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The Effects of Desoxycorticosterone, Hydrocortisone, and Prednisone on Myogenesis in Vitro

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THE EFFECTS OF DESOXYCORTICOSTERONE, HYDROCORTISONE, AND PREDNISONE ON MYOGENESIS IN VITRO

(TITLE)

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ABSTRACT

In order to obtain proper muscle development in tissue culture, certain conditions must prevail. There is a delicate balance of factors, and elimination of even one of these factors leads to poor and improper development. Muscle cells grown in the absence of fibroblasts usually fail to differentiate. Therefore, proper development depends upon the presence of two cell types: myoblasts and fibroblasts. Investigators have demonstrated that the fibroblasts are necessary in that they produce collagen which is necessary for proper muscle development. The present study has shown that desoxycorticosterone, hydrocortisone, and prednisone at concentrations of 10, 1, and 0.1 ug/ml of culture medium alter muscle development to varying degrees. Hydrocortisone has proven to be the most inhibitory steroid. Desoxycorticosterone treated cultures have yielded very poor muscle development at 10 ug/ml and allowed development to a contracting state at 0.1 ug/ml. Prednisone treated cultures have developed the best at all three concentrations.

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INTRODUCTION

A technique used extensively by tissue culturists was developed by Moscona (1952) whereby monolayer cultures were prepared from embryonic or neonatal skeletal muscle. Embryonic muscle consists of uninucleated muscle precursors and partially differentiated multinucleated cells. By treatment with a trypsin solution, the tissue can be dissociated into a suspension of single cells. During this treatment, most of the myofibers are destroyed or lost. Once the trypsin is removed, and the larger debris filtered from the suspension, the cells can be innoculated into culture chambers. In the chambers, the cells soon form a monolayer of uninucleated spindle-shaped myoblasts and fibroblast-like cells.

For the first few days, the cells multiply. By definition, a myoblast is a mesenchyme cell, capable of mitosis, occurring in the areas of the embryo where the muscle will eventually appear. These myoblasts eventually give rise to myosin-synthesizing cells: myocytes.

When there is a confluent layer of cells, the myocytes begin to aggregate, then fuse into multinucleated cells called myotubes. Several days later cross-striated myofibrils and contractions can be observed in cells referred to as myofibers.

Many experiments have been carried out to support the above description of skeletal muscle differentiation. Okazaki and Holtzer (1966) have studied DNA synthesis and myosin formation by using H^3 thymine. Their findings together with the fact that cells producing myosin do not display metaphase plates lead to these conclusions: 1) at some critical point,

sets of genes in the presumptive myoblasts must be repressed; 2) sets of genes associated with myosin synthesis must be activated. DNA synthesis and the formation of myosin do not take place at the same time in muscle precursor cells. Myosin synthesis occurs only after mitosis has ceased.

The actual events in the formation of myotubes, multinucleate cells, have been a controversial topic for many years. Early investigators have felt that the multinucleate state arose from repeated amitotic divisions without subsequent cytokineses or that it was unicellular in origin (Altschul and Lee, 1960; Boyd, 1960; Godman, 1957; Moscona, 1958; Murray, 1960; Pogogeff and Murray, 1946). Other investigators have found evidence to support the multicellular theory. That is, they feel the multinucleate cells arise from the fusion of many individual myoblasts (Bischoff and Holtzer, 1969; Bischoff and Holtzer, 1970; Holtzer, Abbott, and Lash, 1958; Konigsberg, McElvain, Tootle, and Herrman, 1960; Okazaki and Holtzer, 1966; Pyblylski and Blumberg, 1966; Stockdale and Holtzer, 1961). Experimental evidence supporting cell fusion as a mechanism of myotube formation was given by Konigsberg, et al., (1960). Into chick embryonic skeletal muscle in monolayer culture, they introduced nitrogen mustard, which inhibited DNA synthesis, just before massive formation of multinucleate cells. Later examination of the cultures showed that multinucleate cells still formed. Therefore, the multinucleate condition was assumed to result from cell fusion.

Twitching of myotubes, once they are formed, is a common occurrence in these monolayer muscle cultures. As early as 1915 Lewis reported spontaneous contractions in chick skeletal muscle cultures. Konigsberg (1959) has also observed spontaneous twitches in multinucleated cells after cross-striations have formed. Often there is a series of rapid twitches

lasting several seconds and then suddenly stopping. This activity seems to be confined to a single cell and apparently the stimulus for contraction arises within the cell itself.

Myogenesis in tissue culture then begins with a population of fibroblasts and mononucleate myoblasts. Both cells proliferate for several days (log phase) but soon the myoblasts discontinue DNA synthesis and the now myocytes begin synthesizing myosin and actin molecules (stationary phase). Myocyte fusion leads to multinucleate cells, myotubes, in which cross-striated myofibrils are eventually formed. Contraction is then apparent in the myofibers. Sometimes even before the cross-striations are apparent, contractions are observed.

Although tissue culture allows direct analysis of many in vivo phenomena, the system is an in vitro technique with understandable limitations. For example, with dissociation of the tissues being cultured, it is difficult to determine the actual cellular damage created by mechanical factors, enzymatic digestion, and exposure to solutions which might not be exactly physiological (Rinaldini, 1958; Phillips and Terryberry, 1957; Magee, Sheek, and Sagik, 1958; Levine, 1960; Hebb and Chu, 1960). Therefore, a proper medium to maintain the integrity of the cells is essential.

For muscle differentiation, a medium with amino acids, balanced salts, embryo extract, serum, and antibiotics has often been used with varying concentrations of each component. The amino acids were usually supplied in a synthetic mixture called Medium 199. Resseguie (1961), in his studies with chick embryo skeletal muscle, found that increasing concentrations of Medium 199 up to the highest level tested gave improvements in chick muscle fiber formation. This lead Resseguie to believe that

muscle fiber formation was favored by an environment of high nutrient content. Myogenesis involving an abundance of specific protein syntheses would certainly require an ample supply of amino acids.

Some investigators have found embryo extract to be inhibitory (Goodman and Murray, 1953; Szepsenwol, 1946), but Resseguie (1961) in his experiments has observed stimulation of myogenesis with embryo extract. He suggested that embryo extract contains one or more substances which are inhibitory to differentiation when in combination with other medium components.

The optimum growth of most mammalian cells requires serum in the culture medium. Rappaport, Poole, and Rappaport (1960) have found that the efficiency of attachment of HeLa cells is directly correlated with the total ionic charge on the glass surface on which the cells are to grow. Their results suggest that a given cell is dependent upon a critical number of negative charges on the glass surface. Therefore, in a serum-containing medium, the attachment of a cell to the glass surface is derived from a protein to protein interaction between the serum protein and the protein of the cell itself. Such a protein double layer (glass, serum protein, then cell protein) has been estimated to hold the cell 50Å from the glass surface. This distance probably allows a more rapid equilibration of the cell with the medium. De LaHaba, Cooper, and Elting (1966), using chick embryos, have found that in the absence of serum in culture medium there is little fusion of myoblasts to form myotubes.

Optimum medium, however, is of no avail if other conditions are not maintained. For example, Przybylski and Bullaro (1971), using monolayer cultures of twelve-day chick embryo skeletal muscle, have found myotubes

on day three when grown in a CO_2 atmosphere and in either Leibovitz's L-15 or Ham's F-10 medium (both contain embryo extract and serum). When the same conditions are employed, but without CO_2, the cultures in L-15 show a 90% decrease in myoblast fusion. However, Ham's F-10 cultures develop normally. Therefore, Ham's F-10 contains some factors which allows development in the absence of CO_2 while L-15 medium requires CO_2. Analysis of the F-10 medium has shown that it contains twelve more components than L-15 and, in general, has a much lower concentration of each amino acid which they have in common. In the experiments of Resseguie (1961), the cultures show best differentiation when exposing them to CO_2 only at the time of initiation of the cultures. It cannot be clearly shown whether in his experiments the inhibition of myogenesis is directly caused by the increased CO_2 concentration or indirectly by reduced pH when the cultures are exposed to CO_2 daily.

In order to obtain proper muscle development in tissue culture, certain conditions must prevail. There is a delicate balance of factors, and elimination of even one of these factors leads to poor and improper development. Muscle cells grown in the absence of fibroblasts usually fail to differentiate. Therefore, proper development seems to depend upon the presence of two cell types: myoblasts and fibroblasts. Using chick embryos, Konigsberg (1963) has demonstrated that single embryonic muscle cells isolated in culture are capable of forming a macroscopic muscle colony within which the differentiation of the multinucleated, cross-striated muscle fibers occurs. However, the development of such muscle clones depended upon the use of culture medium which had been previously exposed, for several days, to a dense population of fibroblast cells. This type of medium

is referred to as "conditioned medium" and likely has been altered by the metabolic activities of the cells.

Goldberg and Green (1964), using established mouse fibroblast lines, have demonstrated with electron microscopy and hydorxyproline determination that collagen is produced by fibroblasts. This fact has been known for some time, but the time and mechanism has been in need of further study (Gerarde and Jones, 1953; Green and Goldberg, 1963). Goldberg and Green (1964) have indeed found that the time of collagen formation was not during the log phase when the cells were rapidly multiplying. This was demonstrated by the fact that cells examined during this period lacked well-developed ergastoplasm and Golgi systems. However, during the stationary phase the hydroxproline content was high and electron micrographs revealed highly developed smooth and rough ergastoplasm. Actual synthesis of collagen appeared to take place in the rough-surfaced endoplasmic reticulum, and transportation of the collagen to the cell surface as a vesicle of soluble protein. Actual fibers form once the collagen is deposited outside the cell. Using human connective tissue cells from synovial membrane, Castor and Muriden (1964) have obtained results similar to those of Goldberg and Green. Furthermore, Castor and Muriden have found that collagen formed in vitro has the same content of amino acids as found in collagen formed in vivo.

Implications that development of muscle in tissue culture requires the metabolic product of an associated cell type, the fibroblast, led to the experiment of Hauschka and Konigsberg (1966). They compared development of muscle grown in "conditioned medium" and in cultures where the glass surfaces were coated with preipitated collagen from rat-tail tendons. They

found that muscle development was the same in both systems and that the development was complete. Therefore, they concluded that indeed the metabolic product of fibroblasts essential for muscle development was collagen.

The relationship between various organs, tissues, and cells has been the subject of investigation for many years. Any given steroid can elicit a spectrum of responses depending upon the cell on which it is acting. Corticosteroids show a great influence on fibroblasts (Ruhmann and Berliner, 1965). For example, recently it has been shown that corticosteroids induce morphological changes in fibroblasts both in vivo and in vitro (Berliner, Gallegos, and Schneebeli, 1967) and inhibit collagen synthesis (Castor and Muriden, 1964; Panagiotis, 1966).

The studies of many investigators have revealed the techniques and their limitations for establishing an in vitro differentiation of muscle from mononucleate cell precursors to multinucleate, cross-striated, contracting fibers. There is an apparent dependency of proper muscle development on the presence of a second cell type, the fibroblast, or at least a metabolic product of this cell, collagen. Other investigators of disease-related phenomena, such as anti-inflammatory responses, have shown that fibroblasts are altered greatly in their morphology and metabolism by various corticosteroids. Mainly, the fibroblasts tend to become rounded and discontinue the production of collagen. However, not all steroids elicit the same response.

The present experimental approach has been formed on the hypothesis that if primary mammalian skeletal muscle tissue cultures are exposed to various steroid hormones, the response will vary depending upon the hormone

being used. Therefore, primary muscle culture techniques have been employed and the developing muscle cultures treated with concentrations of 10, 1, and 0.1 ug/ml of prednisone, desoxycorticosterone, and hydrocortisone. Normal muscle development of controls in tissue culture has been quantitated with respect to fiber development, fiber width, and fibroblast population. The hormone-treated cultures have been examined in the same manner and compared to the criteria of normal development attained by the controls.

MATERIALS AND METHODS

Seven female and seven male select breeding albino rats were obtained from the Holtzman Co. in Madison, Wisconsin. Ear identification punctures were made and the rats paired so that they mated with the same mate each time. A breeding schedule was established so that a new litter was available every twelve days. Each litter consisted of at least ten newborns. Five newborns were used within the first twenty-four hours and the other five within the first seventy-two hours.

All glassware and instruments were soaked in 1% Bioclean-X-100 (Isolab, Inc.) for at least twenty-four hours, after which each piece was rinsed ten times in glass distilled water, air dried, wrapped, and sterilized. The 22x22 mm #1 coverslips were soaked in a 5% solution of Bioclean-X-100 for twenty-four hours, then individually cleaned further, rinsed, and stored in 100% ethyl alcohol for later use.

Culture chambers were prepared by flaming the alcohol from a coverslip, then placing it centered over the one-half inch hole in a glass microscope slide. Melted Paraplast* (melting point 56° C.) was run along the edges of the coverslip, and one end of the slide was then placed in a flame and the Paraplast allowed to flow under the coverslip. Each slide was then placed on two wooden splints in a petri dish and the Paraplast allowed to cool with the coverslip on the top of the slide. Once cooled, the slide was turned over and oxygenated mineral oil was added to fill the

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*Supplied by GIBCO.

chamber. The top coverslip was added and sealed the chamber after the cells were innoculated.

The culture medium consisted of 44% Minimum Essential Medium (Eagle, 1959), 40% Medium 199 (Morgan, Morton and Parker, 1950; Parker, Morgan, and Morton, 1950), 10% horse serum, 5% embryo extract, and 1% penicillin-streptomycin.*

The conticosteroids** were prepared in the above medium at 10, 1, and 0.1 ug/ml of medium. Solution of the corticosteroids was accomplished by dispersion of the appropriate amount in 0.2 ml of 100% methonol to which was then added 0.2 ml of propylene glycol. This was done according to Ruhmann and Berliner (1965).

The solution used in the trypsinizing procedure consisted of 65% Puck's Saline G, 30% of 2.5% trypsin, and 5% of 4% methyl cellulose (Konigsberg, 1959).

The skeletal muscles of the posterior limbs were removed after the newborn had been given a cervical dislocation. All skin and bone was removed. Dissections were done in Puck's Saline G warmed to 37° C. Following dissection, the small pieces of muscle were placed in the trypsin solution at 37° C. After five minutes of incubation at 37° C., the flask was placed on a magnetic stirrer to agitate the muscle. Reincubation and continued agitation lasted twenty-five minutes. The tissue-trypsin mixture was then passed thru a Swinney adaptor containing a piece of fitted 200-mesh bolting silk, to remove all of the undissociated tissue (Konigsberg

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*supplied by GIBCO.

**Supplied by NBC.

et al., 1960). Immediately, 1 ml of culture medium was added to the tube containing the cells and trypsin. This mixture was then centrifuged for seven minutes at 500 rpm. The pellet was saved and the supernate discarded. One ml of culture medium was then again added to the pellet and the cells suspended uniformally in the medium. Viable cell count was determined by the method of Phillips and Terryberry (1957) with Trypan Blue Stain.

To innoculate the cells, a micropipet was placed through the mineral oil to the coverslip and 3×10^3 cells were dispersed into the chamber by measuring the diameter of the sphere of cell suspension before the cells flowed onto the coverslip (Bollinger, 1971). Twice as much medium (regular or corticosteroid treated), which had been in a 5% CO_2 atmosphere for thirty minutes at 37° C., was then mixed with the cells in the chamber. Once the cells and medium were added, the chambers were placed in a 5% CO_2 atmosphere at 37° C. for thirty minutes. Every forty-eight hours half of the medium was removed and replaced with fresh medium (Resseguie, 1961). Before each feeding, the medium was treated with 5% CO_2 as above, and once the medium was replaced, the chambers were treated in 5% CO_2 as above.

Once each day, cultures were examined by phase contrast microscopy. By examination of five microscopic fields with a 160X total magnitization an objective, the number of myotubes formed and their size were categorized on the basis of the criteria given in Table 1. Fibroblast development, as well, was categorized each day, beginning on the third day (see Table 1).

Table 1

Index of Myogenesis and Fibroblast Growth

Myotube Development:

Category A = no multinucleate cells or less than one per field

Category B = an average of at least one per field but less than six

Category C = at least six per field but less than twelve

Category D = more than twelve per field.

Myotube Width:

Class I = narrower than nuclei

Class II = wider than nuclei but less than 10u

Class III = wider than 10 u but less than 20u

Class IV = wider than 20u

Fibroblast Population:

(-) = very poor; possessed no confluency and highly vacuolar

(+) = fibroblasts healthy looking but scattered and sparse

(++) = very good; possessed confluency and lacked high vacuolization

RESULTS

Under the conditions employed in this study, myogenesis followed a definite pattern. Within twelve hours most of the cells innoculated into the chambers were spread out on the surface of the coverslip. Mass proliferation was apparent for the first two days and by the end of day two in culture, some multinucleate cells were beginning to form. In the cultures that developed into the contracting state, there was a confluent layer of fibroblasts by the end of day three. Branching of multinucleate cells was often observed as early as day four. By day eight contractions were apparent in many of the multinucleate cells, but no striations were observed before day nine. Most of the cultures were terminated by day twelve, but one control was maintained for eighteen days. This culture appeared healthy and would probably have survived longer if the feeding schedule had been continued.

Identification of the cell types was relatively easy since the fibroblasts and myoblasts were quite morphologically distinct. Fibroblasts usually possessed more than two radiating cytoplasmic projections and contained round nuclei (Figure 2). Myoblasts were invariably spindle-shaped, containing elliptical rather than round nuclei (Figure 1), and often had many small cytoplasmic extensions at the terminal (positions) of the cells. Blebbing was also much more apparent in myoblasts than in fibroblasts (Figures 8 and 10).

Changing the medium appeared to be critical in establishing cultures with good muscle differentiation. When the medium was changed every day

and the cells were not treated in a 5% CO_2 atmosphere at 37° C., differentiation was the poorest. If the cells were treated in a 5% CO_2 atmosphere at 37° C. for thirty minutes but the medium was still changed every day, differentiation was somewhat better. However, the best differentiation occurred when the cells were treated in a 5% CO_2 atmosphere at 37° C. for thirty minutes and the medium changed every forty-eight hours.

Two hundred ten cultures were used to establish the relationship between various corticosteroid treated and control cultures. The three months spent in learning and varying the procedures used in this experiment entailed the study of approximately another 120 cultures. In all of these cultures, never once were there observed any mitotic figures in any of the multinucleate cells.

Multinucleate cells often bulged at the ends and narrowed between (the ends). Often the tapering was one-fifth of the diameter of the bulging ends. Juxtaposition of a myoblast alongside a multinucleate cell, as if fusing, was observed between the ends of the multinucleate cell, but most often the myoblasts were juxtaposed at the ends of the multinucleate cell (Figures 9 and 10). Also, the bulging ends usually contained a group of clustered nuclei (Figures 6, 7, and 8). Actual positions of the nuclei in the multinucleate cells varied from peripheral to medial with relation to the plasma membrane of the cell. In quite wide multinucleate cells containing striations, the nuclei were generally in a peripheral position (Figure 12). However, medially located nuclei in cells containing striations were also observed.

Branching of multinucleate cells was a common phenomenon in these cultures and was observed as early as day four (Figure 4). Every culture

that eventually contained contracting cells invariably contained branching of multinucleate cells. However, branching was more extensive in some cultures than in others. There appeared to be no correlation between the amount of branching and the extent of differentiation. When contractions were observed, movement of the entire multinucleate cell was often observed, even along the branched portions. Frequently, contractions were observed in multinucleate cells containing no apparent cross-striations. In one multinucleate cell which was 1700 u long and 20 u wide at the widest point, there were signs of contraction all along the cell. However, no cross-striations were apparent at any point along the cell. Cross-striations appeared in various portions of a multinucleate cell, but the most common position for the first appearance of cross-striations was along the periphery. The width of a multinucleate cell where cross-striations were evident varied from 7 u to 20 u.

Prednisone, desoxycorticosterone, and hydrocortisone affected myogenesis in different manners (Figures 13 and 14). Corticosteroid treated cultures were compared to control cultures. Table 2 shows the compiled results. Using the control as a standard for muscle development in this type of primary culture, cultures with 0.1 ug/ml of prednisone and 0.1 ug/ml of desoxycorticosterone (DOC) showed development similar to the controls. However, in the prednisone cultures treated with 0.1 ug/ml the contractions of multinucleate cells were not observed before day ten and no cross-striations were seen until day twelve. In the DOC cultures treated with 0.1 ug/ml there were contractions on day nine but there were no cross-striations observed until day twelve. As was mentioned earlier in the result section, the control cultures showed signs of contractions by day eight and

Table 2

The Extent of Myogenesis in Control and Corticosteroid Treated Primary Tissue Cultures

Day in Culture	1	2	3	4	5	6	7	8	9	10	11	12
Control	AI	BII	BIII ++	CIII ++	CIII ++	CIII ++	DIII ++	DIII ++*	DIV ++**	DIV ++	DIV ++	DIV ++
Propylene glycon treated	AI	BII	BII ++	CIII ++	CII ++	CIII ++	DIII ++	DIII ++	DIV ++*	DIV ++	DIV ++**	DIV ++
Prednisone												
0.1 ug/ml	AI	BII	BIII ++	CIII ++	CIII ++	CIII ++	DIII ++	DIII ++	DIV ++	DIV ++*	DIV ++	DIV ++**
1.0 ug/ml	AI	BII	BIII ++	CIII ++	CIII ++	CIII ++	CIII ++	DIII ++	DIII ++	DIV ++*	DIV ++	DIV ++
10 ug/ml	AI	BII	BII +	BII +	BIII +	CIII +	CIII +	CIII +	CIII +	CIII +	CIV +	CIV
DOC												
0.1 ug/ml	AI	BII	BIII ++	CIII ++	CIII ++	CIII ++	CIII ++	DIV ++	DIV ++*	DIV ++	DIV ++	DIV ++*
1.0 ug/ml	AI	BII	BII +	BII +	CII +	CII +	CII +	CII +	CIII +	CIII +	CIII +	CIII +
10 ug/ml	AI	AI	AI -	AI -	AI -							
Hydorcorticone												
0.1 ug/ml	AI	AI	AI -	AI -	BI -	BI -	BI -	BI -	BI -	BI -	BI -	BI -
1.0 ug/ml	AI	AI	AI -	AI -	AI -							
10 ug/ml	AI	AI	AI -	AI -	AI -							

* = first time contractions observed
** = first appearance of cross-striations

cross-striations by day nine. In both prednisone and DOC cultures treated with 0.1 ug/ml the fibroblast population was a confluent layer of cells as in the control cultures.

Prednisone treated cultures decidedly showed the best development of multinucleate cells since at 1 and 0.1 ug/ml the development by day eleven was the same as the best development of the control cultures. However, the multinucleate cell concentrations were somewhat sparse at prednisone concentrations of 10 ug/ml. As was already mentioned, the 0.1 ug/ml DOC treated cultures developed to the contracting and cross-striated stage. However, cultures containing 1 ug/ml of DOC developed only to the CIII stage and had only a sparse fibroblast population. At a concentration of 10 ug/ml of DOC, there was no development beyond the initial AI stage.

Hydrocortisone treated cultures showed the strongest inhibition of fibroblasts. Even at 0.1 ug/ml of hydrocortisone the fibroblasts were inhibited. At 10 and 1 ug/ml there was no multinucleate cell development beyond the initial AI stage. However, the cultures containing 0.1 ug/ml developed to the BI stage by day six but they did not develop any further up thru day twelve.

DISCUSSION

Inhibition of inflammation and depression of collagen synthesis was considered an effect of cortisone, but investigators like Castor and Muriden (1964) have shown that these phenomena were due rather to the action of cortisol or hydrocortisone. Berliner and Ruhmann (1966), in metabolic studies using radioactive compounds, found that there was no interconversion between cortisone and cortisol when incubated with active fibroblasts (Clone 929, Strain L) in tissue culture. These investigators, therefore, deduced that *in vivo* fibroblasts possess an active 11 B-OH dehydrogenase system to convert cortisone to cortisol. However, fibroblasts grown in tissue culture lack this enzymatic activity to yield the biotransformation of cortisone to cortisol. Further experimentation by Berliner and Ruhmann revealed that the 11 B-OH corticosteroids like cortisol, 2-methylcortisol, prednisolone, and corticosterone markedly inhibited fibroblastic proliferation. Yet, the 11-keto compounds like cortisone, 2-methylcortisone, prednisone and 11-dehydrocorticosterone often allowed fibroblast growth equal to or greater than the controls. More specifically, 0.1 ug/ml cortisol inhibited fibroblast growth and 0.1 ug/ml 11-desoxycorticosterone had some growth inhibitory activity. Even at 10 ug/ml prednisone was without effect on the fibroblast population. Berliner and Ruhmann (1967) in another study concluded that fibroblasts in tissue culture closely mimic, in their reaction to various corticosteroids, the response of inflammed tissue *in vivo*. That is, the fibroblasts responded with decreased growth and morphological changes similar to those exhibited by connective tissue fibroblasts *in vivo* under

corticosteroid treatment.

The formation of multinucleate cells and the overall development of the primary cultures in this study were greatly affected by the various corticosteroids. Since the only variable in the cultures studied was the cortiosteroid, an inherent property such as the molecular structure of the various corticosteroids might well be the factor which produced varying results in the skeletal muscle culture development. Hydrocortisone possesses an 11B-OH group which has been shown by many investigators to be active in inhibiting fibroblastic growth and collagen synthesis (Berliner, Gallegro, and Schneebeli, 1967; Castor and Muriden, 1964; Panogiotis, 1966; Berliner and Ruhmann, 1966). However, prednisone and desoxycorticosterone lack this 11-BOH group. Prednisone possesses an 11-keto and desoxycorticosterone is deoxygenated at the C-11 position. Berliner and Ruhmann (1966) have found that the C-11 deoxygenated steroids like desoxycorticosterone possess only some fibroblastic inhibition and that prednisone suppresses fibroblastic growth even less. The present study has shown that indeed the general trend for inhibition of fibroblasts based on molecular structure does exist. While hydrocortisone is the most inhibitive for fibroblasts, prednisone is the least, and desoxycorticosterone is between these extremes.

To determine the chemical mechanism behind these observed phenomena is beyond the scope of this experiment. However, certain relationships based on morphological and developmental observations can help explain some results. Table 2 shows that when fibroblast development is poor, the overall muscle differentiation is likewise poor. Konigsberg (1963) has shown that, indeed, when muscle cells are grown in the absence of fibroblasts they usually fail to differentiate. Subsequent to Konigsberg's study, Goldberg

and Green (1964) have shown with electron microscopy that mouse fibroblast lines produce collagen. Furthermore, it has been shown that the necessity of fibroblasts for differentiation of muscle in tissue culture can be replaced by the use of culture medium "preconditioned" by confluent fibroblast cultures (Konigsberg, 1963) or by the use of a collagen substrate (Hauska and Konigsberg, 1966). Therefore, they conclude that the metabolic product of fibroblasts essential for muscle development is collagen.

It seems likely that the results obtained in these primary skeletal muscle cultures depend on an integrated function of the corticosteroid, fibroblast, collagen and myoblast. That is, the corticosteroids inhibit the fibroblast population dependent upon their ability due to their inherent molucular structure. Any inhibition in fibroblast growth alters the number of cells capable of producing collagen.

Important in this system is the concentration of the steroid. The results indicate that hydrocortisone is the most inhibitive of the three steroids examined. Even at 0.1 ug/ml hydrocortisone inhibits the fibroblast population and permits only very poor muscle differentiation. The concentration factor is quite striking in the DOC treated cultures. At 0.1 ug/ml DOC a very good fibroblast population develops and contractions are observed in multinucleate cells by day nine. However, at 10 ug/ml DOC the fibroblast development and muscle development is very poor. Prednisone also shows variations in development of the cultures dependent upon the concentration. Since the development with prednisone is best at 0.1 ug/ml, it is assumed that for these culture conditions, prednisone is non-inhibitory at low concentrations but inhibitory at the higher concentrations. Therefore, all three steroids elicit varying cell responses at varying concentrations.

Changing the medium and treating the cells with 5% CO_2 seemed to be very critical for this type of primary culture. Resseguie (1961) got best results when he treated his cultures with CO_2 only on the first day and simply changed the medium every forty-eight hours. In the present experiment it was initially felt that no CO_2 treatment would be necessary since the medium contained HEPES buffer. However, the medium proved to be quite basic after several days if it was not exposed to a 5% CO_2 atmosphere. Therefore, the CO_2 treatment was carried out each time the medium was changed. The optimum time period between medium changes proved to be forty-eight hours. The time between changes of medium seems to vary with the technique being used. Resseguie (1961) has found that the least differentiation occurs in those cultures which have no medium change at all. He suggests that the poor development might be due to the reduction of oxygen tension, accumulation of metabolic wastes, or, most likely, the depletion of the medium nutrients. For his experiments, the optimum time between medium changes appeared to be every two days. Changes of medium every day seemed to inhibit development in these experiments. It was felt that daily changes wash essential substances out of the cells.

The results of extensive trypsinization indicated that long exposures to trypsin could alter muscle differentiation. Therefore, in order to attain experimental standards which allowed repeatable results, the trypsinization time was held at twenty-five minutes. Skeletal muscles from newborn rats seventy-two hours old seemed to be the cutoff point where trypsinization could be effective in dissociating the tissue in twenty-five minutes. The need for longer trypsinization times in older rats appeared explainable by the fact that more connective tissue would have developed in the newborn

and the tissue, therefore, would be less likely to dissociate under short (twenty-five minutes) trypsinization periods.

Primary cultures of mammalian skeletal muscle seem to follow a pattern in their differentiation. However, before any differentiation can occur in vitro, the cells must attach to the glass surface of the culture chamber floor. Such cell attachment and subsequent spreading out occurs within the first twelve hours following the cellular innoculation into the culture chambers. Presumably attachment of the cells not only allows the spreading out of the cell but also cellular movement on the glass surface.

Morphological analysis of myogenesis in tissue culture has shown (Konigsberg, 1959) that the terminal ends of myotubes appear to be the less differentiated zones of the cell. At these terminal regions there are usually many closely packed nuclei. It has been suggested that perhaps this is a preferential zone for cell fusion. Furthermore, Cooper and Konigsberg (1959) have shown histochemically that the succinic dehydrogenase in the terminal regions is more like the mononucleated cells that the more central regions of the myotube.

Location of nuclei in the myofibers of the chick embryo has been reported by Resseguie (1961) to be generally peripheral. Konigsberg (1959), also using chick muscle in monolayer cultures, has reported the nuclei to be mainly located in the center of the myotubes and fibers in short term cultures. Both of these investigators have reported branching or anastomosis of the muscle fibers. This condition is thought to be the result of the formation of multinucleate fibers in the absence of a connective tissue framework which is found in vivo (Resseguie, 1961). In other words, random fusion of already formed myotubes probably accounts for the branching systems.

Branching in tissue culture has also been reported by Murray (1960).

The pattern of muscle differentiation in these cultures seemed to support the now accepted multicellular theory as opposed to the amitotic theory. First, no mitotic figures were observed in any of the multinucleate cells formed in approximately 300 cultures. Secondly, often the bulging ends of the multinucleate cells contained a group of many nuclei with myoblasts juxtaposed along side the terminal end of the multinucleate cell, as if fusing. Thirdly, the branching phenomenon, observed as early as day four, seemed explainable by random fusion of already formed multinucleate cells. Fourthly, the formation of multinucleate cells followed mass proliferation of the mononucleate cells and as the number of mononucleate myoblasts decreased, the number of multinucleate cells increased.

This study has proven interesting and beneficial in that I have learned many techniques and obtained a much better understanding of muscle development. The relationship between muscle development, fibroblasts, and corticosteroids has revealed a definite pattern with hydrocortisone being the most inhibitory, and prednisone the least inhibitory. Further work might include extensive histochemistry of the cultures, and hydroyproline assays.

ILLUSTRATIONS

Figure 1.--Myoblast (A) twenty-four hours after the inoculation of a control culture. Note the spindle shape of the cell and the centrally located nucleus. Photographs taken at an initial magnification of 400X; phase-contrast optics were used.

Figure 2.--Myoblast (A) and fibroblast (B) twenty-four hours after the inoculation of a control culture. Note the difference in shape between the myoblast and fibroblast. Same magnification and optic system as Figure 1.

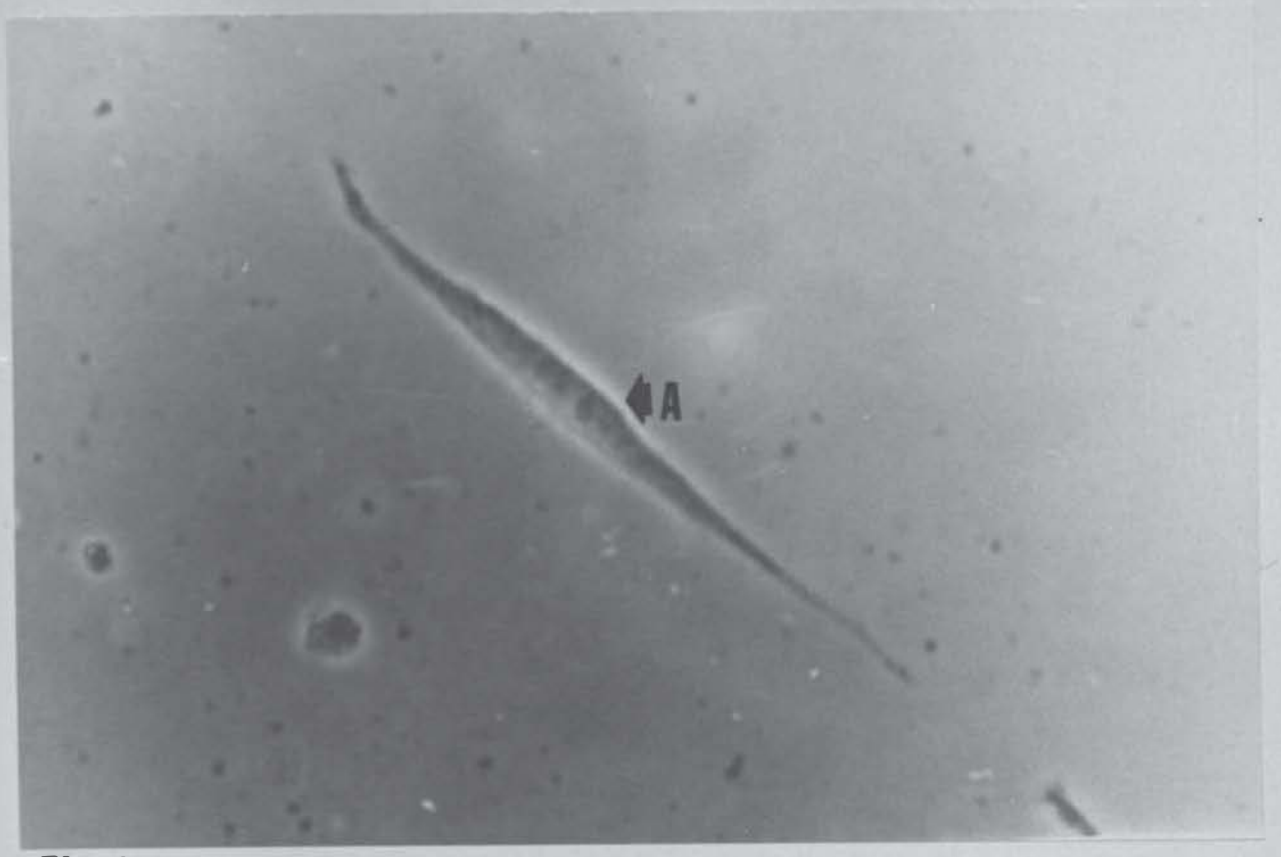

Fig.1

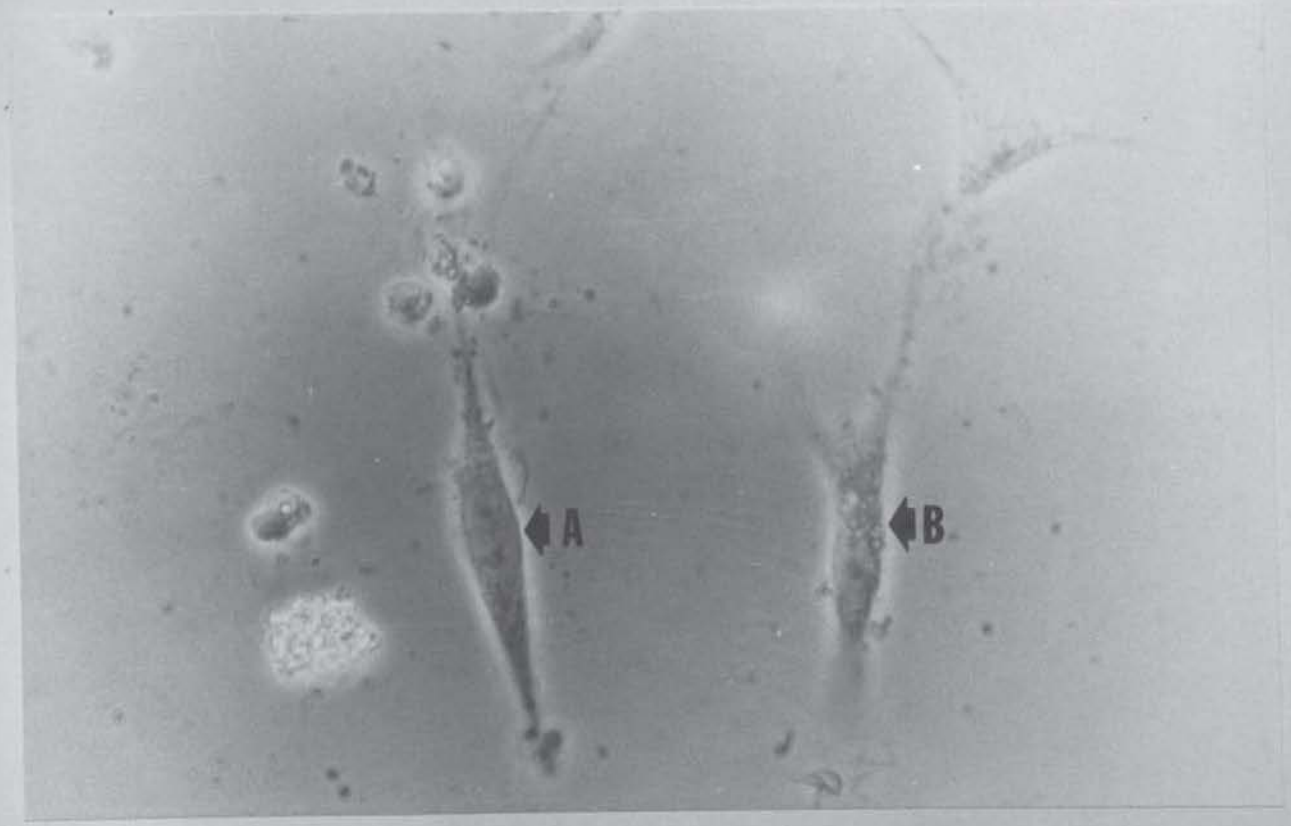

Fig.2

Figure 3.--Group of myoblasts and fibroblasts in a control culture twenty-four hours after the initial inoculation. Note in region A there is a group of aggregating myoblasts. Phase contrast optics with an initial magnification of 160X.

Figure 4.--Control culture four days after the initial inoculation showing myotubes which appear about to fuse (A) and branched myotubes (B). Phase contrast optics at a 160X original magnification.

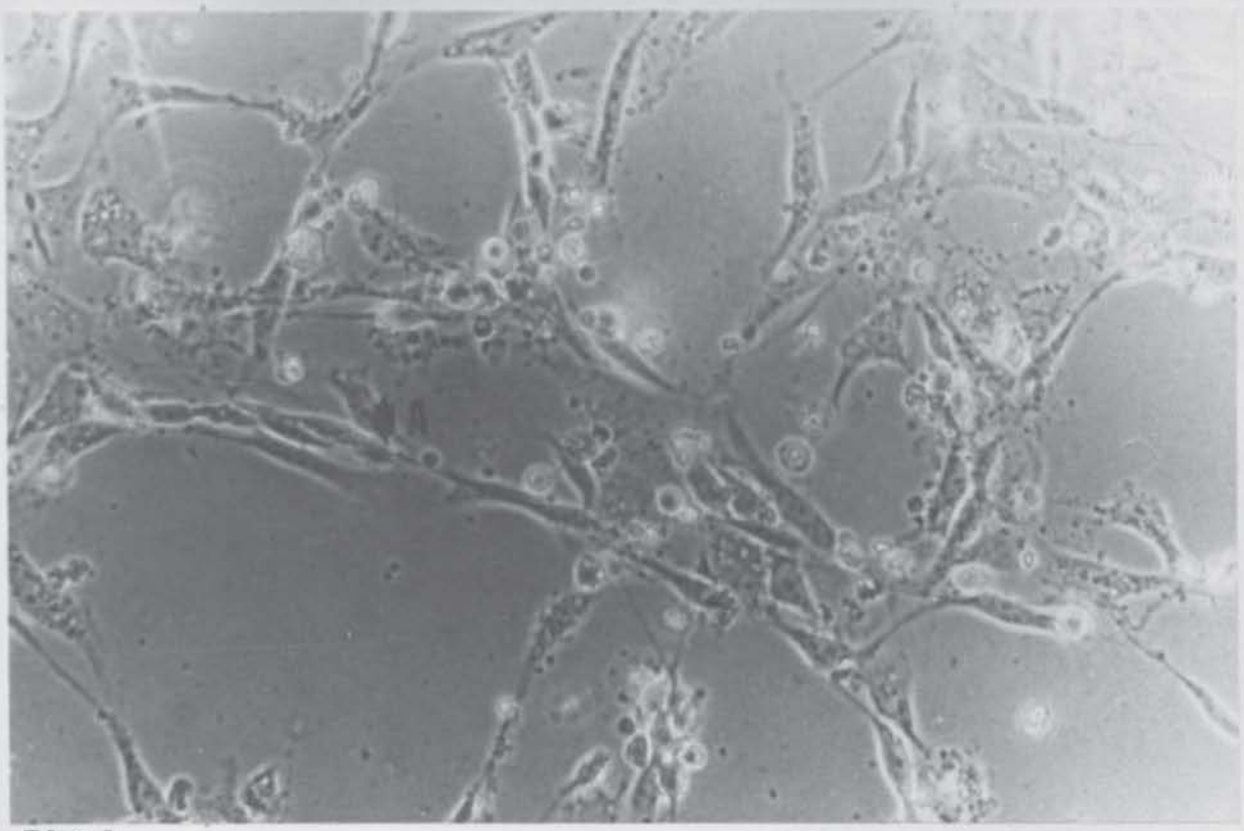

Fig.3

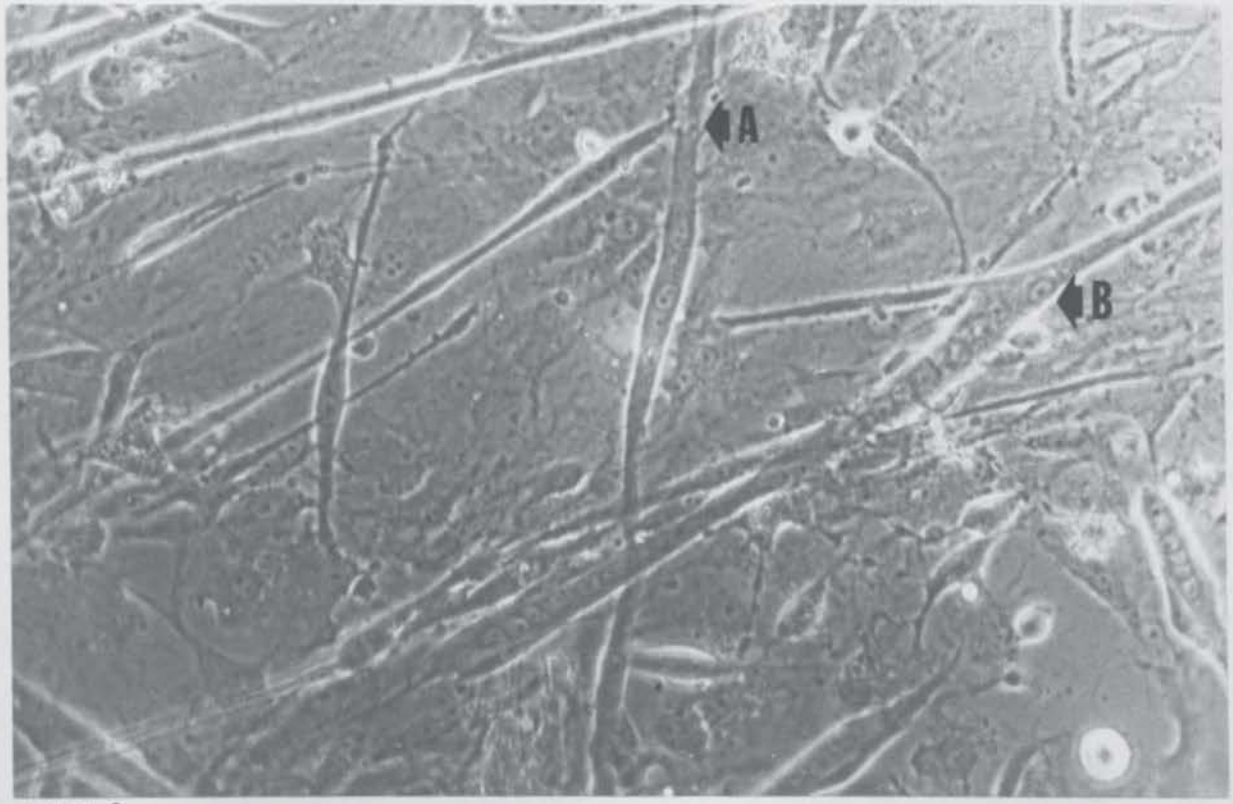

Fig.4

Figure 5.--Control culture showing the extent of myotube formation five days after the initial inoculation. Phase contrast optics at a 160X original magnification.

Figure 6.--Control culture showing the extent of myotube formation six days after the initial inoculation. Phase contrast optics at a 160X original magnification.

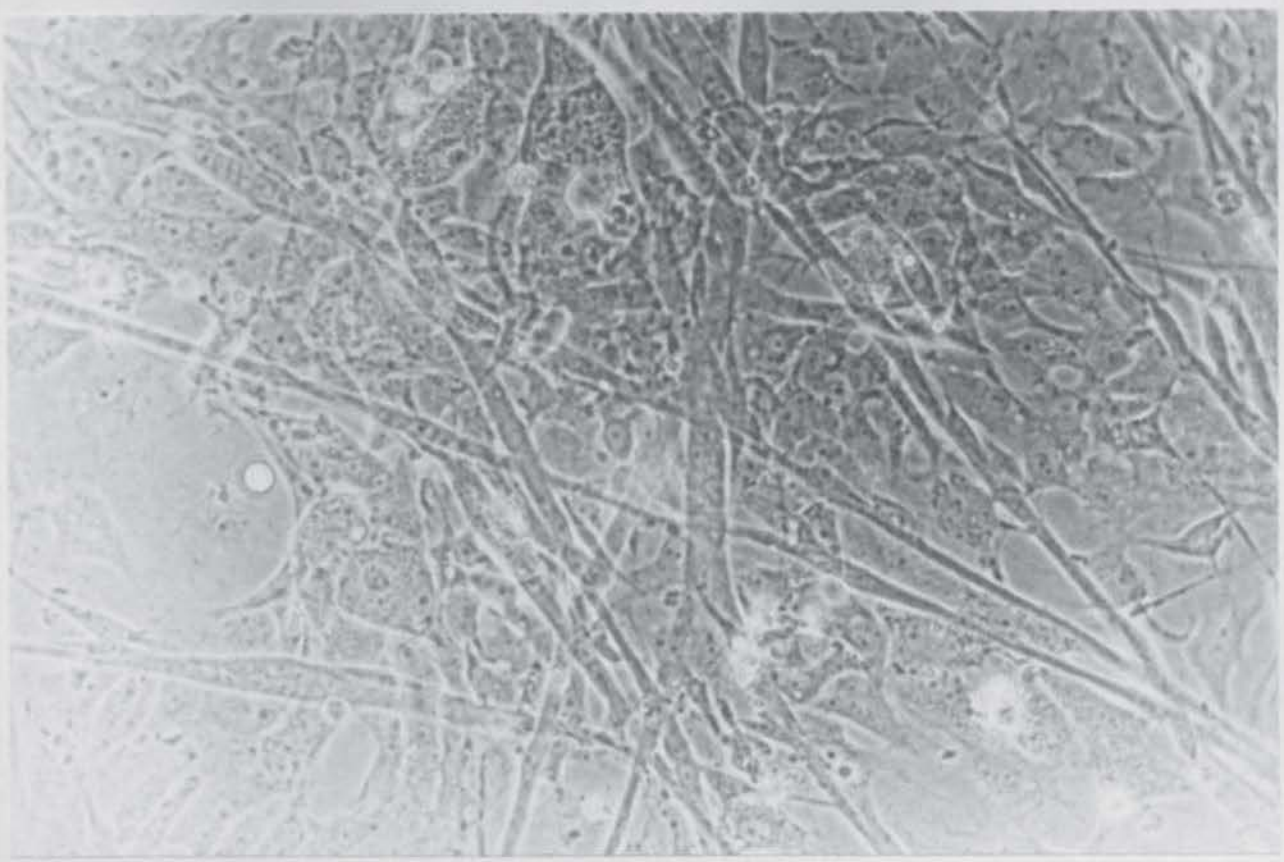

Fig.5

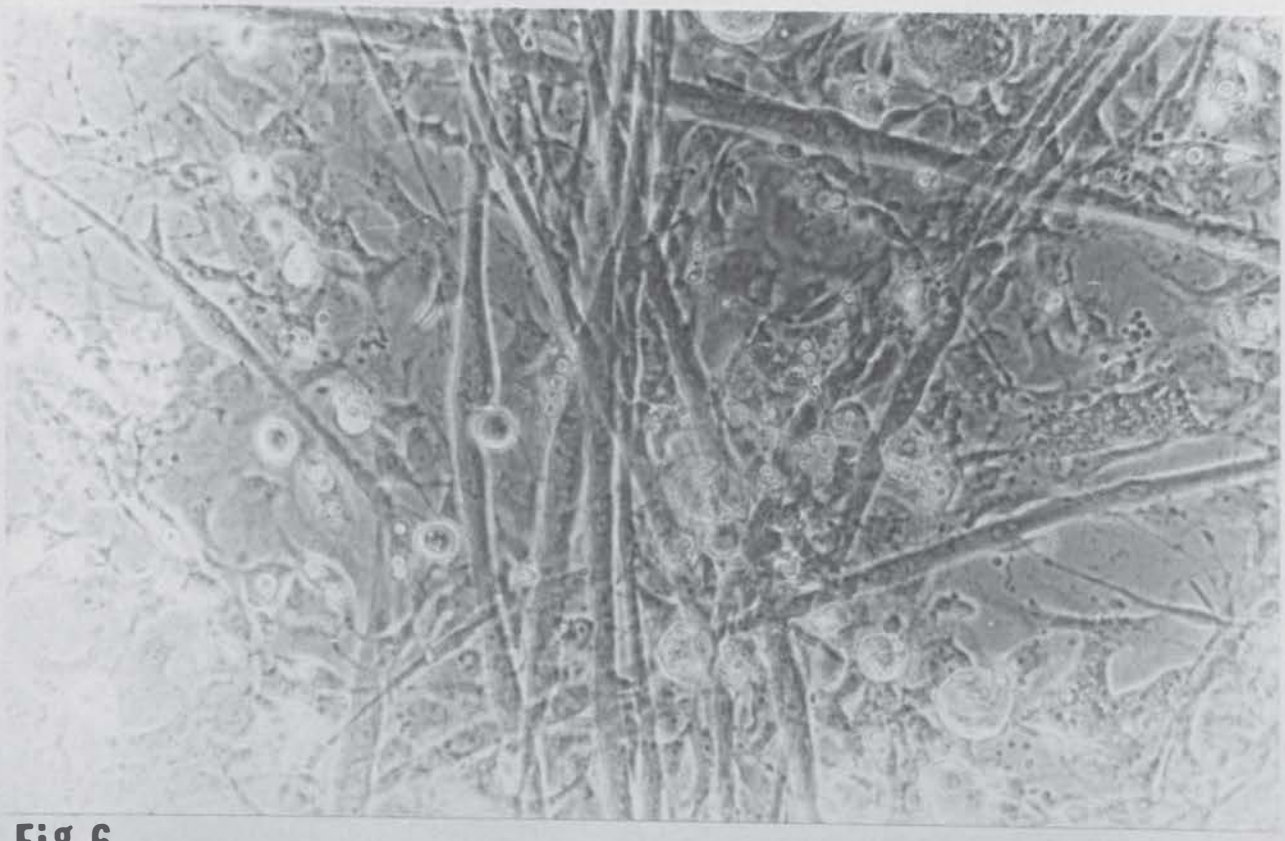

Fig.6

Figure 7.--Control culture seven days after the initial inoculation showing terminal nuclei in various myotubes. Phase contrast optics at a 160X original magnification.

Figure 8.--Higher magnification of the same culture referred to in Figure 7 showing cytoplasmic projections (A) and terminal nuclei (B). Also, note the blebbing. Phase contrast optics at a 400X original magnification.

Fig.7

Fig.8

Figure 9.--Control culture six days after the original inoculation showing what appears to be fusion (A). Phase contrast optics at a 160X original magnification.

Figure 10.--Same culture as in Figure 9, only at 400X magnification. Note the blebbing.

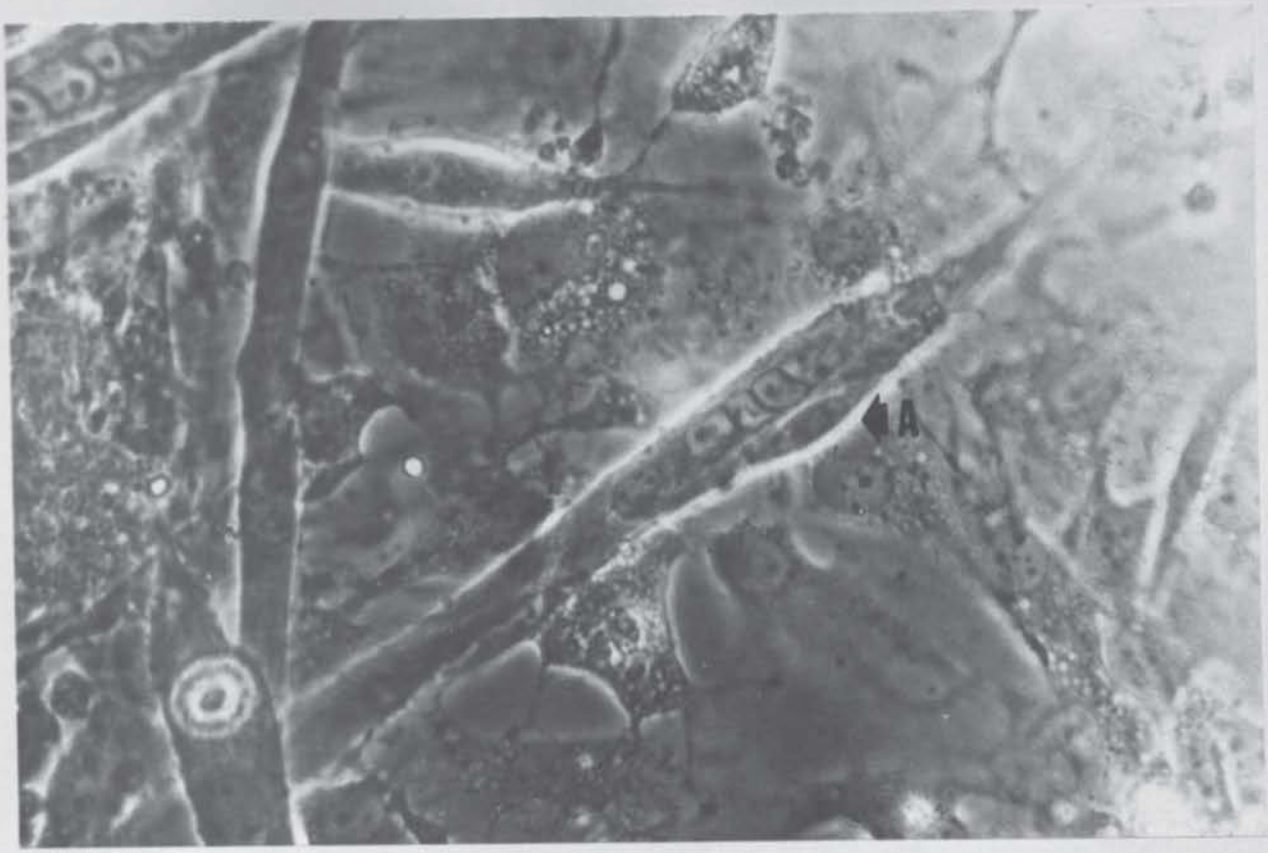

Fig.9

Fig.10

Figure 11.--Control culture six days after the initial inoculation showing the branching phenomenon. Phase contrast optics at a 1000X original magnification.

Figure 12.--Control culture nine days after the initial inoculation showing striations (A) and a nucleus (B) of the myofiber. Phase contrast optics at a 1000X original magnification.

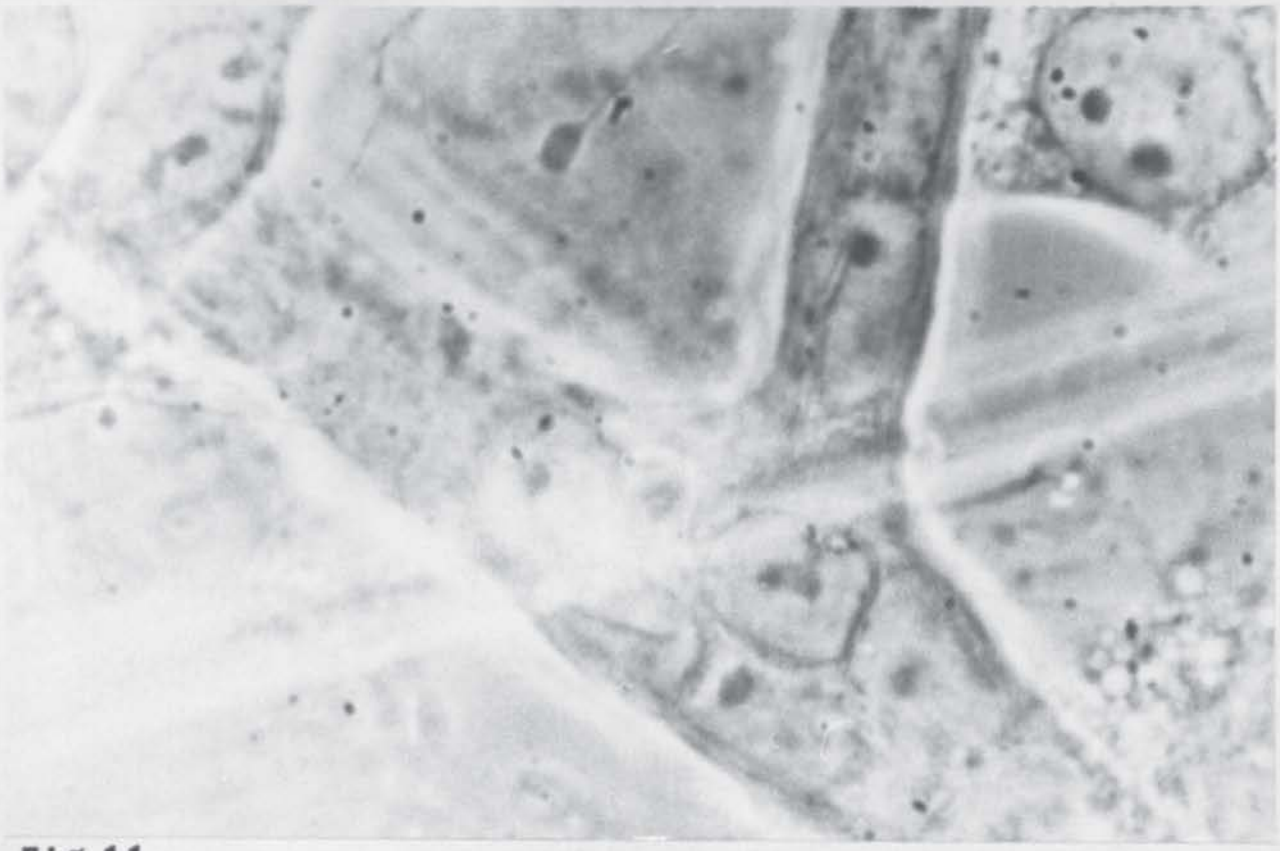

Fig.11

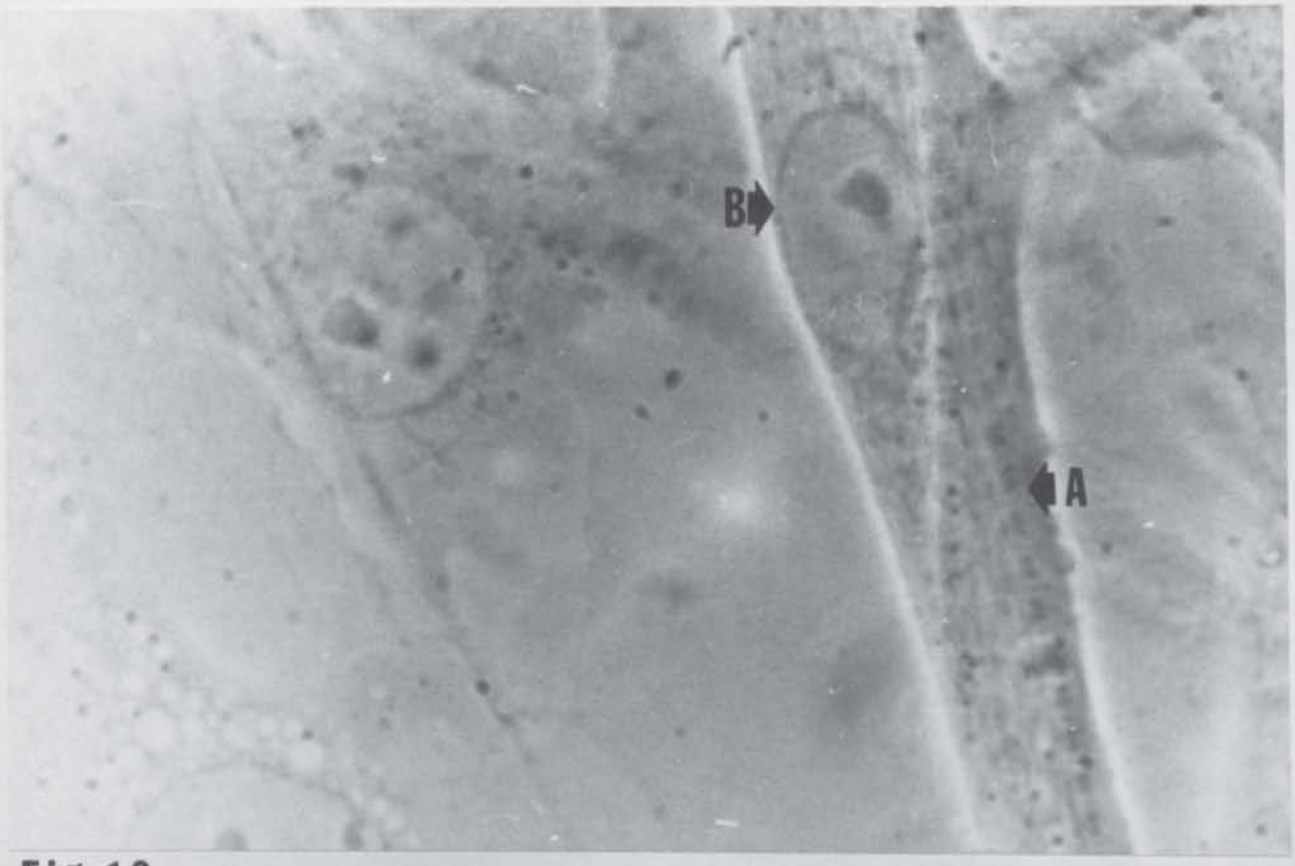

Fig.12

Figure 13.--Hydrocortisone (0.1 ug/ml) treated culture twelve days after the initial inoculation showing an aggregate of rounded, unattached cells (A) and single myoblasts (B). Phase contrast optics at a 160X original magnification.

Figure 14.--Prednisone (10 ug/ml) treated culture twelve days after the initial inoculation showing the slight growth. Phase contrast optics at a 400X original magnification.

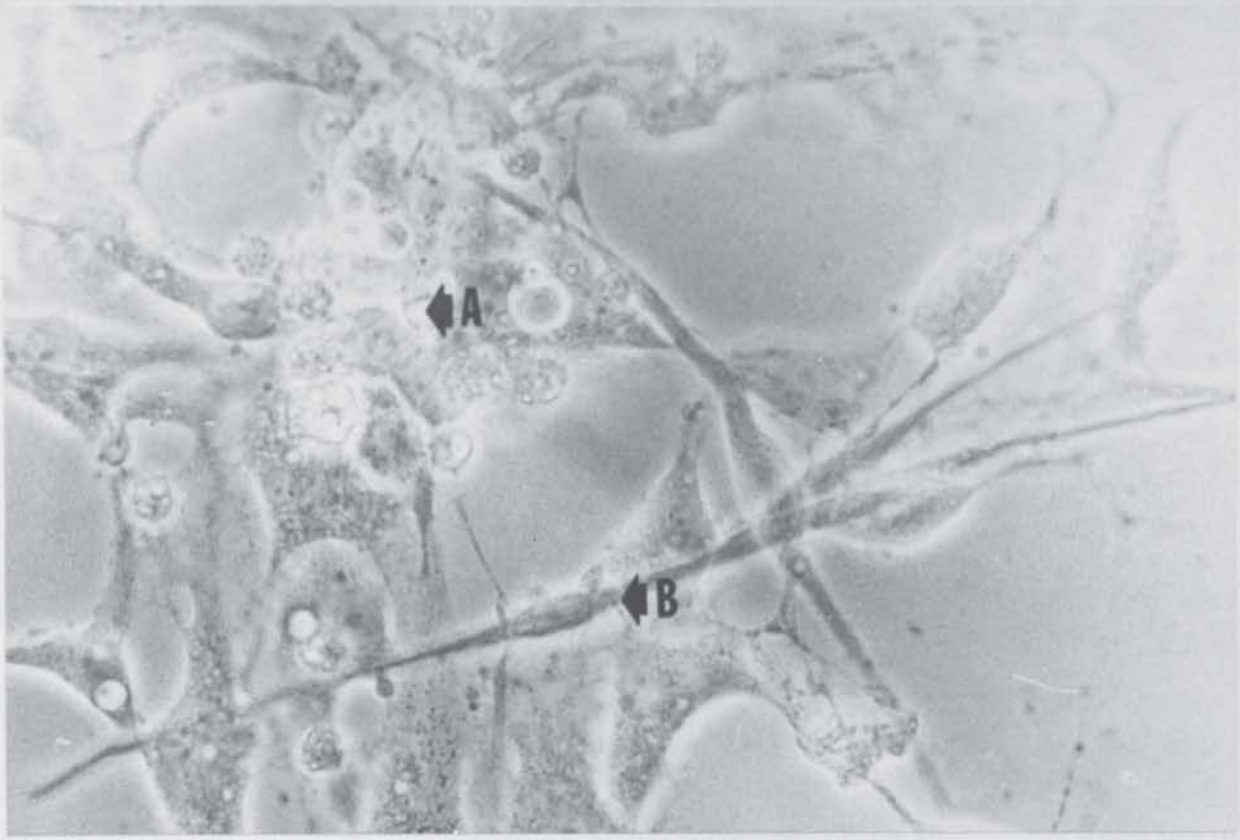

Fig. 13

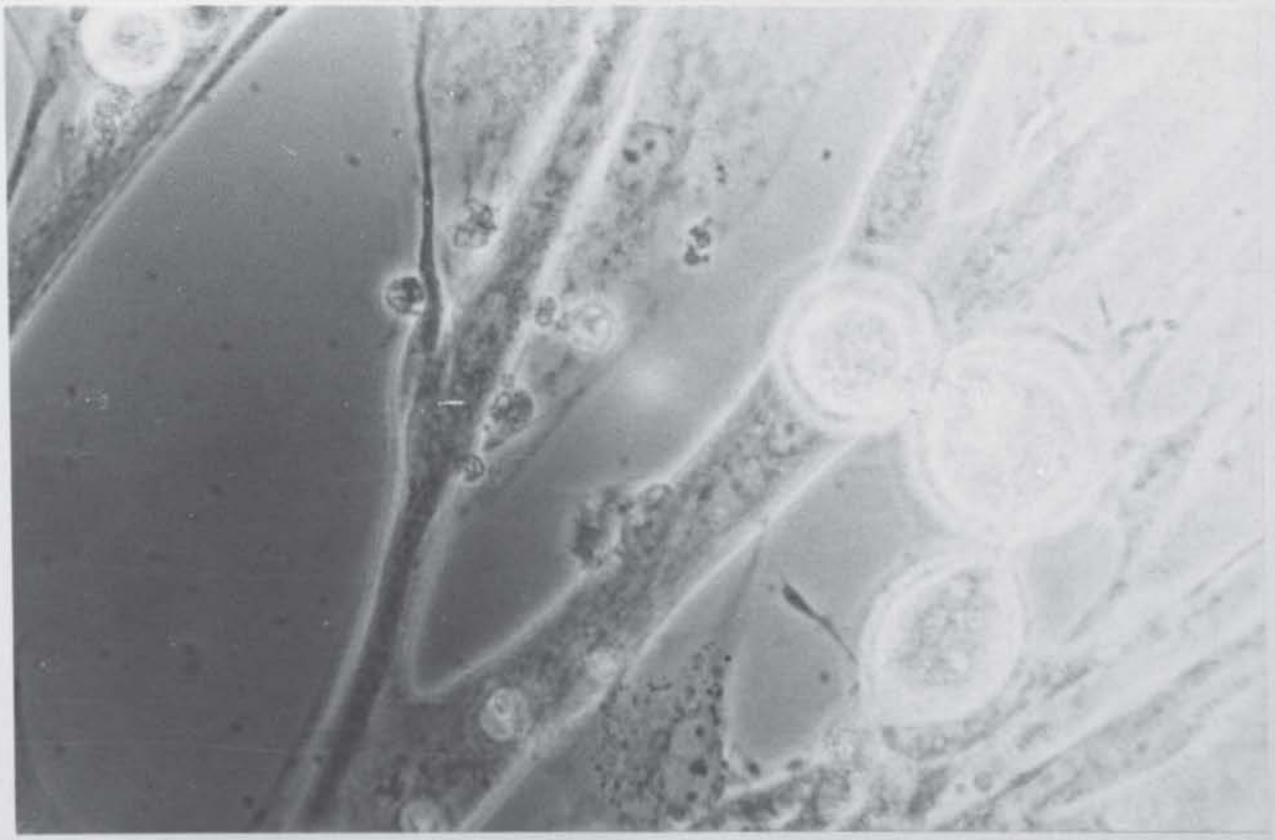

Fig. 14

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