of the anterior lobe of the hypophysis would be one component. The superior system of this theory was proven by Larsen in 1949 on investigations of recessive anterior pituitary hypoplasia.

Tier injected an aqueous extract of mashed outer orbital gland tissue intraperitoneally into rats. This tissue was used because the mitotic ratio is very high at birth but decreases during the growth period, so that mitosis are very seldom found after the rats have become full grown. From an extract of the right outer orbital gland of white rats, he observed stimulation of mitotic cell division both in the left outer orbital gland and also in the right and left inner orbital glands consisting of similar tissue. However, the age of the donors and the receivers, and the quantity of the gland tissue were of decisive importance. Tier concluded that growth promoting agents are re- leased or formed in the treated organ which influence, probably by way of the blood, mitotic cell division in homologous tissues. The mitosis stimulating effect of the tissue extract was interpreted as a proof of the correctness of his growth theory.

Later in the same year, Tier continued his studies by injecting extracts of skin of newborn rats into two month old rats and obtained clear stimulation of mitotic cell division in the skin. To further confirm his assumption of organ and species specificity, Tier chose extracts of embryonic skin from man and mouse, the latter because of the close relation of this species with the rat. With intraperitoneal injections of skin extracts of heterologous species no increase of the mitotic ratio was obtained in the skin of two month old rats as had earlier been observed, with similar skin extracts of newborn rats. Embryonic skin of men and of mice also gave negative results. Here again it was shown that the agent which stimulated mitotic cell division activity, when a tissue is mashed, is apparently species specific in homologous tissue.

Again in 1961, by using homologous skin homogenates he was able to estab-

lish a significant stimulation of epithelization when the skin homogenates were applied both pretraumatically and during the healing process. The specific healing factor in rat skin homogenate was assumed to influence primarily the epithelization.

Kelly and Jones (1953), studying the influence of homologous tissue factors on DNA turn over and radiation protection, found that DNA turnover can be increased, at least in liver and spleen of normal mice, by repeated injections of various fractions of homologous tissue mashes and extracts of tissue mashes. Although these authors were interested in the question of protection after exposure to radiation, they showed evidence that substances capable of stimulating DNA formation can be obtained from mashes and extracts of rapidly growing tissues. The increased DNA synthesis was interpreted by them as increased cell growth.

Prudden et. al., (1957) used heterologous cartilage powder to accelerate wound healing. Parenteral methionine also was used under his test conditions but it had no significant effect. Again in 1963, he was able to extract a repair-stimulating principle from cartilage in sufficient concentrations so that parenteral administration caused a marked acceleration of wound healing. It was suggested that a protein, probably associated with an acid mucopolysaccharide, was responsible for the activity. In 1965, Prudden showed documentary proof that bovine tracheal cartilage contained a repair-stimulating principle or wound accelerator. His experimental evidence was carried out in guinea pigs, mice, dogs, rats and men. The material can be applied topically or extracts from acid-pepsin digested bovine tracheal cartilage with isotonic saline solution can be injected subcutaneously. The cartilage preparations will ac-

celerate the healing of clinical wounds over a period of time ranging from the sixth to the fourteenth post operative days. The behavior of the material prior to seven days was not investigated but such an investigation is currently being conducted.

In 1960, Edwards et. al., inserted a polyvinyl sponge (Ivalon) into the subcutaneous tissues of animals. The sponge acted as a framework for granulation tissue which permeated its pores. This method permitted them to study only one aspect of the healing process, namely, the growth and differentiation of granulation tissue in a special medium within a standard environment. The pores of control sponges were filled with equal parts of heparinized autologous plasma and Tyrode's solution, while those of experimental sponges were filled with the plasma plus a tissue extract. The extracts were prepared by extracting homogenized chick embryos, autologous spleen or autologous sterile post-traumatic granulation tissue with Tyrode's solution. The sponges were embedded on each side of the midline in intramuscular wounds in erector spinae muscles of rabbits. Microscopic sections showed an increased number of fibroblasts and a deeper penetration of all components of granulation tissue into the pores of the experimental sponges. Also, there was an increased rate of maturation of collagenous fibrils in the sponges containing clots permeated with the tissue extracts.

Edwards concluded that it was possible to increase the rate of fibroblastic proliferation, capillary formation, and maturation of newly formed collagen in granulation tissue growing into a closed plasma clot system within a wound by addition of tissue extracts to that system.

TISSUE CULTURE MEDIA

The problem of duplicating the environment or of providing a suitable substitute environment for cells was first attempted by Arnold in 1887. He used slices of elder pith soaked in aqueous humor from the frog's eye and implanted them in the peritoneal cavities of other frogs. After a few days the slices of pith were removed, placed in a dish containing a second supply of aqueous humor and under the microscope Arnold was able to observe migration of leukocytes, their division and the process by which they engulfed debris.

Harrison (1907), using essentially the same method as that of Arnold, isolated bits of embryonic frog ganglia in drops of lymph drawn from the frog and again under the microscope observed and was able to prove that nerve fibers arise solely by growth from the ganglia.

These two experiments were essentially the beginning of tissue culture work and since that time many methods have been employed to provide an adequate surrounding or environment similar to the aqueous humor used by Arnold, the lymph by Harrison and the blood plasma or serum by Burrows in 1911.

The most widely used organic complexes are tissue extracts, especially extracts of chicken as introduced by Carrel in 1913.

The extracts were made of chick embryos from six to twenty days old, and of spleen, kidney, muscle, etc., of the adult chicken. The tissues were either cut into small fragments or ground, and to one volume of tissue, one-third of a volume to four or five volumes of Ringer's solution was added. The culture medium was composed of one volume of extract and two volumes of hypotonic plasma.

The majority of Carrel's experiments were performed on hearts of chick

embryos from seven to fifteen days old. In every experiment the fragments of heart, cultivated in plasma containing an extract, grew more rapidly than their controls. His experiments showed that extracts of tissues and tissue juices, under certain conditions such as dilution, heating and filtration, accelerate the growth in vitro of the connective tissue from about three to forty times.

Doljanski and Hoffman (1943) used adult chicken heart muscle and adult chicken brain to maintain their cell colonies. The tissues were minced and suspended in four times its volume of Tyrode's solution. Tissue cultures were made from heart muscle of adult fowls. The medium was composed of 0.5 cc. adult fowl plasma diluted with 1 cc. Tyrode's solution. Two drops of adult heart extract were added in order to coagulate the plasma. The supernatant fluid phase was composed of 0.5 cc. tissue extract. It proved possible to cultivate colonies of fibroblasts derived from adult heart muscle in media composed of adult fowl plasma and extracts of adult animal tissue continuously.

The complexity and variability of these natural substrates made it impossible to employ them in experiments designed to determine the substances that were actually utilized by the cells for survival and multiplication.

Morgan, Morgan and Parker (1950) devised a synthetic medium of chemically known composition that would promote continuous cell multiplication of small amounts of tissue in the absence of blood serum and embryo extract. These workers first prepared a number of basic stock solutions and combined these in various proportions. In the preparation of the synthetic mixture, 25 ml. of an amino acid basal mixture were taken and to this were added appropriate volumes of other stock solutions. Each new ingredient, or group of ingredients added to the basal solution, was first studied over a wide range of concentrations

to detect possible toxic effects, and were finally incorporated into the mixture at a level which was either beneficial or, at least, non-toxic. Approximately 3,000 cultures were used to test all of the mixtures studied.

The contents of the final mixture, 199, can be visualized from the chart which follows.

Tissues cultivated in mixture 199 were found to be supported as extensive areas of new growth, and cell life was maintained for an average of four-five weeks.

MORGAN, MORGAN AND PARKER'S MEDIUM NO. 199

	Milligrams per 1000 ml.		Milligrams per 1000 ml
L-Arginine	70.0	Riboflavin	0.01
L-Histidine	20.0	Pyridoxine	0.025
L-Lysine	70.0	Pyridoxal	0.025
L-Tyrosine	40.0	Niacin	0.025
DL-Tryptophan	20.0	Niacinamide	0.025
DL-Phenylalanine	50.0	Pantothenate	0.01
L-Cystine	20.0	Biotin	0.01
DL-Methionine	30.0	Folic Acid	0.01
DL-Serine	50.0	Choline	0.50
DL-Threonine	60.0	Inositol	0.05
DL-Leucine	120.0	P-Aminobenzoic Acid	0.05
DL-Isoleucine	40.0	Vitamin A	0.10
DL-Valine	50.0	Vitamin D	0.10
DL-Glutamic Acid	150.0	Vitamin K	0.01
DL-Aspartic Acid	60.0	Vitamin E	0.01
DL-Alanine	50.0	Ascorbic Acid	0.05
L-Proline	40.0	Glutathione	0.05
L-Hydroxyproline	10.0	Cholesterol	0.2
Glycine	50.0	Tween 80	20.0
Cysteine	0.1	Sodium Acetate	50.0
Adenine	10.0	L-Glutamine	100.0
Guanine	0.3	Adenosine Triphosphate	10.0
Xanthine	0.3	Adenylic Acid	0.2
Hypoxanthine	0.3	Ferric Nitrate	0.1
Thymine	0.3	Ribose	0.5
Uracil	0.3	Deoxyribose	0.5
Thiamin	0.01		

WOUND HEALING

The local tissue reaction to non-specific injury is inflammation. When injury occurs or a lesion is produced in loose connective tissue, there is an extravascular accumulation of fluid and cells. The fluid component of the exudate tends to dilute the causal agent, thus lowering its concentration in tissues, and to provide antibodies to antagonize the effect of toxins and favor phagocytosis. This component also contains fibrinogen which may be transformed into fibrin thus creating a barrier to the inflammatory process, a blockage of the lymphatic capillaries, and a scaffolding upon which the repair process may begin. Menkin (1946) suggests that fibrin is deposited in tissues and lymphatic capillaries to act as a filter and stop the spread of the inflammatory process. The cellular components of the exudate are responsible for phagocytosis and the removal of foreign organisms and debris.

Tamayo (1961) divides the sequences of wound healing into three separate sections: a) cellular activity, b) vascular proliferation, and c) deposit of intercellular substances.

Cells present in the area of injury, such as macrophages and monocytes, clear the injured area of hemorrhage and necrotic debris. As the cellular exudation of acute inflammation subsides, the fibroblast rapidly becomes the dominant cell. The origin of proliferating fibroblasts is widely disputed. Allgower (1956) claims that they are derived from undifferentiated histiocytes while McDonald (1959) postulates that fibroblasts can originate only from other fibroblasts. However, in all probability these fibroblasts are derived from undifferentiated connective tissue cells present in the area.

The first stage of cellular activity is one of mobilization and prolif-

eration of fibroblasts. These cells are irregular in distribution but are more numerous around blood vessels. From approximately the fourth day, the fibroblasts show very fine protoplasmic prolongations which are condensations of intracellular fibrils. From this time on, the cells are functionally engaged in laying down interstitial fibrils. The second stage of fibroblast activity is dominated by collagen fiber formation. The cells become less conspicuous and are replaced by fibers so that at the end of eight to ten days fewer elements are seen between the fully developed collagen bundles (Edwards and Dunphy, 1958). When this stage is reached, the cells are known as fibrocytes. Studies in the rabbit ear chamber by Wasserman (1954) show that connective tissue fibers appear both within and without the cytoplasm of fibroblasts as a very fine reticulum. When intracellular, the fibrils become thicker as they approach the cellular membrane and acquire the staining characteristics and the 650A° periodicity, which is fully developed when they leave the cell. However, Jackson (1956) showed that in the growing chick embryo collagen fibers appear first in the intercellular space and that the cell secretes a globular, soluble protein which becomes insoluble through the effect of acid mucopolysaccharides.

The first vascular buds begin to appear two or three days after injury, originating from pre-existing vessels close to the lesion. These sproutings anastomose with each other to form arches at different levels between the lesion and the periphery (Tamayo, 1961). When there is loss of substance, the capillaries bulge on the surface of the lesion and can be seen as small reddish granulations, so the term "granulation tissue."

During the first stages of repair, the interstitial spaces show edema which is the manifestation of increased capillary permeability. Chemical an-

alysis of edematous fluid from an implanted plastic sponge showed an accumulation of hexoseamines, gamma globulins, lipoprotein, cholesterol, phopholipids and amino acids, especially glycine, lysine and proline (Edwards and Dunphy, 1958). The edema which results from capillary permeability increases in the following two or three days following injury during which it acquires a progressively increased concentration of mucopolysaccharides (Taylor and Saunders, 1957). These compounds form a part of the mucoproteins of connective tissue ground substance. Four to six days after injury the highest concentrations of these carbohydrate complexes are reached and from then on they decrease until at the end of ten to twelve days the wound is found to have normal or even lower concentrations of these substances (Tamayo, 1961).

When the protoplasmic prolongations of fibroblasts become apparent as very fine fibrils in the intercellular space, they become arranged perpendicular to the capillaries. The thickness of the fibers is increased and their straight course is changed to a more wavy one characteristic of mature collagen bundles.

At the end of ten to twelve days, with completion of healing, the intercellular substance is made up of fully developed collagen fibers, few blood vessels and fibrocytes scattered in between.

In the normal pattern of wound healing, the concept of Dunphy (1956) illustrates the sequence of events just described. This pattern consists of two different stages. 1) Productive or substrate phase which begins shortly after wounding and lasts for about five days. During this period mucopolysaccharides and soluble protein precursors of collagen, the building blocks of repair, are produced. 2) The collagen phase in which normal collagen fibers are formed.

This phase begins about the fifth day and continues until healing is complete. Dunphy's concept postulates an active and preparatory period with intense accumulation of building materials deposited by the cells and blood in preparation for the collagen phase of wound healing.

METHODS AND MATERIALS

Five male albino mice weighing between 25-35 grams were used in this study. The animals were obtained commercially and housed in wire bottom cages. They were maintained on a Purina Chow diet and water ad libitum.

Three animals were used for the test condition while the remaining two served as controls. Using ethyl ether inhalation anesthesia, an air pouch was formed on the back of each of the five animals simulating the granuloma pouch technique of Selye. The pouch is designed to permit the objective quantitative analysis of factors regulating inflammation and wound healing. With it an almost perfectly symmetrical, ellipsoidal air space of any desired size can be created by injecting a given amount of air into the loose subcutaneous connective tissue. The cavity thus formed acts as a "mold" for the subsequent formation of a granulamatous wall or capsule.

Fifteen (15) cc. of air were injected into the loose connective tissue, between the shoulder blades, through a No. 25 hypodermic needle. After a 24 hour period each of the test animals received an injection of 5 cc. of the artificial substrate, T199,[1] directly into the preformed pouch. As the needle was withdrawn, the injection site was held for an undetermined period of time so as not to allow the escape of any of the fluid media. The two control animals each received injections of 5 cc. sterile saline in a like manner.

Twenty-four hours following the first series of injections the test animals were again given 5 cc. of the fluid substrate directly into the air pouch

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1. Obtained from Grand Island Biological Company, Grand Island, New York.

while the controls received 5 cc. sterile saline.

Seventy-two hours following the formation of the pouch, each of the five animals received intraperitoneal injections of tritiated thymidine (Sp. Act. 1.9c m/mole) [1] at the dose rate of 1 microcurie per gram body weight. The thymidine was administered by tuberculin syringes to insure accurate measurement.

One hour following thymidine injections, all animals were sacrificed by placing them in a large jar which contained a cotton pad saturated with ethyl ether.

The fluid filled pouches were dissected in toto from each of the specimens and samples of tissue were placed in 10% formalin for autoradiographic analysis.

These tissue samples were embedded in parrafin and sectioned at three to six microns. To prepare the autoradiographs, NTB₃ Emulsion [2] was used. The autoradiographic emulsions were exposed for four weeks at 4° C., developed, washed, fixed and stained with nuclear fast red and indigo carmin.

The number of labelled nuclei as well as non-labelled cells in the tissue adjacent the fluid filled pouches in each of ten random oil immersion fields were counted from each section of tissue received. The mean of labelled and non-labelled nuclei were calculated and a table of the percentages of labelled in comparison to unlabelled cells prepared. The criteria for labelled nuclei was based upon the number of grains found above each cell. A minimum of five grains above background was selected as the basis for counting.

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1. Obtained from Schwarz Bioresearch Inc., Orangeburg, New York.

2. Research Division, Special Products, Rochester, New York.

The remaining samples of tissue were prepared for liquid scintillation counting. Sections of tissue were air dried and weighed on the same analytical balance and the weights recorded for subsequent determination of tritium activity. Each sample was placed in a 20 milliliter glass screw top vial which was made of glass having a low radioactive potassium (K^{40}) content in order to minimize the effect of the natural radioisotope.[1] The samples were then brought into solution by means of one milliliter of a one molar solution of hyamine hydroxide in methanol. This process was hastened by placing the vials in an incubator, temperature ranging from 58-60° C., for a period of 72 hours.

Twenty milliliters of scintillation solution were added to each of the counting vials containing dissolved tissue as well as to a background blank vial which contained only hyamine hydroxide, after they had been cooled to room temperature. This solution was prepared by dissolving: (1) 1.5 grams of 2-5 diphenyloxazole B (PPO), (2) 50 mg. of 1,4-D1 2-(5-Phenyloxazole) benzene (POPOB) into 500 ml. toluene.

The samples were then mixed and placed in the dark at 4° C. for one day until counted. Counting was done at 4° C. with a Packard Tri-Carb liquid scintillator [2] and ten minute counts were made on each sample. All samples were counted four times.

In order to determine the internal quenching effect of the tissue samples, 0.01 ml. of a 0.02 microcurie tritium solution was added to the blank and the representative samples to act as an internal standard. The vials were again

1. Obtained from Nuclear Chicago Corp., Des Plaines, Ill.

2. Packard Tri-Carb Scintillation Counter, Courtesy Dental Research Dept., Hines V.A. Hospital, Hines, Ill.

recounted four times.

The results of these series of counts were then expressed as counts per minute per milligram of tissue. The relative disintegration rate per minute of the samples was determined. This allows for internal quenching and internal standards.

EXPERIMENTAL RESULTS

The extent of radiolabelling of cells surrounding the fluid filled pouches was determined by autoradiographic analysis of labelled cells adjacent to the wall of the pouch along with liquid scintillation counting. Since the air-filled pouches in both test and control animals were created in the same manner, a histologic description of the wall or capsule surrounding the fluid substrate and adjacent tissue will suffice for both animals.

The tissue immediately adjacent to the pouch contained several layers of mononuclear round and fusiform cells with large, ovoid nuclei. These cells appeared undifferentiated and constituted the majority of labelled cells. There were also a few scattered polymorphonuclear neutrophils and blood lymphocytes seen in this layer.

Immediately adjacent was a moderately thin band of striated muscle bundles, the panniculus carnosus. This layer also shows dense connective tissue containing an abundance of collagen fiber bundles and fibroblasts. Many of the fibroblasts were observed to be labelled. A few scattered mononuclear inflammatory cells and histiocytes also were present.

Numerous hair follicles and a few sebaceous glands were seen in the corium underlying the epithelium which contained spindle shaped fibroblasts and scattered collagen fibers.

The surface layer presented a rather thin layer of keratinized stratified squamous epithelium with heavy labelling of many of the basal cells.

Autoradiographic analysis indicated a highly significant difference in the number of labelled cells found along the wall of the pouch in test animals as compared with controls (Table 1). Table 2 shows the percentage of labelled in

comparison with unlabelled cells in three test and two control animals. The percentage index of labelled cells was 8.5%, 7.7% and 7.5% for the animals receiving injections of T199 basic substrate whereas saline injected animals only showed a 6.0% and 6.3% labelled cells. By obtaining a grand mean of labelled cells counted for both test and control animals, the standard deviation was then determined along with the standard "t" test. The significance of difference was shown to be 10.7 between test animals receiving T199 and saline control animals.

The data collected from liquid scintillation counting failed to demonstrate a statistically significant difference in the uptake of tritiated thymidine in the tissues surrounding the fluid filled pouches in test and control populations. The significance of difference from these results was 1.5.

Autoradiographic analysis of tissues found along wall of air pouches.

Table 1

SAMPLE	MEAN	SUMS OF SQUARES	STANDARD DEVIATION
TESTS	6.13	18.63	0.99
CONTROLS	2.90	4.17	0.56

$$\text{Standard Error Difference} = \sqrt{\frac{(0.98)^2}{20} + \frac{(0.56)^2}{14}}$$

$$= \sqrt{0.05 + 0.04}$$

$$= \sqrt{0.09}$$

$$= 0.3$$

$$t = \frac{6.13 - 2.90}{0.3} = \frac{3.23}{0.3} = 10.7$$

Table 2   Percentage Labelled Cells

	LABELLED CELLS	UNLABELLED CELLS	PERCENTAGE
Test 1	5.87	63.1	8.5
Test 2	6.40	76.7	7.7
Test 3	5.96	78.6	7.5
Control 1	2.80	43.2	6.0
Control 2	3.00	44.5	6.3

RESULTS OF SCINTILLATION COUNTING PROCEDURE

Table 3

SAMPLE	UNCORRECTED COUNT/mg./minute	CORRECTED COUNT/mg./minute	DIFFERENCE FROM MEAN	DIFFERENCES SQUARED
T1-1	86.4	100.6	13.2	174.24
T1-2	106.7	110.9	2.9	8.41
T1-3	77.8	89.5	25.3	740.09
T1-4	107.0	159.2	45.4	2051.16
T1-5	88.6	99.8	14.0	196.00
T1-6	120.5	136.2	22.4	501.76
T1-7	97.6	100.8	13.0	169.00
T2-1	90.5	99.1	4.3	18.49
T2-2	57.4	73.9	29.5	870.25
T2-3	108.1	119.0	15.6	243.36
T2-4	95.3	175.2	71.8	5155.24
T2-5	63.0	86.8	16.6	275.56
T2-6	64.2	74.4	29.0	841.00
T2-7	79.5	95.6	7.8	60.84
T3-1	69.3	85.7	11.2	125.44
T3-2	52.5	65.7	31.2	973.44
T3-3	98.7	115.1	18.2	331.24
T3-4	85.6	86.1	10.8	116.64
T3-5	105.7	125.6	28.7	823.69
T3-6	87.6	103.7	6.8	46.24
C1-1	77.5	92.7	11.6	134.56
C1-2	75.3	93.9	10.4	108.16
C1-3	112.1	158.1	53.8	2894.28
C1-4	88.0	102.2	1.9	3.61
C1-5	113.4	127.3	23.0	529.00
C1-6	100.0	146.1	41.8	1747.24
C1-7	97.5	109.9	5.6	31.36
C2-1	76.5	87.5	4.1	15.81
C2-2	72.5	85.7	2.3	5.29
C2-3	89.3	98.2	14.8	219.04
C2-4	71.4	78.9	4.5	20.25
C2-5	76.2	94.4	11.0	121.00
C2-6	66.9	74.2	9.2	84.64
C2-7	59.3	65.3	17.9	320.41

RESULTS OF SCINTILLATION COUNTING PROCEDURE

Table 4

SAMPLE	MEAN	SUMS OF SQUARES	STANDARD DEVIATION
TEST 1	113.8	5450.66	30.1
TEST 2	103.4	7464.74	35.3
TEST 3	96.9	2416.69	22.0
CONTROL 1	104.3	5448.21	30.1
CONTROL 2	83.4	787.44	11.5

$$\text{Standard Error of Difference} = \sqrt{\frac{(29.1)^2}{20} + \frac{(20.8)^2}{14}}$$

$$= \sqrt{\frac{846.81}{20} + \frac{432.64}{14}}$$

$$= \sqrt{42.34 + 30.9}$$

$$= \sqrt{73.24}$$

$$= 8.5$$

$$t = \frac{105.1 - 93.8}{8.5} = \frac{11.3}{8.5} = 1.5$$

CALCULATION OF QUENCHING EFFECT

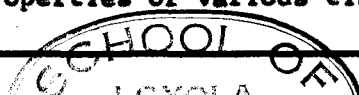$$\text{Quenching} = \frac{(B+IS) - B \;(T+IS) - T}{Twt} \quad \times \quad \frac{100}{IS} \quad \text{per cent}$$

B	background blank count
IS	internal standard count
T	tissue sample count
(B+IS)	background blank with internal standard added
(T+IS)	tissue sample with internal standard added
Twt	tissue weight

DISCUSSION

The use of an artificial substrate, T199, to stimulate DNA synthesis to a great extent simulates the growth promoting action of embryonic extract as introduced by Carrel (1913). It is well known that culture medium supplies the nutritional requirements for cell growth, and cells cultured in such a medium have the ability to carry out a wide variety of biosynthetic processes leading to the formation of the structural elements of cells and to their replication. The results of this study clearly indicate that T199 has a stimulating effect of DNA synthesis in vivo. This is reasonably anticipated as T199 supports connective tissue cell proliferation in vitro. These same connective tissue cells in vivo may be expected to proliferate when T199 is introduced into their environment. Thus, it appears that one of the regulatory mechanisms for growth of connective tissue must lie in the provision of an adequate substrate capable of stimulating DNA synthesis and cellular proliferation.

It is clearly evident that T199 has such a capacity for stimulation of DNA synthesis. Moreover, the significant increase in the number of such cells under the influence of T199 preparing to enter mitosis suggests that a locally applied artificial substrate indeed by itself is a growth stimulant. It is self evident that in vivo the production of new cells can occur only at the least by providing the raw materials for new cell formation. What effects other somatic growth stimulants and regulators, for example, growth promoting hormone (STH) and thyroxine, may have, are also dependent upon the in vivo substrate the reactive cells have in their environment. The results of this experiment support this concept of cell proliferation dependence upon a substrate.

In tissue cultures, the growth promoting properties of various tissue ex-

tracts, especially embryonic, have long been recognized, though the exact na-
ture of active ingredients has remained obscure (Carrel, Baker, Fisher). Sim-
ilar objections relate to attempts by a number of investigators studying vari-
ous extracts and natural substances locally applied (Waugh, Auerbach, Doljan-
ski, Teir).

The development of the chemically defined medium, 199, made it possible to
cultivate tissues for prolonged periods of time and under precisely controlled
conditions. Since the first studies on Medium 199, considerable improvements
and simplifications in its preparation have been made. It has found wide ap-
plication as one of the most adequate synthetic media for supporting cell sur-
vival for periods of 40-60 days. Cultures maintained on 199 initially exhibit
extensive areas of proliferation and these areas are subsequently maintained
intact for prolonged periods until degeneration and death ensue (Morgan, 1956).

The introduction of this chemically defined media into the preformed air
pouches under study presented a means of including as many as possible of the
nutritional factors already shown to be necessary for man and animals. Thus,
a basic substrate containing all of the elements essential for cell growth and
proliferation were present.

It is known that mitosis is preceded by a synthesis of deoxyribonucleic
acid (DNA). If a radioactive DNA precursor is administered at that time, the
nucleus becomes radioactive and may be recognized by radiographic means, even
before mitosis takes place. Such nuclei retain the label during the actual mi-
tosis and pass some of it on to the daughter cells. In Hughes' (1958) dis-
cussion of tritium labelled thymidine, he points out that, a) thymidine is a
specific precursor of DNA, which is available for only a short time after in-

jection; b) thymidine is incorporated into DNA only at the time of synthesis in preparation for cell division, and the activity is distributed to the daughter cells at the time of mitosis; c) in a non-dividing cell, formed from a labelled cell, the activity is stable for the life of the cell.

The specific advantage of tritium for autoradiography lies in the very high resolution which can be obtained because of the very weak energy and consequently short range of its Beta radiation. The maximum range in tissue of a Beta ray from tritium is only six microns, and half of the Beta's will travel less than one micron. The net distances from origin to terminus of tracks will be even less because the direction of travel changes frequently. For this reason, the activated silver grains of an autoradiogram should largely lie within one micron of their source.

Hughes suggests that thymidine may enter the cell via phosphorylation to thymidylic acid and that the thymidylic activity of tissue falls during the next hour; thereby the concentration of tritium reaches a maximum within about 45 minutes following intravenous injection or within one hour following intraperitoneal injection. Consequently, the precursor pool for CNA snythesis must become simultaneously exhausted, suggesting that all labelling of nuclei occurs during the first hour after injection.

Ficq (1959) points out that the quantitative estimation of specific radioactivity and the number of labelled molecules depends on several factors: 1) the dilution of the radioisotope in the labelled medium; 2) the concentration of the labelled substance in the organism and cells under study; 3) the content in different precursors of the substance; 4) the particular turnover of these precursors and the labelled substance; 5) the actual synthesis of the precursors and the complex molecule under study.

The extent of DNA synthesis and mitosis occurring in the tissue adjacent the T199 and saline pouches was determined following previous treatment with tritiated thymidine and subsequent autoradiography. The significance of differences between test and control animals was calculated by the standard "t" test (Table 1). Statistical analysis of the data indicated that labelling of cells in the wall of the test pouches is highly significant and clearly shows that T199 is a growth stimulating agent. The percentage labelled cells in both populations can be visualized from Table 2.

The liquid scintillation counter depends upon the production of fluorescence in a phosphor by the absorption of ionizing radiation (Bell and Hayes, 1958). The radioactive substance is dissolved directly in the liquid scintillator and Beta particles which are emitted as radioactive decay interact with the liquid scintillator to produce small flashes of light. These light flashes are detected by a photomultiplier tube.

Though liquid scintillation counting is ideal for many applications, there is one substantial disadvantage. Quenching, either by virtue of the light filtering effect of colored substances or interference by colorless substances may cause reduction of counting efficiencies (Rapkin, 1964). This problem seems to be more severe when working with the least energetic isotopes such as Beta particles, but is of little consequence when working with highly energetic nuclides. Thermal quenching occurs when the solvent absorbs Beta energy, while color quenching occurs if the solvent absorbs the light emitted by the liquid scintillator (Bell and Hayes, 1958). Packard (1958) found that for the most part the effect of quenching in tritium counting is complete elimination of individual low energy pulses since they are barely at the limits of detection.

Brown (1961) found that Hyamine tends to cause yellowing of many substances during the course of solubilization, especially if heating is required. They demonstrated that sonically solubilized samples had less color and counted with as much as twice the efficiency of samples solubilized by heating.

Data obtained from liquid scintillation counting failed to demonstrate a statistically significant difference in the uptake of tritiated thymidine in the tissues surrounding the pouches (Table 4). However, the results were sensitive enough to appreciate an increase in thymidine uptake between populations. The lack of statistical significance in scintillation values was perhaps due to one or more of several factors which are unknown at present: 1) random sampling of tissue which may not have been a representative fraction of specific area under study; 2) quenching effect of tissues in solution; 3) some unknown human error. The results of the scintillation data indicate that it is essential that autoradiography be performed when dealing with studies of this nature.

From the over-all data thus collected, it appears that the substrate, T199, in some manner augments cells for synthesis of DNA. It is strongly felt that one of the regulatory mechanisms of growth of connective tissue must therefore simply be the amount of available substrate present for cells undergoing synthesis and division. One also may state that not only is this basic substrate essential for the growth of cells, but in fact has the capacity to stimulate growth and cellular proliferation.

SUMMARY

An ellipsoidal air pouch was formed on the back of five young mice by in-
jecting 15 cc. of air into the loose subcutaneous connective tissue between the
shoulder blades. After a 24 hour period each of three test animals received
an injection of 5 cc. of artificial substrate, T199, directly into the pre-
formed pouch. Two control animals each received injections of 5 cc. sterile
saline in like manner.

Twenty-four hours following the first series of injections the test ani-
mals were again given 5 cc. of the fluid substrate directly into the pouch
while the controls received 5 cc. sterile saline.

Seventy-two hours following the formation of the pouch each of the five
animals received intraperitoneal injections of tritiated thymidine (Sp. Act.
1.9 curies/millimole) at the rate of 1 microcurie per gram body weight. One
hour following thymidine injections, all animals were sacrificed.

Radiolabelled cells and radioactivity were observed in the wall or cap-
sule of the pouches by means of autoradiographs and a liquid scintillation
counter.

Autoradiographic analysis of tissue surrounding the pouches showed a sta-
tistically significant increase in labelled cells of animals receiving injec-
tions of the basic substrate, T199, as compared with their saline controls.
Liquid scintillation data showed an increase in thymidine uptake between the
two populations.

The results of this study clearly indicated that the basic tissue culture
substrate, T199, had a stimulating effect on DNA synthesis in vivo. This was
in support of the concept of cell proliferation dependence upon an available

substrate. It was concluded that one of the regulatory mechanisms for growth

of connective tissue must lie in the provision of an adequate substrate capable

of stimulating DNA synthesis and cellular proliferation.

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APPENDIX

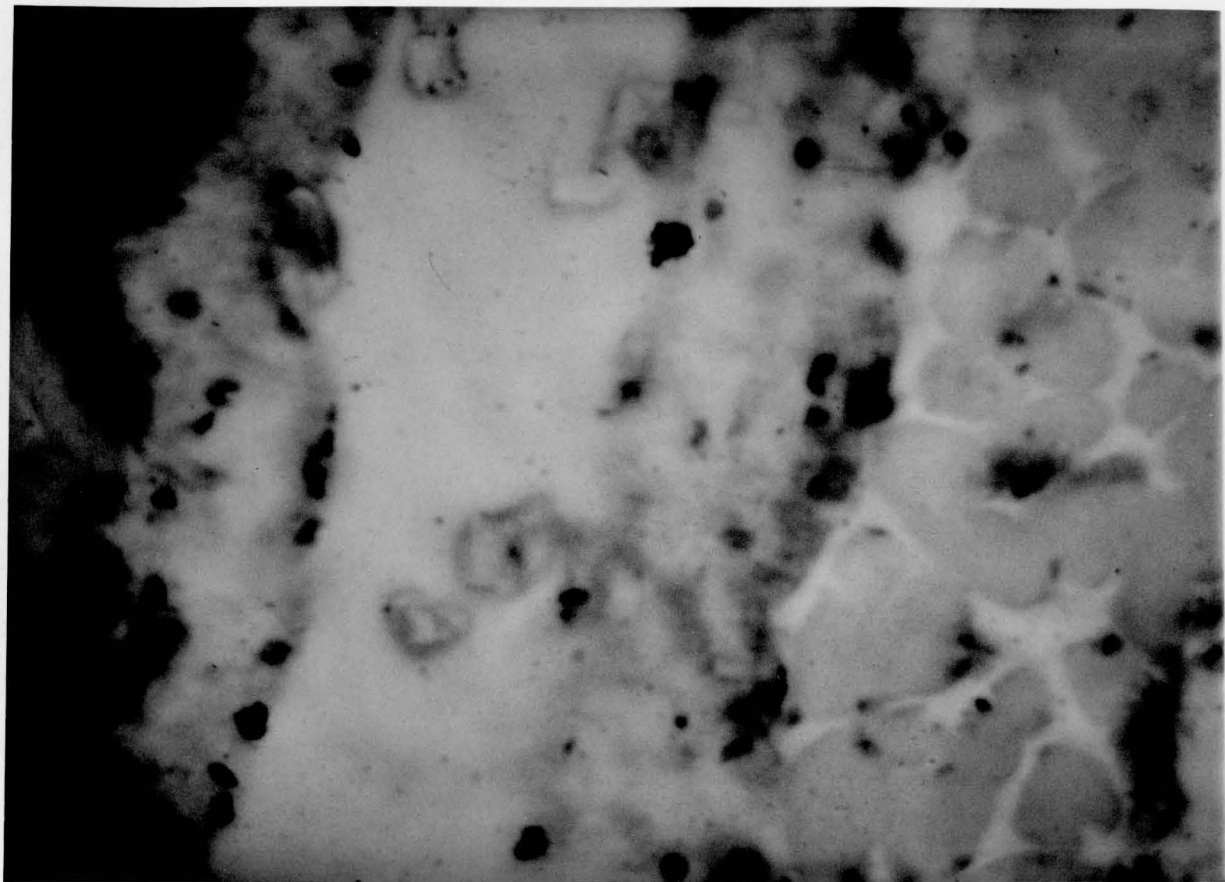FIGURE 1 Example of fluid filled pouch on back of young mouse.

FIGURE 2 Representative example of labelled cells within
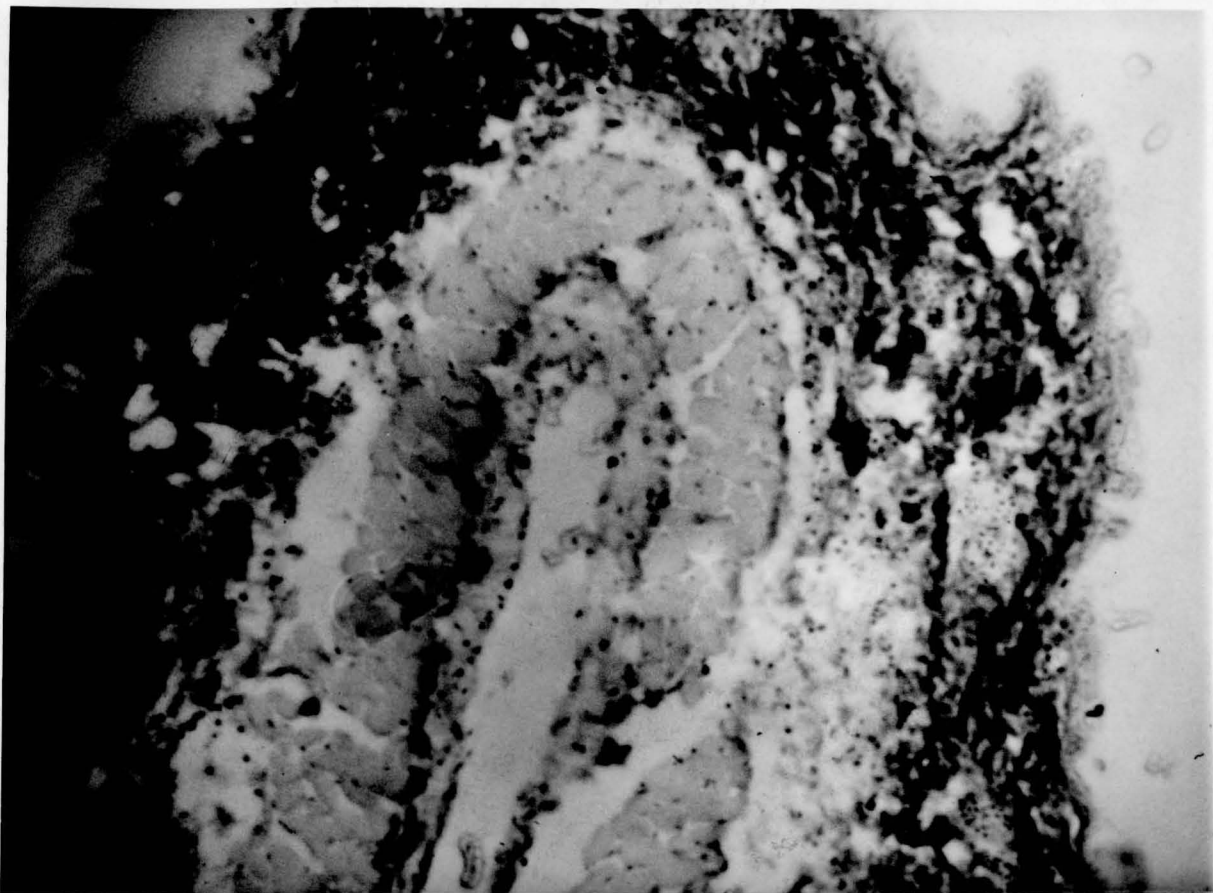the wall of fluid filled pouch of test animal. X 1000.

FIGURE 3 Histologic section showing wall of fluid
filled pouch of test animal. X 100.

APPROVAL SHEET

The thesis submitted by Dr. Edward E. Black has been read and approved by three members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 16, 1966

Date

Patrick D Toto

Signature of Advisor