A STUDY OF LEPTOSPIRA POMONA IN TISSUE CULTURE

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ABSTRACT

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by Daniel Dale Harrington

Bovine fetal kidney cells were grown in vitro and infected with leptospira pomona. Medium pH fluctuations, kidney cell responses, leptospiral morphologic changes and/or growth were studied in infected cultures using three types of media. Complementary experiments were conducted to determine pH changes, alterations of organism morphology and/or growth in the absence of cells.

The media used in this study were (1) Eagle's Minimum Essential Medium prepared with Earle's balanced salt solution and containing ten per cent calf serum (2) the above medium prepared with Hanks' balanced salt solution, and (3) a composite medium of equal parts of Stuart's medium with ten per cent rabbit serum and number 2 medium above. The first medium was found to be superior in that pH fell less rapidly and was more easily controlled.

Incubation temperatures of 37° C. were found to be detrimental to the leptospires. Organisms became longer than normal, apparently were unable to divide, underwent degenerative changes, and decreased in numbers after three days incubation.

A modified Warthin-Starry silver-stain technique was used to demonstrate leptospires in the tissue cultures. Care was required in the interpretation of stained filaments since cytoplasmic filaments and junctions of adjacent cells at times stained and simulated leptospires.

Oil-Red-O stain for fat was used with satisfactory results in this study. Stained fats were confined to the cytoplasm of the cells but not to the vacuoles within the cytoplasm. In one experiment, a simultaneous increase in cytoplasmic fat occurred with the progressive decrease in leptospires. This suggested that leptospires utilized the lipid materials which were ordinarily deposited in the cells.

Free granules and coiled leptospires were frequently observed in association with the infected cells and sometimes appeared to be intracytoplasmic. Ring or coiled forms at times contained a granular internal structure. Similar structures were observed in some granule-with-tail forms of leptospires. Other leptospires were beaded and frequently segments of protoplasmic cylinders joined by axial filaments were found. It was not known whether the structures or forms of leptospires observed were part of the life cycle, a resting phase or merely a degenerate stage of the organisms.

Nuclear aberrations were more frequent among some cells in the infected cultures. Further investigations are needed to determine if a relationship exists between the occurrence of these aberrations and the infected state of a cell culture.

The cytopathogenic effects which occurred were inhibition of cellular growth and degeneration with apparent cell death. Some cells were apparently more resistant to these changes than others. Cytoplasmic vacuolation was not considered part of the cytopathogenic effects since it occurred with equal frequency in both the infected and control cultures.

The severity and rapidity of cytopathogenic effects was greater in those cultures which had monolayers of 50 per cent or less prior to infection. This was believed to be due to an organism-cell ratio which favored the leptospires in these cultures.

Two causes for the cytopathogenic effects observed were considered. These were (1) the production of toxins by the organism and (2) direct trauma to the cells by the leptospires.

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Dedicated to

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INTRODUCTION

Though not recorded in the literature, several attempts to study the effects of leptospires on cultured cells have been made. Lundberg (1960) successfully infected porcine kidney cells for two days, and Sleight (1960) investigated the possibility of *in vitro* production of antibodies by pig spleen cells in the presence of *Leptospira pomona*. Miller (1962) indicated that he has utilized hamster and monkey kidney cells, fetal calf skin, HeLa and human amnion cells in some of his work.

The following study was initiated for the purpose of observing and recording the cellular alterations that occur when *in vitro* bovine fetal kidney cells were exposed to *Leptospira pomona*.

REVIEW OF LITERATURE

Lesions of Leptospirosis

Numerous and diverse renal responses have been recorded in naturally occurring and artificially induced leptospiral infections *in vivo*. The character of the lesions varied with the species of animal, the seretype of organism, and the stage and severity of the infection. Both primary and secondary effects contributed to the total pathology, but most workers made no attempt to pinpoint specific cellular alterations that could be attributed to the presence of the organism or its "toxic" products.

The tubular epithelium frequently manifested degenerative changes such as cloudy swelling, atrophy, and vacuolation (Arean, 1962; Boulanger et al., 1959; Cordy and Jasper, 1952; Jungherr, 1944; Langham *et al.*, 1958, a.; Marsh, 1945; Menlux, 1948; Morter *et al.*, 1960; Reinhard and Hadlow, 1954; Runnells *et al.*, 1960; Seibold *et al.*, 1961; Sleight *et al.*, 1960; Smith and Jones, 1961). A more severe finding was necrosis as evidenced by pyknosis, karyolysis, and sloughing (Arean, 1962; Boulanger *et al.*, 1959; Cordy and Jasper, 1952; Jungherr, 1944; Langham *et al.*, 1958, a.; Morter *et al.*, 1960; Reinhard and Hadlow, 1954; Runnells *et al.*, 1960; Seibold *et al.*, 1961; Smith and Jones, 1961). Secondary changes included interstitial and some tubular infiltrations by lymphocytes, plasma cells, heterophils, macrophages, and rarely eosinophils (Boulanger *et al.*, 1959; Burnstein and Baker, 1954; Cordy

and Jasper, 1952; Hadlow and Stoenner, 1955; Langham et al., 1958, a.; Monlux, 1948; Morter et al., 1960; Seibold et al., 1961; Sleight et al., 1960; Smith and Jones, 1961). Tubular epithelial regeneration and fibrosis occurred in some subacute and chronic infections (Burnstein and Baker, 1954; Jungherr, 1944; Langham et al., 1958, a.; Morter et al., 1960; Runnells et al., 1960; Seibold et al., 1961; Smith and Jones, 1961). The significance and/or occasional presence of syncytial and Langhan's type giant cells was discussed by several authors (Gsell, 1946; Hadlow and Stoenner, 1955; Seibold et al., 1961).

Descriptions of the locations of the organisms in the kidneys were not consistent. Many investigators were able to demonstrate the spirochetes within the lumens of the renal tubules or the interstitial tissues (Bernkopf et al., 1947; Boulanger et al., 1959; Cordy and Jasper, 1952; Jungherr, 1944; Langham et al., 1958, a.; Little and Baker, 1950; Marsh, 1945; Reinhard and Hadlow, 1954; Seibold et al., 1961; Smith and Jones, 1961). Intracellular parasitism has been described by a few (Miller and Wilson, 1962; Smith and Jones, 1961), while others preferred to use the term "apparently" intracellular. (Reinhard and Hadlow, 1954).

Microorganisms in Tissue Culture

In their review "A Bibliography of the Research in Tissue Culture • 1884-1950", Murray and Kopeck (1953) have indexed 15,000 original articles, and by a process of cross-indexing, have expanded this to some 86,000 entries. If Murray and Kopeck's work accurately indicates the volume of material written on tissue culture (up to 1950), one can readily realize that a comprehensive literature review on the

bacteria in *in vitro* cell systems could develop into a formidable task. This review is then limited to those reports, which in the author's opinion, contribute directly or indirectly to the study presented.

Bacteria other than Spirochetes

Using different technics, Smith (1955) and Mackaness (1962) reported the infection of tissue cultures with *Listeria monocytogenes*. Smith stated that, with splenic explants, no inhibition of growth was exhibited, but that there was a granulomatous type of inflammation. Mackaness, however, allowed rabbit macrophages from the peritoneal cavity to form a monolayer *in vitro* and then infected them with *L. monocytogenes*. He indicated that each infected cell became separated from the next by uninfected cells. Organisms capable of multiplying within the cells gave rise to circumscribed "plaques" which, if left undisturbed, became larger and more discrete. A "corona" of cells soon surrounded the older plaques. This corona was formed by a migration of cells toward the "infectious center". Plaque-forming efficiency was increased by serial passages of the macrophages.

According to Lewis (1919), "Typhoid bacilli seemed to hasten markedly some process which results in vacuolation". He claimed that the formation of vacuoles was a degenerative change. A difference existed in the pattern of vacuolation that occurred in "normal" degenerating cells and those infected with *Bacillus typhosa*. In noninfected chicken embryo explants, not all cells became vacuolated at the same time nor to the same extent. This differed from infected cultures in that all the cells and all cell types (connective tissue, mesothelium, and endoderm of the intestinal lining) showed this change, and were in

the same stage of vacuolation simultaneously. Vacuoles were always confined to the cytoplasm, and neither the vacuoles nor organisms were found in the nucleus. Both bacilli and granules were found in the vacuoles, but Lewis did not discuss the significance of the latter.

Cytopathogenic effects have been observed by Eaton et al., (1962) when atypical pneumonia organisms were used to infect human amnion cells. It was only after one to three weeks post-inoculation that definite cytopathogenic effects were seen. Early changes began with degeneration of the epithelial cells. Cells eventually fell off the glass leaving open areas which on continued incubation gradually enlarged. Like Lewis (1919), these workers also observed organisms within the vacuoles in the cytoplasm. The organisms often took the form of "granules" (microcolonies) which appeared pinkish with May-Grunwald stain. They were frequently surrounded by a halo.

At Michigan State University, Richardson (1959) studied cultures of bovine fetal kidneys and skin, as well as adult bovine uterine mucosa, testes, spleen, bone marrow, and lungs in the presence of Brucella species. Though no differences in the intracellular growth of Br. abortus in either uterine mucosa or fetal skin cultures were noted, only a fraction of the cells were invaded. Since isolated parasitised cells are routinely found among cells of the same type in vivo, Richardson suggested that invaded cells represent differences in physiological state. The amount of intracellular parasitism was dependent upon the number of Brucella organisms present and the length of time to which the cells were exposed to them. This was in accordance with similar findings by Shepard (1959) and Merriott et al. (1961). The cytoplasmic Brucella organisms completely obscured the nucleus just prior to rupturing the cytoplasm.

Richardson stated that, "As long as the nucleus was visible, no intranuclear multiplication was seen." The filling of the cytoplasm by other bacteria has been mentioned by Shepard (1957) and Gerber et al. (1961). Shepard suggested that the relative innocuousness of mycobacteria in tissue cultures may be a necessary concomitant of their plentiful growth in living cells. Both Lewis (1919) and Richardson described infected cells undergoing mitosis, but the former investigator stated that once completed, no further division occurred.

Both pathogenic and nonpathogenic mycobacteria and non-acid-fast organisms have been observed by Shepard (1957, a., b., 1959) in cultivated mammalian cells. Results indicated that several factors may govern intracellular growth. The presence or absence of phagocytosis was of importance. Sera from different animal species did not possess the same ability to promote phagocytosis and subsequent intracellular multiplication. Merriott et al. (1961) made similar observations and also found that serum concentration played a significant role with Pasteurella tularensis. Besides Shepard, several others (Gerber and Watkins, 1961; Merriott et al., 1961) have noted that intracellular growth was more or less directly proportional to the virulence of the organism. Shepard also stated that temperature, composition of the medium, cell size and activity all influence the multiplication of some organisms. Observations with HeLa cells directed attention to the pH, oxygen tension and other nonspecific biochemical qualities that exist around the bacteria within the metabolizing cell. He thought it possible that a dependent relationship occurs when an organism develops intracellularly.

Spirochetes

According to Murray and Kopeck (1953), work has been done with the spirochetes in tissue culture. They have indexed reports on Borrelia duttoni (Constantinesco, 1931, 1932; Mantenfel and Dressler, 1933-1934; Yokayama, 1933), Treponema pallidum (Bessemans, 1934; Bessemans and de Gesst, 1933, 1934; Bessemans and Haelst, 1934; Foldvari, 1932, 1933; Haagen and Schlossberger, 1932; Kast and Kalmer, 1933; Levaditi, 1920; Levaditi and Constantinesco, 1932; Perry, 1948; Haagen et al., 1932 and Treponema gallinarum (Hemmelweit, 1933, 1934; Levaditi and Stoel, 1931). The inclusion of Ungermann's work (1916) in Murray and Kopeck's bibliography on tissue culture would seem to indicate that he had worked with leptospires in cultured cells. On reviewing this article, it was found that Ungermann did not in fact use tissue culture, but had incorporated liver cells and bile into a medium used for the propagation of L. icterohemorrhagiae.

Perry (1948) had limited success in the maintenance in vitro of T. pallidum using testicular explants from infected rabbits. Three experiments were run but only five out of 80 explants grew. Phase contrast microscopy examination revealed living organisms after five and seven days in two of the five cultures. Treponemas were never seen in cultures that had not grown, nor were they demonstrated in any cultures using Levaditi's method of staining. Rabbits were infected when media from cultures that had been growing ten days were pooled and used as an inoculum. Perry concluded that the survival of the spirochete for ten days was apparently dependent upon the presence of living testicular tissue.

In some preliminary work, Steinhardt (1913) reported multiplication of T. pallidum after 25 days in cell culture. These organisms were obtained from Noguchi who stated that he had kept them growing for some time and that they had lost most of their virulence. Prior to incubation, Steinhardt could not demonstrate the organisms in the preparation by dark-field microscopy. After 25 days, many motile treponemas were seen. The presence of short forms was strongly suggestive of multiplication according to Steinhardt. The author said that it might be possible to restore pathogenicity to avirulent organisms by growing them with living cells and transferring them to fresh cultures.

Bacterial Toxins in Cell Cultures

The production or elaboration of substances which do or may potentially contribute to the pathogenicity of leptospires has been demonstrated. Alexander et al. (1956) first reported the production of hemolysin by L. pomona and other workers (Bauer et al., 1961; and Sleight and Langham, 1962) have demonstrated lesions caused by it in lambs, ewes, and cows. Lacey (1963) exposed bovine fetal kidney monolayers to crude hemolysin extract from L. pomona. He observed complete sloughing of the cells within 24 hours. Stained preparations revealed pyknotic nuclei and shrunken cytoplasm. Based on his work with tissue culture and embryonating chicken eggs, Lacey (1963) concluded that a toxic factor, other than hemolysin, was present in ammonium sulfate fractions of culture filtrates of both hemolytic and nonhemolytic strains of L. pomona. Zemskov (1962) tested 16 strains of leptospires, including L. pomona. He found that 14 produced plasma coagulase and 11 exhibited fibrinolytic properties.

It does not seem illogical that other unidentified "banes" (toxins) might be present. Gourley and Low (1962) described *in vitro* platelet aggregation and liquefaction of blood clots by extracts from *L. icterohemorrhagiae* and *L. canicola*. They suggested that these leptospiral extracts may contain an endotoxin. According to Alston (1961), "Nothing certain is known of the elaboration by leptospires of toxic substances which produce specific skin reaction in infected hosts or which might be responsible for important lesions of the disease." Since these substances, or others, may also affect *in vitro* cells exposed to *L. pomona*, a review of at least some of the investigations on other bacterial "toxins" in tissue culture seemed advisable.

Several authors have reported the effects of diphtheria toxin on the metabolism of cultured cells (Bonventre, 1961; Kato and Pappenheimer, 1960; and Strauss and Hendee, 1959). Strauss *et al.* (1959) observed that HeLa cells became somewhat rounded and granular and assumed a raspberry contour due to the appearance of numerous swellings on the cell surface. Eventually the cell outline disappeared leaving an apparently intact nucleus surrounded by shreds of cytoplasm. He indicated that although glycolysis and aerobic respiration were not interfered with, protein synthesis was completely inhibited two hours before morphological changes were seen.

Fischer (1927) studied the effects of avian tuberculin on fibroblast cultures from the pericardium of tuberculous and nontuberculous chickens. He found that fibroblasts from infected chickens grew more extensively in the presence of tuberculin than did those from the "normal" chickens. Rich *et al.* (1927-1928), however, found that human

tuberculin had no effect on explants from the spleen or leukocytes of tuberculous guinea pigs.

Aronson et al. (1931) pointed out that tissues from various guinea pigs differed in their sensitivity to tuberculin. He also noted that bone marrow cells from infected guinea pigs failed to migrate and multiply in the presence of certain concentrations of human and bovine tuberculin, or the few that did migrate were degenerative or dying as indicated by their round, shrunken or vacuolated appearance. Dilution of the tuberculin resulted in a decrease in the severity of the changes. Tuberculin from 16 other species of mycobacteria had no effect on the growth and migration of marrow cells from either infected or noninfected guinea pigs. Splenic macrophages and testicular fibroblasts from infected guinea pigs also showed inhibition of migration and multiplication by human and bovine tuberculin; however, dilutions greater than 1:15 did not inhibit the explants from nontuberculous animals.

Leptospires in Culture

Cultural and Metabolic Characteristics

Merchant and Packer (1956) stated that the spirochetes are devoid of marked biochemical activity and are not cultivated with ease on artificial media. Several investigators have studied the nutritional requirements of L. icterohemorrhagiae (Faine, 1958, 1959; Fulton and Spooner, 1956; Helprin and Hiatt, 1957; Marshall, 1949; and Ungermann, 1916), but most of the work, according to Vanseltine et al. (1961), has been done on L. canicola (Gerhart and Ball, 1958; Schneiderman, Green, Schieler, McClure, and Dunn, 1953; Vogel and Hutner, 1961).

Faine (1958) indicated that tests for a cytochrome system were positive and that catalase activity was present in cultures of L. pomona. Lacey (1963) and Bertók et al. (1960) confirmed the production of lipase by the same organism.

Many authorities, according to Faine (1959), recommended the addition of "laked" erythrocytes to media as a "source of hemoglobin" for the cultivation of pathogenic leptospires. Vanseltine et al. (1961) stated that pathogenic leptospires require blood or blood serum. In his report on the requirements of L. pomona, Vanseltine suggested that the major function of serum was to provide some lipid material and that fatty-acids were involved.

Vogel et al. (1961) indicated that thiamine was essential for all leptospires, and Ellinghausen (1962) suggested that media be supplemented with vitamin B_{12} when serum low in this vitamin was used.

Ellinghausen (1960) studied several factors which influence the propagation of L. pomona in vitro. Using various buffers in Stuart's medium, he found that both Na_2HPO_4 and KH_2PO_4 were satisfactory, but that when only one buffer was employed, Na_2HPO_4 was superior to KH_2PO_4. Little or no growth occurred when rabbit serum was not incorporated into the medium. At least two per cent serum was required, but maximum cell crops were produced when eight, ten or 12 per cent serum was present. The incorporation of 14 per cent serum caused depression of leptospiral growth at four days. Rabbit serum was superior to bovine serum and pooled rabbit serum was better than serum from a single animal.

Minimum pH and Eh fluctuations are characteristic of this genus, according to Ellinghausen (1960). Optimum starting pH was 7.4

and growth became less above this or below 6.7. The author also stated that no significant difference in the growth was observed when three or eight day old cultures were used for inoculation, but with old cultures (15 days) a lag period was noted. The density of the leptospires was more important than the age of the culture. Low density inoculums resulted in a growth lag which, with increasing density, disappeared and total growth approached equality at nine to ten days regardless of the size of the inoculum.

Ellinghausen (1960) indicated that optimum temperature for total leptospiral crops and maximum growth rate was 28.5° to 29.5° C. Though temperatures above this were stimulatory to the synthesis of protoplasm and faster initial growth, they were not necessarily conducive to the process of cell division. When held at higher temperatures (31° to 37° C.) for prolonged periods of time, a detrimental effect on the process of cell division was noted. Such thermal sensitivity suggested, according to Ellinghausen, that the enzymes which controlled the process of cell division were progressively inactivated at temperatures above 29° C. At 37° C., cell lysis became a factor.

"Granules" in Leptospiral Cultures

The leptospires are slender spiral microorganisms which multiply by transverse fission (Merchant and Packer, 1956). Granules are often observed in old cultures (Alston and Broom, 1958; Swain, 1955; Takeya and Mori, 1953) and a controversy has arisen as to their possible role in the life cycle of the organisms.

A variety of names have been used to describe these bodies, including "leptospirogenes" (Jakob, 1949), "plaques" (Varpholomeeva et

al., 1958), "cysts" (Swain, 1955), and "pseudospores" (Babudieri, 1958).

In their review of the literature, Czekalowski and Eaves (1954) found that some authors considered granules to be products of degenerating or dying leptospires (Gonder and Goss, 1919; Marchoux and Couvy, 1913; Uhlenhuth and Fromme, 1930), while others believed them to play a part in the life cycle of the organism (Sargent and Foley, 1908; Balfour, 1911, 1912; Fantham, 1911; Meirowsky, 1914; Nicolle and Blanc, 1914; Leishman, 1918, 1920; Leipold, 1926; Besseman, 1942; Gastinel and Mollinedo, 1942).

Babudieri (1949) stated that he caused the formation of granules in young cultures using physical and chemical agents and that those which appear spontaneously in old cultures are products of dead or dying leptospires. He said that the "leptospirogenes" discussed by Jakob (1949) were not part of the life cycle, and that the "S" shaped oval bodies described by the same author, were actually contaminants. Timmerman (1927) and Heneweghe (1943), according to Czekalowski et al. (1954), have also produced "granulation" artificially in young cultures of leptospires.

Swain (1955) admitted that the formation of "cysts" observed in his cultures of L. icterohemorrhagiae could have resulted from osmotic effects on the dying organisms. However, he believed it more likely that they were examples of degenerating forms in old cultures or a resting phase in the life of the spirochete.

Czekalowski and Eaves (1954), Jakob (1949), Santos and Muth (1955), and Varpholomeeva and Stanislavsky (1958) all believed that these bodies represent a resting phase or process in the life cycle of the organism.

Some researchers (Bessemans et al., 1949) have isolated granules from nonpathogenic leptospires and grown single cell cultures of the identical organism, according to Czekalowski et al. (1954).

In their studies of the life cycle of the spirochete T. pallidum, De Lamater, Wiggal and Hannes (1950, a.) described "gammae" or "buds". These gammae occurred at any point along the body of the spirochete. They were sessile or stipitate, and appeared both singly and in multiples. De Lamater et al. indicated that a small vesicle or cyst developed from the body of the spirochete and sometimes became free in the medium. Various stages of the organism could be seen developing within the vesicle and later they emerged as filamentous spirochetes.

Miller and Wilson (1962) studied the morphology of L. pomona both in vivo and in vitro using the elctron microscope. Coiled spirochetes were demonstrated in vesicles within the cytoplasm of hepatic parenchymal and renal tubular epithelial cells. They believed that the vesicles were derived from the host, but were unable to determine if this was a defense mechanism of the animal or if it represented a resting or reproductive phase in the life of the leptospires.

MATERIALS AND METHODS

I. Procedures and Preparation of Materials

Cultivation of Stock Leptospires

The LW strain of *Leptospira pomona*, previously described by Bauer *et al.* (1961), was used as the infective agent. Cultures were maintained by weekly passage in Stuart's media (Difco Laboratories, Detroit, Michigan) at 30° C.

Preparation of Equipment

A detergent suspension of "Tide" (Proctor and Gamble, New York) in tap water was used on all metal equipment and most glassware. The routine rinse procedure for all equipment consisted of six rinses in tap water followed by three in distilled water.

Glassware which was to have contact with cells or media was washed in the detergent cleaning solution, rinsed several times and immersed in a potassium dichromate-sulfuric acid cleaning solution (100 ml. saturated $K_2Cr_2O_7$ solution : 9 pounds concentrated H_2SO_4) for at least 24 hours. A second detergent wash was followed by the routine rinse.

Metal instruments received only the detergent wash and a routine rinse, whereas rubber and plastic equipment (stoppers and caps) were soaked 24 hours in Haemo-Sol (Meinecke and Company, New York), scrubbed, and then given the routine rinse.

Cover-slips, 11 x 11 mm., were cut into three pieces approximately 3.5 x 11 mm. and washed in a solution of equal parts ether and alcohol. They were polished with cheesecloth and placed in clean, screw-capped Leighton tubes (Kimble Glass Company, Toledo, Ohio).

All equipment was sterilized by autoclaving for 15 minutes at 121° C. and 15 pounds pressure.

Preparation of Solutions

Antibiotic Solution

A 100x antibiotic concentrate was used in some experiments. Two hundred thousand units of penicillin G potassium, and one gram of dihydrostreptomycin sulfate were dissolved in 100 ml. of sterile double distilled (DD) water. One ml. of this concentrate was added to each 100 ml. of Eagle's Minimum Essential Medium or balanced salt solution when antibiotics were desired.

Trypsin Solution

All trypsinization was done with a 0.25 per cent solution which was prepared in the following manner:

NaCl	8.00 Gm.
KCl	0.29 Gm.
Na_2HPO_4	1.15 Gm.
KH_2PO_4	0.29 Gm.
Trypsin*	2.50 Gm.

* (Trypsin 1:250, Difco Laboratories, Detroit, Michigan)

The various ingredients were dissolved in one liter of DD water using a magnetic stirrer. Enough Phenol Red (which did not contain chloroform) was added to make the solution pink. The solution was filtered through No. 1 Whatman filter paper (W. & R. Balston, Ltd., England), sterilized by pressure filtration using ST-1 asbestos pads (Hercules Filter Corporation, Paterson, New Jersey), dispensed in 200 ml. amounts and stored at -20° C.

Phenol Red Solution

Phenol Red, 0.4 Gm., was dissolved in 150 ml. of DD water and titrated to pH 7 with N/20 NaOH. It was diluted to 200 ml. with DD water. When used with Hank's Balanced Salt Solution (BSS), 1-2 ml. of chloroform were added as a preservative.

Balanced Salt Solutions

A. Earle's Balanced Salt Solution (BSS)

Earle's Balanced Salt Solution was found to be most satisfactory. It was prepared as follows:

Unit #1

NaCl	6.80 Gm.
KCl	0.40 Gm.
$MgSO_4$	0.10 Gm.
NaH_2PO_4	0.125 Gm.
$NaHCO_3$	2.20 Gm.
Glucose	1.00 Gm.

The above ingredients were dissolved in 500 ml. of DD water.

Unit #2

$CaCl_2$ . . . . . 10.0 Gm.

H_2O (double distilled) . .100.0 ml.

For use, 2.0 ml. of Unit #2 were dissolved in 200 ml. of DD water and added to Unit #1.

B. Hank's Balanced Salt Solution (BSS)

In some experiments Hank's Balanced Salt Solution was used. It was prepared in a 10x concentration as follows:

Unit #1

$NaHCO_3$ . . . . . 3.5 Gm.

Sodium bicarbonate was dissolved in 250 ml. of DD water, dispensed in prescription bottles in 50 ml. aliquots, sterilized by autoclaving at 121° C. for 15 minutes, and stored at room temperature until the medium was prepared.

Unit #2

NaCl . . . . . 80.0 Gm.

KCl . . . . . 4.0 Gm.

$MgSO_4 \cdot 7H_2O$ . . . . . 2.0 Gm.

$Na_2HPO_4 \cdot 2H_2O$ . . . . . 0.6 Gm.

Glucose . . . . . 10.0 Gm.

KH_2PO_4 . . . . . 0.6 Gm.

The components of Unit #2 were dissolved in 800 ml. of DD water.

Unit #3

$CaCl_2$ . . . . . 1.4 Gm.

Calcium chloride was dissolved in 100 ml. of DD water.

One hundred ml. of Phenol Red solution was added to Unit #2. Unit #3 was then added to the latter to make 1000 ml.

Preparation of Serum

Two-to nine-month-old calves were used as the source of serum. These animals were negative to the agglutination-lysis test using L. pomona (strain Johnson) as the antigen (Morse et al., 1955). The blood was allowed to clot and set for 18-24 hours at 4° C. and then was centrifuged at 3000 r.p.m. for ten minutes. The collected serum from at least three animals was pooled, sterilized by filtration using Seitz ST-1 asbestos pads, and stored at -20° C.

Preparation of Media

Eagle's Minimum Essential Medium (EMEM) containing ten per cent calf serum was used in this study. The medium was prepared in a slightly different manner depending upon which BSS was used. The components and their order of addition were as follows:

Eagle's Minimum Essential Medium with Earle's BSS (EMEM-E)

Earle's BSS . . . . . 700 ml.

Phenol Red solution . . . . . 100 ml.

The above combination was autoclaved to drive off chloroform and allowed to cool at room temperature.

Amino Acids (50x concentration)* . . . . . 20 ml.

Vitamins (100x concentration)* . . . . . 10 ml.

Calf serum . . . . . 100 ml.

Double distilled water . . . . . q.s. 1 liter

* (Microbiological Associates, Inc., Bethesda, Maryland)

The medium was sterilized by pressure filtration using Seitz ST-1 asbestos filter pads, dispensed in 100 ml. amounts, and stored at -20°C. One ml. (200 micromoles) of glutamine (Microbiological Associates, Inc., Bethesda, Maryland) was added to each 100 ml. of medium just prior to use. Antibiotics, when used, were also added at this time. The pH of the medium was adjusted to 7.2 - 7.4 by providing a five per cent CO_2-in-air atmosphere.

Eagle's Minimum Essential Medium with Hank's BSS (EMEM-H)

Hank's BSS (10x) . . . . . 100 ml.

Double distilled water . . . . . 700 ml.

To remove the chloroform (Phenol Red in Hank's concentrate contains chloroform as a preservative) the diluted salt solution was autoclaved and allowed to cool at room temperature.

Amino Acids (50x concentration)* . . . . . 20 ml.

Vitamins (100x concentration)* . . . . . 10 ml.

Calf serum . . . . . 100 ml.

* (Microbiological Assoc. Inc., Bethesda, Maryland)

The medium was then diluted with DD water to a final volume of one liter. The processing of the medium from this point on was exactly the same as that for EMEM-E. Just prior to use, sodium bicarbonate (Unit #1 in Hank's BSS) was added to adjust the hydrogen ion concentration of the medium to pH 7.2 - 7.4. This usually required 8-12 ml.

50-50 Medium

When the medium of a culture in some experiments was changed, it was replaced with a "50-50" medium. This medium was prepared by combining equal volumes of Stuart's medium (containing ten per cent rabbit serum) and EMEM. Depending upon the BSS used, $NaHCO_3$ (Unit #1 of Hank's BSS) or five per cent CO_2-in-air was used to adjust the pH of the medium.

Tissue Culture Preparations

Procurement and Processing of Tissue

Kidneys from four- to six-month-old bovine fetuses obtained from a local slaughtering plant were used in this study. The dam's uterus was washed with 1:2000 Roccal (Sterwin Chemicals Inc., New York, New York) followed by 70 per cent alcohol. Using sterile instruments, the uterus was opened and the fetus removed. After Roccal and alcohol were poured over the flank of the fetus, a paralumbar incision was made and the kidneys removed by blunt dissection with a sterile-gloved hand. Kidneys were immediately placed in sterile Hank's BSS solution. In the initial experiments, antibiotics were incorporated into the BSS. Antibiotics were found to be unnecessary in later studies.

At the laboratory, the kidneys were placed in Petri dishes and the capsules removed. Using curved scissors, the cortical tissue was separated and placed in a 100 ml. mincing-washing beaker. Two or three kidneys were used depending upon their size. The tissues were washed two to three times with Hank's BSS or EMEM, minced with scissors into pieces approximately 3 x 3 mm., and again washed with BSS or media.

After the supernatant washing solution was poured off, the minced tissue was placed in a 500 ml. trypsinization flask (Bellco Company, Vineland, New Jersey), trypsin added to the 80 ml. graduation, and the tissue trypsinized for five to ten minutes at room temperature using a magnetic stirrer. The supernatant was again poured off, fresh trypsin added to the 150 ml. graduation, and trypsinization continued for 30 to 40 minutes.

The cell suspension was filtered through four layers of cheesecloth, the filtrate placed in 13 x 100 ml. screw-capped test tubes, and centrifuged at 3000 r.p.m. for five minutes. The cell "plug" was resuspended in EMEM and recentrifuged. This latter step was repeated at least twice.

A modified viable cell count was made using a hemocytometer as described by Merchant et al. (1961). No crystal violet was used. Aliquots of 100,000 to 200,000 cells in ten ml. of EMEM were dispensed into 99 ml. milk dilution bottles. If EMEM-E was used, the bottles were flushed with five per cent CO_2-in-air. Incubation was at 37° C. Media was changed the next day and subsequently at three- to five-day intervals. Antibiotics, when used, were sometimes omitted with the first medium change. Seven to ten days were usually required for a monolayer to develop.

Subcultures were used in all experiments. The medium was poured off and the cells removed from the glass by trypsinizing at room temperature with occasional shaking. Fifteen to 20 minutes exposure to the trypsin was usually sufficient to harvest the cells. The cell-trypsin suspension was centrifuged at 3000 r.p.m. for five minutes, resuspended in EMEM and a viable cell count made. Eighty thousand to 100,000 cells in one ml. of media were dispensed in Leighton tubes, and if EMEM-E was used, a five per cent CO_2-in-air atmosphere provided. Cultures were incubated at 37° C. in Leighton tube racks (Bellco Company, Vineland, New Jersey).

Determination of Monolayer "Status"

Early in this study it was observed that the amount of monolayering varied from culture to culture, and that the interpretation of the results were in part dependent upon this variation. A classification system of the amount of monolayering was therefore developed.

When a sufficient monolayer had developed, the cell cultures were examined with a light microscope and the approximate amount of monolayering (status) recorded for each culture. A plus (+) status indicated at least a five per cent monolayer. Status I, II, and III represented monolayers of 25, 50, and 75 per cent respectively. A 95 to 100 per cent monolayer was indicated as a status IV. The cultures were numbered and distributed so that representative statuses were present in both the control and infected groups.

Fixation and Staining Procedures

All staining procedures were modifications of those described in the "Manual of Histologic and Special Staining Technics" (1957), except that for May-Grunwald-Giemsa. The latter was adapted from Jacobson (1952).

Warthin-Starry Silver Stain for Spirochetes

Fixation of cell cultures: The medium of the cell culture was poured off. To avoid precipitation of the remaining medium, the cover-slips were washed once with two ml. of saline and twice with two ml. of ten per cent formalin. (In some experiments no wash was given prior to fixation of the cultures to be stained by the Warthin-Starry technique.) Two additional ml. of ten per cent formalin were added and the cultures stored until stained.

Staining of cell cultures: Since paraffin was not employed it was not necessary to pass the cover-slips through xylene and alcohols. They were washed twice with distilled water and stained starting with one per cent $AgNO_3$. After completion of the silver staining, the cover-slips were either passed through alcohols to xylene and mounted with "Permount" (Fischer Scientific Company, Fair Lawn, New Jersey), or counter-stained with Delafield's hematoxylin for one to two minutes and then mounted in the usual manner.

Oil Red O Stain for Fat

The fixation of the cell cultures was the same as for the Warthin-Starry silver stain. No modifications were made in the staining procedure.

May-Grunwald-Giemsa Stain

Fixation of cell cultures: The cell cultures were fixed with 100 per cent methyl alcohol using the same procedure as described for Warthin-Starry silver stain.

Staining of cell cultures: The cover-slips were placed in May-Grunwald solution for ten minutes and then transferred to diluted Giemsa (1:10) for ten additional minutes. They were rinsed quickly in distilled water and rapidly dehydrated in two changes each of acetone and acetone-xylene (equal parts). The cover-slip monolayers were cleared in xylene and mounted in "Permount".

Other Procedures

Dark-field Microscopy

Frequently it was necessary to make direct observations on living leptospires as in the morphology studies and enumeration of the organisms. A Baush and Lomb dark-field microscope was found to be satisfactory for this work.

Enumeration of Leptospires

The following method of determining the number of leptospires in a culture medium was used: One-tenth ml. of medium was diluted 1:10 or 1:20 with two per cent formalin. A small quantity of the diluted medium was then placed in a Petroff-Hausser Bacterial Counting Chamber (Chicago Apparatus Company, Chicago, Illinois) and the number of organisms in the small squares counted using a dark-field microscope. The following formula was then employed:

$$K \frac{a\ b}{c} = d$$

where:

K = 2×10^7 (conversion factor for the chamber).

a = the number of leptospires in the small squares.

b = the dilution (10 or 20).

c = the number of small squares counted.

d = the number of leptospires in one ml. of diluted medium.

II. Experimental Procedures

Growth and Morphology Studies of Leptospires in Various Media

Three growth and morphology study (GMS) experiments were set up to determine the growth rates and/or morphological changes that occurred when L. pomona was grown in EMEM-H, EMEM-E, or 50-50 medium.

GMS #1: GMS Studies with EMEM and Hank's BSS (EMEM-H)

Three groups of tubes, designated as #1, #2, and #3, were set up and received one ml., 0.5 ml., and 0.2 ml., respectively, of a seven-day-old culture of L. pomona. This culture contained approximately 294 million organisms per ml. EMEM-H was added so that a total of three ml. was present in each of the cultures. The cultures were incubated at 37° C. and the pH of the medium and the growth rate recorded daily for six days. The hydrogen-ion concentration was determined by comparison with Phenol Red standards. Leptospiral morphologic observations were made on post-inoculation (PI) days four and five.

GMS #2: GMS Studies with EMEM and Earle's BSS (EMEM-E)

One ml. of a seven-day-old culture of L. pomona containing approximately 100 million leptospires was inoculated into one ml. of EMEM-H, a five per cent CO_2-in-air atmosphere provided and the tubes incubated at 37° C. The pH of the medium and the morphology and number of organisms were checked daily for five days and recorded.

GMS #3: GMS Studies with 50-50 Medium

In this experiment a three-day-old culture of L. pomona served as the inoculum. One ml. of the culture, containing approximately 240 million leptospires, was inoculated into one ml. of EMEM-H and incubated at 37° C. Three days after infection the medium was poured off, except for approximately 0.3 ml. which acted as "seed", and replaced with two ml. of 50-50 medium. Incubation was continued and at PI day six the morphology and activity of the leptospires were observed.

Leptospira-Infected Cell Culture Studies

A series of infected cell culture study (ICS) experiments were set up to study L. pomona-infected cell cultures. Because of the results in some experiments, observations were confined primarily to the morphology, number, and distribution of the leptospires. Limited observations were, however, made on the cells. In other experiments, both the cells' and leptospires' morphological changes and/or growth in the medium were studied in detail. The experiments are therefore not necessarily numbered (ICS) or listed in the sequence in which they were performed, but rather are grouped according to the types of observations made in the experiment. For the convenience of the reader, a number in parentheses was placed after the ICS number. Example: ICS #2 (4). This indicates that experiment ICS #2 was the fourth experiment in the series of experiments performed. These ICS numbers will be referred to in the "Experimental Procedures", "Results" and "Discussion".

It will also be noted that the experimental method varied somewhat from experiment to experiment. This is explained by the fact

that the findings in one experiment suggested modifications in those which were to follow. A summary of the more important variations and similarities in the experimental methods of the ICS experiments are shown in TABLE I.

Group A: Observations on Leptospires in Infected Cultures with Limited Cell Studies

ICS #1 (1)

The cell cultures were in EMEM-H with antibiotics for five days prior to infection. To remove the antibiotics, the medium was poured off, replaced with fresh EMEM-H without antibiotics and the cultures incubated for 24 hours at 37° C.

To infect the cells, the medium was poured off and a total of two ml. of EMEM-H-Stuart's medium added. The latter medium was prepared by adding 0.1 ml. of a five-day-old leptospiral culture containing approximately 440 million organisms per ml., to 1.9 ml. of EMEM-H. Controls received only two ml. of EMEM-H. The cultures were divided into four groups and incubated at 37° C.

PI Day 2: Due to a lowered pH of the medium, Group 1, which was to be exposed to the infective medium for three days, was processed at two days by washing three times with GKN (Merchant et al., 1960), and then formalin-fixed. At the same time the medium in Groups 2 and 4 was poured off, the cultures washed three times with sterile GKN, two ml. of fresh EMEM-H added and incubation continued at 37° C. Group 3, however, was transferred to a 30° C. incubator without a change of the medium.

TABLE I. Variations and similarities in infected culture (ICS) experimental methods

EXPERIMENT NUMBER	INFECTION OF SUBCULTURES				
	Antibiotics in original subcultures	Monolayer status determined	Media and atmosphere	Leptospiral inoculum	
				Age of lepto. culture (in days)	No. of lepto. used (in millions)
ICS# 1 (1)	Yes	No	Air EMEM-H	5	440
ICS# 3 (2)	Yes	No	5% CO_2 EMEM-H	3	42
ICS# 4 (3)	Yes	Yes	Air EMEM-H	5	440
ICS# 2 (4)	Yes	Yes	Air EMEM-H	2	150
ICS# 5 (5)	No	Yes	5% CO_2 EMEM-E	8	126
ICS# 6 (6)	Yes	Yes	Air EMEM-E	9	404
ICS# 7 (7)	Yes	Yes	Air EMEM-E	9	404

TABLE I. (cont'd.)

ROUTINE MEDIA CHANGE				TERMINATION OF EXPERIMENT	
PI Days	Media checked for lepto.	50-50 medium used to replace EMEM	Seed media left	PI days cultures were fixed	Stains used *
2	No	No	No	2,4,5	WS
3	Yes	No	No	3,6	WS + H
2,4, 6	Yes	Yes	No	2,4, 6,8	WS
3	No	Yes	Yes	3,8	WS + H
3,6	Yes	No	Yes	3,6,9	WS, MGG, ORO
3	No	No	Yes	1,2,3, 4,5,6	WS, MGG, ORO
3	Yes	No	Yes	6	WS, MGG

*WS: Warthin-Starry
WS + H: Warthin-Starry + Hematoxylin
MGG: May-Grunwald-Giemsa
ORO: Oil Red O

PI Day 4: The pH continued to fall and Groups 2 and 3 were formalin-fixed without a GKN wash. Group 4 was allowed to continue incubation for one additional day.

PI Day 5: The monolayered cells in Group 4 began to slough and therefore the cultures were formalin-fixed. All groups were stained using the modified Warthin-Starry silver technique.

ICS #2 (4)

This group of cell cultures had been grown in an antibiotic-containing EMEM-H. To eliminate the antibiotics, the cultures were processed in the same manner as those in ICS #1 (1). The status of each monolayer was determined, the culture tubes numbered and distributed so that representative statuses were present in both the control and infected cultures. The cell cultures were divided into two groups and infected with one ml. of a two-day-old Stuart's culture of leptospires containing approximately 150 million organisms, and one ml. of EMEM-H added.

PI Day 3: Group 1 was formalin-fixed. The medium in Group 2 was poured off leaving approximately 0.1 ml. of medium to act as a "seed" and two ml. of EMEM-H added. The pH of the medium was adjusted with $NaHCO_3$ (Unit #1 of Hank's BSS) and the cultures incubated for five additional days at 37° C.

PI Day 8: Group 2 was fixed in the same manner as Group 1. All cultures were stained using the modified Warthin-Starry silver technique and Delafield's hematoxylin as a counter-stain.

Group B: Observations on Cells and Leptospires in Infected Cultures

ICS #3 (2)

The cell cultures, which had been maintained in an antibiotic-containing EMEM-H, were washed twice with antibiotic-free EMEM-H; the cell cultures infected with 0.2 ml. of a three-day-old leptospiral culture containing approximately 42 million organisms; two ml. of EMEM-H added; and the tubes flushed with five per cent CO_2-in-air. Controls received 0.2 ml. of noninfected Stuart's medium and two ml. of EMEM-H. The cultures were divided into two groups and incubated at 37° C.

PI Day 3: The medium in group 1 was poured off, the cultures fixed with formalin and the medium examined for the presence of leptospires. The medium in Group 2 was removed, replaced with two ml. of fresh EMEM-H, a five per cent CO_2-in-air atmosphere provided and incubation continued.

PI Day 6: Group 2 was fixed and both groups were stained using the modified Warthin-Starry silver technique and Delafield's hematoxylin as a counter-stain.

ICS #4 (3)

As described in ICS #2 (4), the monolayer status was determined, the cell cultures numbered and distributed into four groups. Cultures were infected with one ml. of a five-day-old leptospiral culture containing approximately 440 million organisms per ml., and one ml. of EMEM-H added. Controls received one ml. each of noninfected Stuart's medium and EMEM-H, and all cultures incubated at 37° C.

PI Day 2: The medium of Group 1 was poured off, the cell cultures washed once with saline, and the cultures fixed with formalin. Groups 2, 3, and 4 were treated in a similar manner except they were washed with sterile saline, two ml. of 50-50 medium added and incubation continued.

PI Day 4: The medium of Group 2 was poured off and examined for the presence of leptospires. The cell cultures were formalin-fixed without a saline wash. Groups 3 and 4 were treated in a similar manner except the medium was replaced with 50-50 medium as on PI day 2 and no examination made for the presence of leptospires. Incubation of the latter groups was continued.

PI Day 6: Group 3 was processed like Group 2 on PI day 4, and Group 4 the same as it was on PI day 4.

PI Day 8: Group 4 was formalin-fixed without a saline wash. All groups were stained using the modified Warthin-Starry silver technique.

ICS #5 (5)

The cell cultures in this experiment had been grown in antibiotic-free EMEM-E. The status of the monolayers of the various cultures was determined, the culture tubes numbered and distributed into three groups. The medium was poured off; the cultures were infected with one ml. of an eight-day-old culture of leptospires containing approximately 126 million organisms per ml.; one ml. of EMEM-E was added; and the cultures were flushed with five per cent CO_2-in-air. Controls were treated in a similar manner except one ml. of noninfected Stuart's medium was substituted for the infective medium. Incubation was at 37° C.

Daily observations on the cell culture monolayers were made with a light microscope, and the pH of the medium noted by comparing with Phenol Red standards.

PI Day 3: Group 1 was processed first removing the medium, flushing with saline, and then fixing the cultures with formalin or alcohol. The medium in Groups 2 and 3 was poured off leaving approximately 0.3 ml. to act as a "seed", two ml. of EMEM-E added and a five per cent CO_2-in-air atmosphere provided.

PI Day 6: The procedure was the same as for PI day 3 except Group 2 was fixed and the medium of Group 3 changed.

PI Day 9: The cell cultures of Group 3 were fixed in the same manner as those in Groups 2 and 3, and all were stained using the Warthin-Starry Silver, May-Grunwald-Giemsa or Oil-Red-O techniques.

ICS #6 (6)

The cell cultures were prepared in the same manner as those in ICS #3 (2) substituting EMEM-E for EMEM-H. The monolayer status was determined and the cultures divided into six groups. They were infected with one ml. of a nine-day-old culture of L. pomona containing approximately 404 million organisms, and one ml. of EMEM-E added. Since the pH of the medium was approximately 7.2, a CO_2 atmosphere was not necessary. Control cultures received one ml. each of noninfected Stuart's medium and EMEM-E. Incubation was at 37° C.

PI Day 1: The pH of the medium in Group 1 was noted and the cultures washed with saline and formalin- or alcohol-fixed.

PI Day 2: Group 2 was processed in the same manner as Group 1.

PI Day 3: Group 3 was fixed in the same manner as Group 1. The medium of groups 4, 5 and 6 was removed, leaving approximately 0.3 ml. to act as a "seed", two ml. of EMEM-E added, and incubation at 37° C. continued.

PI Day 4: Group 4 was processed in the same manner as Group 1.

PI Day 5: Group 5 was processed in the same manner as Group 1.

PI Day 6: Group 6 was processed in the same manner as Group 1., and all cultures were stained using Warthin-Starry, May-Grunwald-Giemsa or Oil-Red-O technique.

ICS #7 (7)

This experiment was run simultaneously with ICS #6 (6) and the cell preparations and infections were the same. In this experiment daily counts of the leptospires were made using the method described under Enumeration of Leptospires and the pH of the medium noted by comparison with Phenol-Red standards. As in ICS #6 (6), the medium was changed on PI day 3 and the observations continued. The cell cultures were formalin- or alcohol-fixed on PI day 6, and all cultures stained using the modified Warthin-Starry silver or May-Grunwald-Giemsa technique.

TERMINOLOGY

Tissue culture descriptive terminology varies somewhat from one investigator to another. For the convenience of the reader, the following terms used in RESULTS and DISCUSSION are defined:

Fibroblast-like cells: Cells which are spindle-shaped or elongated, possess an oval nucleus and a moderate amount of cytoplasm. When in groups, these cells frequently lie parallel to one another (parallelism), as seen in Figure 1.

Epithelial-like cells: Cells which are irregularly polyhedral in shape, possess a round to oval nucleus and a moderate amount of cytoplasm (Figure 2).

Large epithelial-like cells: Cells which are two to ten times the size of epithelial-like cells and possess a nucleus which is round to oval in shape and three to ten times the size of those in epithelial-like cells. These cells have an abundance of cytoplasm which may have processes (Figure 3).

Epithelial-type colonies: Mono- or multilayered colonies of 20 or more epithelial-like cells.

Fibroblast-type colonies: Mono- or multilayered colonies composed of 20 or more fibroblast-like cells.

Mixed-type colonies: Mono- or multilayered colonies composed of 20 or more epithelial-like and fibroblast-like cells.

Sunburst-type colonies: Mixed-type colonies which usually have a centrally located nidus of cells and a corona composed of radiating fibroblast-like cells.

Open Areas: The spaces between cell colonies usually containing large epithelial-like and other cell types.

RESULTS

Growth and Morphology Studies with Leptospires in Various Media

GMS #1: GMS Studies with EMEM-H

The growth rate studies of GMS #1 are graphically shown in Figure 4. Though the rate of growth varied somewhat with the size of the inoculum, all groups exhibited an increase in the number of leptospires and peaked at three to four days. The number of organisms then decreased through PI day six.

A record of the pH of the medium in the various leptospiral cultures is shown in TABLE II

TABLE II. pH studies of Leptospira-infected Eagle's Minimum Essential Medium prepared with Hank's Balanced Salt Solution (EMEM-H).

Group Number	Culture	PI Days*						
		0	1	2	3	4	5	6
1	A	7.4	7.3	6.8**	6.8	6.8	6.8	6.8
	B	7.4	7.3	6.8	6.9	6.9	6.8	6.8
	C	7.4	7.4	6.9	7.5	7.6	7.2	7.0
2	D	7.4	7.6	6.8	6.9	5.8	6.8	6.8
	E	7.4	7.6	6.8	6.9	6.8	6.8	6.8
	F	7.4	7.6	6.8	7.0	7.2	7.2	7.0
3	G	7.4	7.9	7.7	7.5	6.9	7.0	6.8
	H	7.4	7.9	7.7	7.5	6.9	7.0	6.8
	I	7.4	7.9	7.7	7.6	7.2	7.0	6.8

*Post-inoculation Days.

**Phenol Red indicator has a sensitivity range between 6.8-8.4 (Pelczar and Reid, 1958); therefore the pH of the medium could have been lower.

The results of the morphologic observations were as follows:

Day of inoculation: All leptospires appeared morphologically "normal" and were very active.

PI Day 4: The activity of the leptospires in all groups was somewhat reduced by comparison to the day of inoculation.

Group 1 (high inoculum group): Approximately five per cent of the leptospires exhibited a "normal" morphology. The remainder appeared beaded or granular. Dumbbell and comma forms were common.

Group 2 (intermediate inoculum group): Nearly all organisms possessed a "normal" morphology and only one per cent or less appeared granular or beaded.

Group 3 (low inoculum group): With the exception of a few organisms which appeared to be two to three times normal length, all leptospires exhibited a "normal" morphology.

PI Day 5: The activity of all groups was greatly reduced.

Group 1: Nearly all leptospires appeared granular.

Group 2: About 50 per cent of the organisms appeared "normal" and the remainder were beaded or granular.

Group 3: With the exception of a few beaded forms, all leptospires possessed a "normal" morphology.

GMS #2: GMS Studies with EMEM-E

The results of the growth rate studies in GMS #2 are shown graphically in Figure 5, and the pH of the medium on the various days is presented in TABLE III.

TABLE III. pH studies of Leptospira-infected Eagle's Minimum Essential Medium prepared with Earle's Balanced Salt Solution (EMEM-E)

Culture	PI Days*					
	0	1	2	3	4	5
A	7.3	7.3	7.3	7.0	7.0	6.9
B	7.3	7.3	7.3	7.0	7.0	6.9
C	7.3	7.3	7.3	7.0	7.0	6.9

*Post-inoculation Days.

The leptospires appeared "normal" and active up to PI day two. At this time, long forms (two to three times normal length) appeared, and their number increased throughout the remainder of the study. Granular, beaded or comma forms were not seen at any time. The activity of the leptospires decreased progressively from PI day two through PI day five.

GMS #3: GMS Studies with 50-50 Medium

Observations on the morphology of the leptospires on PI day six revealed both "normal" and long forms of the organisms. The activity of the organisms was somewhat less than that seen on the day of inoculation.

Leptospira-Infected Cell Culture Studies

Group A: Observations on Leptospires in Infected Cultures with Limited Cell Studies

ICS #1 (1)

The monolayers in this experiment consisted of large and small epithelial-type colonies. In the open areas there was an uneven distribution

of individual or small groups of epithelial-like and a few large epithelial-like cells.

The cell subcultures had been seeded with a large number of cells. The uncontrollable rapid growth of these cells resulted in monolayer detachment. The rapid fall in pH of the medium and/or detachment of the monolayered cells determined when culture groups were fixed in this experiment. Attempts to control the pH by the addition of $NaHCO_3$ were futile, and it was felt that the excessive amounts required would disrupt the isotonic state of the medium.

Since the monolayer statuses were not determined in this experiment, no valid comparison could be made between the amount of monolayer growth in the infected and control cultures. Though detailed morphologic studies of cells stained using the Warthin-Starry silver technique were not possible, no differences between the cells of the control and infected cultures were detected.

In some cultures, a small quantity of medium remained after the saline washes and precipitated when formalin (used for fixation) was added. As a result, the silver-stained monolayers of these particular cultures were distorted, and nearly impossible to read (Figure 7). Occasionally, filaments in the cytoplasm of cells would stain and resemble leptospires (Figure 8). Examination of the readable silver-stained infected cultures revealed that many leptospires were present in the PI-day-two cultures; very few in the PI-day-four cultures (Groups 2 and 3); and none in the PI-day-five cultures. While a few organisms were found in the open areas, the vast majority were on cell colonies.

Spirochetes, irrespective of the total number seen in a particular cell culture, were distributed so that the greater number were present on the larger epithelial-type colonies. The organisms associated with these colonies were located on the cell surfaces. When a multilayer of cells was present, the leptospires were frequently between the layers.

On oil immersion examination, the spirochetes frequently appeared fragmented. Several pieces of the black-staining protoplasmic cylinders were joined by a thin, brown-staining, sometimes refractile filament (Figure 9). These filaments resembled and were interpreted as axial filaments.

Granules were present in both the infected and control cultures, and some appeared to be intracytoplasmic. These were either brownish-black and refractile or pure black in color. These structures frequently circled and outlined the cell nuclei. Varying numbers of irregularly round, black-staining granules were at times seen free and attached to the leptospires or axial filaments. Attachment to the body of the organisms or filaments was indiscriminate, and sometimes resulted in granule-with-tail or, less commonly, dumbbell-like forms (Figures 10, 11). Some of these forms were apparently formed by a movement of the protoplasmic cylinder to one end of the axial filament.

Ring structures, some of which were apparently intracytoplasmic, were found in the infected cultures (Figure 12). These rings were formed by the coiling of organisms about themselves, since spirochetes with varying degrees of looping were observed (Figure 13).

ICS #2 (4)

The monolayers in this experiment were composed of irregularly shaped, sunburst-type colonies. A nidus of cells was located at the center of each colony from which numerous fibroblast-like cells radiated. These radiating cells bridged from one group to the next and at times gave the monolayer a whorled appearance. Cytoplasmic vacuoles were common in both the infected and control cultures.

On microscopic examination of the silver-stained cultures at PI days three and eight, leptospires, granules or structures similar to those seen in ICS #1 (1) could not be found. The monolayer observations were as follows:

PI Day 3: Examination of the cultures revealed little or no difference between the infected and control monolayers.

PI Day 8: At PI day eight, control and infected monolayers were dense and detachment of the cells was common.

Group B: Observations on Cells and Leptospires in Infected Cultures

ICS #3 (2)

The growth pattern in both the infected and control cultures at PI days three and eight differed from those described in ICS #1 (1) and #2 (4). The monolayers were made up of epithelial-type colonies. Large epithelial- and fibroblast-like cells were found in the open areas. The fibroblast-like cells tended to take on a whorled arrangement and nearly filled the open areas between the cell colonies. Mitotic figures were common only in the fibroblast- and epithelial-like cells.

Vacuoles, however, were present in all cell types, including the large epithelial-like variety. Some nuclear aberrations were present but were limited in distribution to the large epithelial-like cells. Monolayer statuses had not been determined at the onset of this experiment. It was therefore not possible to interpret differences between the infected and control monolayers.

Leptospires were not found in the medium three days after infection, and organisms, granules or other structures similar to those in ICS #1 (1) could not be demonstrated in the silver-stained cultures. A few organisms were observed in one stained culture at PI day six. These spirochetes were smaller in diameter than those seen in ICS #1 (1). Morphologically similar granules were present in both the PI-day-six control and infected cultures.

ICS #4 (3)

The monolayers in this experiment were composed of irregularly shaped epithelial-, fibroblast- and mixed-type colonies. Large epithelial-like cells were rare.

PI Day 2: Little or no cellular growth had occurred in either the infected or control cultures. Cell fragments were more common in the open areas of the infected cultures.

Examination of the medium revealed many leptospires. Their numbers varied directly with the status of the monolayer. In cultures of status III, organisms were most dense in multilayered areas. This was similar to the findings in ICS #1 (1). On oil immersion examination the organisms appeared smaller in diameter than those observed in ICS #1 (1). The protoplasmic cylinders of the spirochetes stained in

such a manner that alternate light and dark segments resulted. Small black granules were sometimes seen along the body of the organisms giving the spirochetes a beaded appearance. Granule-with-tail, ring-with-tail, looped and coiled-ring forms similar to those observed in ICS #1 (1) were present. The coiled-ring forms were most commonly on the cell surface, but sometimes were apparently within the cytoplasm.

In cultures of status I, leptospires were found with equal frequency on the monolayers and in the open areas. Groups of leptospires within masses of nondescript debris were observed in the open areas.

PI Day 4: Examinations of medium revealed that all cultures were infected.

No significant monolayer growth had occurred in either the infected or control cultures. Though the medium observations indicated that cultures were infected, difficulty was encountered in the demonstration of organisms on some stained monolayers. Those organisms which were seen, stained similarly to those described at PI day two and appeared thin and segmented. A few granule-with-tail, ring-with-tail, looped and coiled leptospiral forms were observed. Black granules occurred in both the control and infected cultures.

PI Day 6: The monolayers of some controls had grown markedly. Others, however, showed little change. Studies of the infected culture medium confirmed infection in only one culture. In this culture, numerous organisms were found on the monolayers. Coiled leptospiral rings were seen. Nearly all of these structures were confined to the monolayers and many appeared to be intracytoplasmic. A few of the other forms of leptospires described on PI day two were also present.

PI Day 8: No significant differences in the monolayer status of the control and infected cultures were seen, nor were any spirochetes found.

ICS #5 (5)

Data on the pH of the medium of Groups 2 and 3 (PI days four through nine) are shown in TABLE IV. Except for PI day four, the pH of the control cultures generally ranged lower than that of the infected. This corresponded to the more rapid growth of cells in these cultures.

PI Day 3: Dark-field observations of the medium from infected cultures disclosed the presence of numerous leptospires possessing "normal" morphology and motility. Long forms were also seen.

Stained control cultures had increased in cellular density and possessed a nearly complete even monolayer composed of mixed-type colonies (Figure 14). Between the colonies, many epithelial-, fibroblast- and a few large epithelial-like cells were found. Some multinucleated cells were also present.

Infected monolayers were characterized by sparsely distributed, lacy and ragged appearing mixed-type colonies. Located at the center of the colonies were small dense niduses of cells. Irregularly placed large epithelial-like cells with long, thin cytoplasmic processes surrounded the peripheral portion of these colonies. Large open areas were found. These contained a few epithelial- and fibroblast-like cells; some small individualized cells with contracted and intensely stained cytoplasm; and numerous large epithelial-like cells possessing long, thin cytoplasmic processes (Figure 15). Occasionally a multinucleated

TABLE IV. Hydrogen-ion concentration of medium in control and Leptospira-infected bovine fetal kidney cultures. Infective culture study (ICS) experiment #5 (5)

PI* Day	Type of Culture	Total number of cultures	pH of medium							
			6.8	6.9	7.0	7.1	7.2	7.3	7.4	7.5
Distribution of cultures in Groups 2 and 3 according to pH of media										
4	control	16	-	-	-	-	-	14	2	-
	infected	17	-	-	-	-	-	17	-	-
5	control	16	-	6	7	-	2	1	-	-
	infected	17	-	-	9	5	3	-	-	-
6**	control	15	2	11	1	-	-	1	-	-
	infected	17	-	-	11	1	3	1	1	-
Distribution of cultures in Group 3 according to pH of media										
7	control	8	-	6	1	-	-	-	-	1
	infected	9	-	-	1	2	3	2	1	-
8	control	8	-	7	-	-	-	1	-	-
	infected	9	-	-	5	1	1	2	-	-
9	control	8	-	7	-	-	1	-	-	-
	infected	9	-	3	2	-	2	-	2	-

*Post-inoculation Day.

**One Group 2 control culture was lost due to bacterial contamination and all other Group 2 cultures (infected and control) were fixed for staining. The medium of all Group 3 cultures was changed on PI day 6.

cell was seen. Dense cell niduses from the seed culture were also present (Figures 16, 17).

Both the infected and control cultures contained mitotic figures. These figures were plentiful in all areas of the control monolayers, but were nearly absent in open areas of the infected cultures. Large epithelial-like cells in early prophase or late telophase were occasionally seen in the control cultures (Figure 18).

Cytoplasmic vacuolations were common in all cell types in both the infected and control cultures. The largest vacuoles, however, were consistently found in the large epithelial-like cells (Figure 19).

Round, pink-staining materials were present in the vacuoles of some epithelial-, fibroblast- and large epithelial-like cells. In the fibroblast- and epithelial-like cells, a clear halo occasionally surrounded this material (Figures 20, 21).

Large epithelial-like cells with nuclear buds and bizarre aberrations were found in both infected and control cultures (Figures 22, 23). They were, however, more common in the infected monolayers.

Faulty staining and precipitated medium made observation of the Warthin-Starry silver-stained cultures nearly impossible. It was noted, however, that stained precipitated medium did at times form filaments resembling degenerate leptospires (Figure 24).

PI Days 4 and 5: Leptospires were observed in culture medium and most organisms were actively motile. Degenerate spirochetes and long forms were also present, and attempts at division were noted in the latter forms. This was evidenced by an active whip-like motion of the segments on each side of a "bend" which occurred near the center of the spirochete. In addition, the segments were frequently observed to rotate rapidly about the "bend".

PI Day 6: The results of the observations on the medium from several Group 3 cultures were similar to those made on PI days four and five.

Stained PI-day-six control cultures (Group 2) exhibited a dense whorled multilayer of fibroblast-type colonies. Cellular detachment was seen in some areas (Figure 25). Mitotic figures were common. A few epithelial-like cells were found in less dense areas and between the fibroblast-like cell multilayers. Large epithelial-like cells were rare.

In contrast to the controls, infected cultures (Group 2) had grown only slightly since PI day three. Though cell types and distributions were similar to those described at PI day three, the number of cells present was greater (Figure 26). Cell niduses were absent due to cell outgrowth and the resultant formation of new colonies. As in PI-day-three cultures, small individual or "individualized" groups of cells with small nuclei and scanty, contracted, deeply stained cytoplasm were seen. Degenerate and apparently dying cells, though more common in the open areas, were present in all portions of the monolayers. Two such cells in telophase are shown in Figures 27 and 28.

Cytoplasmic vacuoles were seen in both the infected and control cultures as were mitotic figures. The latter figures, however, were less numerous in the infected groups.

Examination of the silver-stained cultures disclosed a large number of leptospires. The vast majority of these organisms were in close association with the cells and cell colonies. The spirochetes appeared singly, in multiples, or as dense tangled masses (Figure 29).

At times the leptospires were so dense as to obscure portions of the cells.

Morphologic variations of the Leptospira were similar to those seen in ICS #1 (1). Sometimes a ring structure with an internal granule was seen attached to a Leptospira by a short thin filament (Figure 30). Granules, which were apparently intracytoplasmic, outlined the cell nuclei (Figure 31).

In both the control and infected cultures, the cytoplasmic junctions of adjacent cells stained and at times simulated leptospires (Figures 32, 33).

PI Day 9: The general description of the control cultures at PI day six also applies to those observed at PI day nine (Figure 34). Numerous mitotic figures were present in all areas of the monolayer, and monolayer detachment was not uncommon.

Infected cultures approached the controls in density of monolayering. Mitotic figures were also common in these cultures. Large epithelial-like cells were very numerous and frequently were observed being incorporated into the outgrowth areas of the cell colonies. Some dead or degenerate appearing cells were present.

Examination of the silver-stained cultures revealed neither leptospires nor granules; however, the cell junctions and some precipitated medium did stain.

Fat-stained Cultures: Oil-Red-O-staining material was abundant in all control cultures from PI days three to nine (Figures 35, 36, 37). Fat droplets varied in size and were always found in the cytoplasm of the cells. Though some cytoplasmic vacuoles did contain fat, the lipids rarely entirely filled the vacuoles completely.

In contrast to the controls, fat was almost completely absent in the PI-day-three infected cultures (Figure 38). Some increase in fat was noted on PI day six (Figure 39). At PI day nine, infected cultures contained nearly the same quantity of fat as the controls (Figure 40). This progressive increase in fat corresponded remarkably to the decrease in leptospires found in the infected cultures on various PI days. The distribution of lipid on each PI day was the same as that described in the control cultures.

ICS #6 (6)

All monolayers in this experiment were originally classed as status II.

PI Day 1: The character of the cells and monolayers in both the infected and control cultures was similar (Figures 41, 42). Cell colonies were of the mixed-type. Some large epithelial-like, binucleated and degenerate appearing cells were present in the open areas of the infected cultures, but were rare or absent in the controls. Cytoplasmic vacuoles, which sometimes contained amorphous pinkish- or bluish-stained materials, were found. Mitotic figures were common.

Numerous leptospires in association with the cells were seen in Warthin-Starry-stained cultures. Many were segmented and similar to those described in previous experiments. Solid black granules, coiled-ring and ring-with-tail forms of leptospires were observed. The coiled-ring forms and granules commonly outlined the nucleus and frequently appeared to be intracytoplasmic (Figure 43). Oil-immersion examination of the coiled-ring forms frequently disclosed the presence of granular structures within the ring.

PI Day 2: The control cultures had the same general morphology as those at PI day one; however, large epithelial-like cells were more common. In the open areas, single and small groups of epithelial- and fibroblast-like cells were seen. Some of these cells were degenerate, as indicated by their shrunken and deeply-stained cytoplasm. Cytoplasmic vacuoles, similar to those described in the PI-day-one cultures, were observed. Some nuclear aberrations in the large epithelial-like cells were found (Figure 44).

In contrast to PI day one, the infected cultures differed markedly from their controls. In many colonies, the cells possessed little cytoplasm, and that cytoplasm which was present stained intensely and was frayed at the edges (Figure 45). Other findings included dense groups of three or four nuclei which appeared to share the same cytoplasm. Large epithelial-like cells, frequently containing large cytoplasmic vacuoles, were numerous in the open areas. Nuclear aberrations were more common in the infected cultures, but conversely, mitotic figures were fewer in number than in the controls.

The distributions, morphologic variations, and numbers of leptospires were similar to those on PI day one. Granules and coiled-ring forms of leptospires were more numerous (Figure 46).

PI Day 3: The control monolayers grew well, as evidenced by the number of mitotic figures and size of the cell colonies. The fibroblast-like cells had become dominant with the increase in cell density. Large epithelial-like cells were incorporated into the colonial outgrowths and both nuclear aberrations and cytoplasmic vacuoles were common. A few degenerate-appearing small cells were present in the open areas.

Little cell growth had occurred in the infected cultures and individualized cells and cell groups were prevalent. The monolayers generally possessed a lacy, ragged appearance. Many degenerate-appearing cells with shrunken, deeply stained cytoplasm were observed. Numerous large epithelial-like cells were present. As on other days, nuclear aberrations and vacuoles were seen (Figure 47).

Silver-stained, infected cultures resembled those described for the two previous days. Large numbers of leptospires were adhered to the cells and cell colonies. Granules and coiled-ring forms of leptospires in association with the individualized fibroblast- and epithelial-like cells were especially plentiful.

PI Day 4: Control culture monolayers were more dense than on previous days (Figure 48). Mitotic activity was present in all portions of the monolayer. In the open areas some large epithelial-, fibroblast-like and degenerate-appearing cells were observed. Cytoplasmic vacuoles and nuclear aberrations were also seen.

The most outstanding characteristic of the infected monolayer was "individualization" of the single and small groups of cells (Figure 49). These degenerate-appearing cells possessed a deeply stained, sometimes scanty, contracted cytoplasm which was frayed at the edges. The presence of these cells in combination with the apparently healthy large epithelial-like cells with long, thin cytoplasmic processes gave the monolayer a distinctly ragged appearance.

As compared to previous PI days, the numbers of filamentous leptospires were reduced. Apparently intracytoplasmic granules and coiled-ring forms of leptospires which outlined the nucleus of cells were nearly as numerous as on PI day three (Figure 50).

PI Day 5: Except for an increased density of cells, control monolayers resembled those on PI day four.

Like the controls, infected cultures looked like their counterparts at PI day four. While some growth had occurred, "individualization" of cells and cell groups dominated the infected monolayers' morphologic character. Numerous large epithelial-like cells were found.

On examination of the silver-stained cultures, a few segmented, beaded and degenerate leptospires were observed (Figure 51). These organisms were always associated with the cells. The number of granules and coiled-ring forms of leptospires was reduced.

PI Day 6: Control cultures exhibited heavier monolayers than on the previous day. Fibroblast- and epithelial-like cells spread out between the mixed-type cell colonies to fill in many parts of the open areas. Cellular detachment was seen in some areas of the monolayers (Figure 52).

The general description of the infected cultures at PI day five also applied to those at PI day six. Cell "individualization" and degeneration persisted (Figure 53). Degenerate large epithelial-like cells were never seen.

Leptospires were exceedingly difficult to find and those present appeared fragmented or beaded. A small number of granules and coiled-ring forms of leptospires associated with the cells were observed.

Fat-stained Cultures: The Oil-Red-O-stained cultures in this experiment could not be studied due to improper staining. For unknown reasons, a fine film of small stained droplets completely covered the cover-slips. The distribution of these droplets was indiscriminate in that they were found on the cells and in the open areas.

ICS #7 (7)

This experiment was run simultaneously with ICS #6 (6). Stained cell cultures on PI day six conformed in every respect to those described at PI day six in ICS #6 (6).

The results of the leptospiral counts over the six-day period in this experiment are graphically shown in Figure 6. An apparent drop occurred in the number of organisms in the medium at PI day one. However, this may not have been an accurate observation in that some difficulty with media precipitation in the counting chambers was encountered.

At PI day three the number of organisms was determined prior to medium change. Two points are therefore shown in the graph at this day. The second point represents a calculated count based on the amount of "seed" inoculum left in the cultures after the medium was changed. The number of organisms on subsequent days (PI days four and five) decreased and approached zero at PI day six. These results corresponded to the decrease in organisms in the stained cultures.

DISCUSSION

Effects of Antibiotics in Cell Cultures Used for Leptospira Studies

While aseptic techniques were employed during this study, the working conditions under which kidneys for primary culture were obtained were not always conducive to "asepsis". Antibiotics were therefore incorporated into the medium used for the initial growth of primary and/or first subcultures in some experiments.

The inability to demonstrate leptospires or significant cytopathogenic effects (CPE) in some early ICS experiments suggested that the organisms were being inhibited by antibiotic residues. ICS #5 (5) was therefore conducted with cell cultures which had been grown entirely in the absence of antibiotics. A comparison of the ability of the leptospires to survive and produce CPE in ICS #5 (5) and ICS #6 (6) (in which antibiotics were employed) did not disclose any significant differences. This indicated that the use of antibiotics in media for the propagation of cell cultures was not contraindicated as long as subcultures were processed so that antibiotic residues were removed or reduced prior to infection.

The possible explanations for the absence of CPE in early experiments are discussed in more detail below.

Relationship of Temperature to Leptospira-infected Cell Cultures

Since man and the animals commonly affected by leptospirosis normally maintain a body temperature of 37° C. or higher (Lyght, 1961; Duke, 1955), it seemed logical to carry out this study at 37° C. The use of this temperature was, however, considered detrimental to growth of the leptospires, as evidenced by the results of the morphology and activity observations in GMS #3, ICS #3 (2), #4 (3), and #5 (5); and the growth rate studies in GMS #1, #2, and ICS #7 (7).

The occurrence of long forms of leptospires, which were apparently attempting to divide, in some experiments was probably due to an increase in the spirochete's protoplasm and an inhibition of the enzymes which control division, as suggested by Ellinghausen (1960). His observations of thermal damage after three days at temperatures above 30° C. was confirmed in this study by the drop in the number of organisms in the GMS experiments, the occurrence of beaded and degenerate forms and the progressive decrease in the number of spirochetes on the monolayers in ICS #5 (5) and #6 (6) after PI day three.

In light of the observations made in this study, it seems evident that factors other than the mere presence of cells play a part in the survival and propagation of the leptospires in vivo at 37° C. One such factor to consider is the virulence of the organism. Faine (1959a) suggested that the survival of L. icterohemorrhagiae in vivo and in vitro at 37° C. may be related to the virulence of the organism. Whether or not this explains the observations in this study cannot be determined since the virulence of the Leptospira used was not determined. However, it should be noted that they were virulent at one time and that

propagation for several months in artificial medium at 30° C. may have resulted in a loss of "virulence".

pH Control and Responses in Leptospiral Cultures and Infected Cell Studies

During the course of this study difficulty was encountered in the stabilization of media-hydrogen-ion concentrations. The desirable pH range for cell cultures is 7.2 to 7.4 (Paul, 1959; Merchant *et al.*, 1961) and that for *L. pomona* is 6.7 to 7.4 (Ellinghausen, 1960). Because of the necessity of maintaining pH within these optimal ranges, EMEM prepared with both Hank's and Earle's BSS were tried. A comparison of the results in the various GMS and ICS experiments disclosed that EMEM-E was superior to EMEM-H in the control of media pH.

An additional observation made in this study was the wide range of pH change that occurred in the GMS experiments. Fulton and Spooner (1955) reported that no pH change occurred as leptospires "used up" the nutrients in media, and Ellinghausen (1960) stated that "minimal" media pH fluctuations were characteristic of the genus. It is not known why the results of the present work differed from those observed by the above authors. Temperature may be a factor, since Fulton, Spooner, and Ellinghausen all used cultures incubated at 30° C. instead of 37° C.

Serum in Leptospiral Cultures and Infected Cell Studies

The role of serum and necessity of supplying it in a medium used for the propagation of leptospires has been discussed by other investigators (Alston and Broom, 1958). EMEM used for the cultivation of cells also requires and is prepared with serum (Merchant, *et al.*, 1961).

Since bovine fetal calf kidney tissues were used in this study it was felt that better results would be obtained if homologous serum were used. Though rabbit serum is routinely used in leptospiral media, Sleight (1961) adapted L. pomona to cultivation in Stuart's medium using bovine serum.

Because of the difficulty encountered in maintaining leptospires in cell cultures for more than a few days in the first two experiments, 50-50 medium, which contained bovine and rabbit serum in concentrations of ten per cent, was employed in GMS #3, and in ICS experiments #4 (3) and #2 (4). When the morphologic and activity changes of the leptospires and the number of organisms in the silver-stained cultures in these experiments were compared to the findings noted in all other GMS and ICS experiments (in which only bovine serum was used), no advantage to the use of rabbit serum could be found. The significance of this observation, however, must be weighed against the possibility of high incubation temperatures and/or other factors which may have affected the organisms and thereby negated any advantages of the use of rabbit serum.

Lipids in Infected Cell Cultures

The production of lipase by leptospires (Bertok and Kemenes, 1960; Lacey, 1963) indicated the fat-splitting capabilities of these spirochetes. Fulton and Spooner (1956), Helprin and Hiatt (1957), Vogel and Hutner (1961) and Vaneseltine and Staples (1961) all stated that fatty acids or phospholipids were needed by leptospires. This need for lipids and the presence of a lipase may explain the observations made in the infected fat-stained cultures in this study.

Examination of Oil-Red-O-stained control cultures in ICS #5 (5) at PI days three, six, and nine, revealed a constant abundance of cytoplasmic lipid material. At PI day three, however, practically no fat was present in the infected cell cultures. On PI days six through nine, a progressive increase in the amount of cytoplasmic fat was observed. This increase in cytoplasmic fat in the infected cell cultures corresponded to the decrease in the number of leptospires observed in silver-stained cultures during the same time period. This suggested that the lipid materials in these cultures were being metabolized by the organisms, and that as the number of leptospires decreased from day to day, more and more lipid material became available for deposition in the cytoplasm of the cells.

It is unfortunate that the fat stains in ICS #6 (6) were not suitable for reading, since a possible decline in the cytoplasmic fat may have taken place during the first three days of infection when the number of organisms was increasing.

Demonstration of Leptospires

Warthin-Starry silver stain was found suitable for the demonstration of leptospires in tissue cultures. Care, however, was required in the culture fixation procedure, since formalin caused the precipitation of small amounts of media which remained after simple saline washes. These precipitates either obscured portions of the monolayer or stained in such a manner as to simulate leptospires. The author found that two saline washes followed by two rapid formalin washes removed most of the media.

Intracytoplasmic filaments and the cytoplasmic junction of adjacent cells frequently stained and resembled leptospires. To accurately interpret filamentous structures, careful high-power and oil-immersion microscopic examination was required.

The use of hematoxylin as a counter-stain in silver-stained cultures did make identification of nuclei easier. With experience, however, nuclei were identified without counter-staining and this procedure was therefore not used in the last few experiments.

Granules and Other Structures

Microscopic examination suggested that the "normal" leptospires associated with cells were extracellular, and that the coiled organisms and free-granules were frequently intracytoplasmic.

In light of the controversy that exists regarding the significance of "granules" in leptospiral cultures, the occurrence of various "intracytoplasmic" structures in this study was of interest. The coiled leptospires observed resembled those found by Miller and Wilson (1962) within erythrocytes, and renal and hepatic parenchymal cells. In this study, however, the author was unable to demonstrate "vesicles" similar to those described by Miller and Wilson (1962) surrounding the coiled organisms.

The coiled leptospires, rings-with-tails, and other forms seen were similar to those said to be involved in the life cycle of *Treponema pallidum* (De Lamater *et al.*, 1950, a.). It was not possible to determine whether or not the "intracytoplasmic" formations observed in the present study represented resting phases, parts of the life cycle of the organisms, or merely phagocytosis of degenerate forms of leptospires by cells.

Time-lapse photography of living infected-cell cultures and electron microscopy are techniques which would make it possible to more accurately observe and interpret these various phenomena.

Nuclear Aberrations and Multinucleated Cells in Tissue Culture

The nuclear aberrations observed in this study have also been described in "normal" cell cultures from five different animal species (Klein and Melnick, 1958; Larin et al., 1959). While actual counts were not made, there was an apparent higher incidence of these aberrations among the large epithelial-like cells in the infected cultures. This finding suggested the need for further investigation to determine whether or not a relationship exists between the occurrence of cytological aberration and the infected state of *in vitro* cell systems.

Polynucleated cells were found in the cultures of this study. This was not considered abnormal since Klein and Melnick (1958) also reported the occurrence of multinucleated cells in "normal" monkey kidney cultures. The origin of these cells was not certain, but many were assumed to represent cells in which normal mitotic division had occurred without a subsequent separation of the cytoplasm. Another possible explanation considered was amitotic division. Though said to be rare or unimportant *in vivo* (Copenhaver and Johnson, 1958; Trautmann and Feibiger, 1959; Brachet and Mirsky, 1961, a.), some workers have suggested, stated, or shown that amitosis does occur in cell cultures (Maximow, 1949; Gey et al., 1954; Larin et al., 1959).

Cytopathogenic Effects in Leptospira-infected Cell Cultures

The general cytopathogenic effects (CPE) manifested in this study were (1) an apparent cell death and/or degeneration as indicated by pyknosis of nuclei and shrunken, frayed cytoplasm, and (2) an inhibition of cellular growth as evidenced by the less frequent occurrence of mitotic figures and a failure of the infected monolayers to spread over the cover-slips.

No direct relationship between CPE and the number of leptospires used for infection was evident. However, the rapidity and severity of CPE were more apparent when the organism-cell ratio favored the organism. For example, in the last three ICS experiments the monolayers had statuses of II or less prior to infection. Effects were more severe and rapid in these monolayers than in the first four ICS experiments in which monolayers were heavier.

Cells apparently affected most severely by leptospires were the small fibroblast- and epithelial-like cells. Large epithelial-like cells remained apparently healthy and increased in number during the course of the experiment. The more susceptible epithelial- and fibroblast-like cells were likely either two different cell types each susceptible to infection, or morphologic variations of the same cell type. Attempts to determine the origin of these two cell types seemed futile. Bloom (1937) lengthily discussed the "pros" and "cons" of the possible derivations and interpretations of epithelial- and fibroblast-like cells in tissue culture. Berman *et al.* (1957) reported many fibroblastic and epithelial variations occurring in the Ep-L and Fb-L Detroit cell strains over a period of time.

The possibility was considered that the large epithelial-like cells represented mutants and/or morphologic variations of the small fibroblast- or epithelial-like cells which were less susceptible to infection. However, other investigators observed mutants and physiologically changed cells only after many serial passages (Levan, 1956; Richardson, 1959). Since only one subculture was used in the present study, it is quite likely that the large epithelial-like cells were entirely different cells which were less susceptible to the leptospires.

The occurrence of cytoplasmic vacuoles in cells was not considered a CPE though other authors have described the development of vacuoles in cells infected with other microorganisms (Lewis, 1920; Eaton et al., 1962). In the present study vacuolation was equally frequent in both the infected and control cultures.

Two possible explanations for the CPE in Leptospira-infected cultures were examined. These were (1) physical trauma to the cells by the leptospires and/or (2) production of toxic substances detrimental to the cells. Two investigators (Richardson, 1959; Shepard, 1957, a., 1959) have shown that excessive intracytoplasmic growth of bacteria can cause death of cultured cells. Little evidence of such "growth" was, however, observed in this study. "Granules" and other structures which apparently were intracytoplasmic did occur, but their origin and significance in all cases was uncertain.

The toxin-induced-CPE theory was considered, since Lacey (1963) has shown that L. pomona hemolysin will kill in vitro bovine kidney cells. He suggested that other toxins might also be present. Other workers (Strauss and Hendee, 1959; Kato and Peppenheimer, 1960; Vicari et al.,

1960; Bonventre, 1961) have also demonstrated the ability of bacterial toxins to produce CPE in cultured cells. Though toxin-induced-CPE theory was the more easily accepted, physical trauma was not ruled out. More study is obviously needed.

SUMMARY

Bovine fetal kidney cells were grown *in vitro* and infected with *Leptospira pomona*. Medium pH fluctuations, kidney cell responses, leptospiral morphologic changes and/or growth were studied in infected cultures using three types of media. Complementary experiments were conducted to determine pH changes, alterations of organism morphology and/or growth in the absence of cells.

The media used in this study were (1) Eagle's Minimum Essential Medium prepared with Earle's balanced salt solution and containing ten per cent calf serum, (2) the above medium prepared with Hank's balanced salt solution, and (3) a composite medium of equal parts of Stuart's medium with ten per cent rabbit serum and number 2 medium above. The first medium was found to be superior in that pH fell less rapidly and was more easily controlled.

Incubation temperatures of 37° C. were found to be detrimental to the leptospires. Organisms became longer than normal, apparently were unable to divide, underwent degenerative changes, and decreased in numbers after three days' incubation.

A modified Warthin-Starry silver-stain technique was used to demonstrate leptospires in the tissue cultures. Care was required in the interpretation of stained filaments since cytoplasmic filaments and junctions of adjacent cells at times stained and simulated leptospires.

Oil-Red-O stain for fat was used with satisfactory results in this study. Stained fats were confined to the cytoplasm of the cells but not to the vacuoles within the cytoplasm. In one experiment, a simultaneous

increase in cytoplasmic fat occurred with the progressive decrease in leptospires. This suggested that leptospires utilized the lipid materials which were ordinarily deposited in the cells.

Free granules and coiled leptospires were frequently observed in association with the infected cells and sometimes appeared to be intracytoplasmic. Ring or coiled forms at times contained a granular internal structure. Similar structures were observed in some granule-with-tail forms of leptospires. Other leptospires were beaded and frequently segments of protoplasmic cylinders joined by axial filaments were found. It was not known whether the structures or forms of leptospires observed were part of the life cycle, a resting phase or merely a degenerate stage of the organisms.

Nuclear aberrations were more frequent among some cells in the infected cultures. Further investigations are needed to determine if a relationship exists between the occurrence of these aberrations and the infected state of a cell culture.

The cytopathogenic effects which occurred were inhibition of cellular growth and degeneration with apparent cell death. Some cells were apparently more resistant to these changes than others. Cytoplasmic vacuolation was not considered part of the cytopathogenic effects since it occurred with equal frequency in both the infected and control cultures.

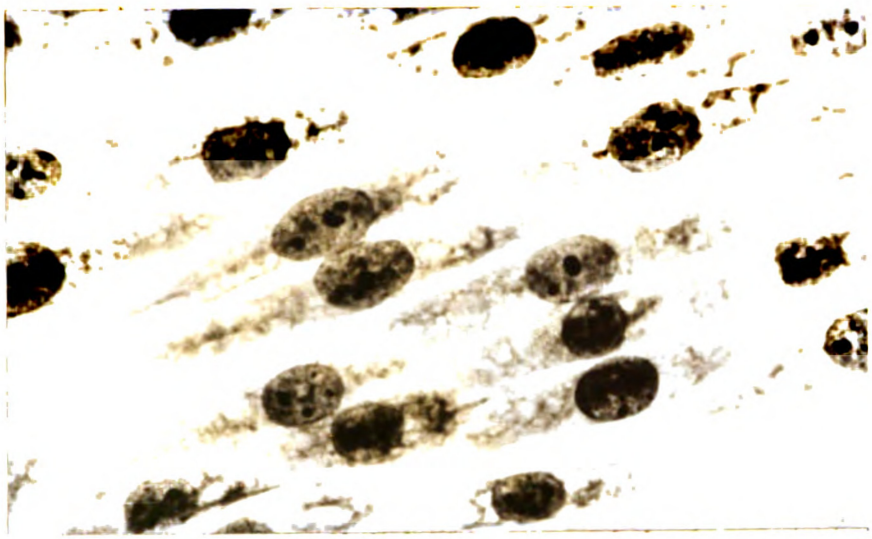
The severity and rapidity of cytopathogenic effects was greater in those cultures which had monolayers of 50 per cent or less prior to infection. This was believed to be due to an organism-cell ratio which favored the leptospires in these cultures.

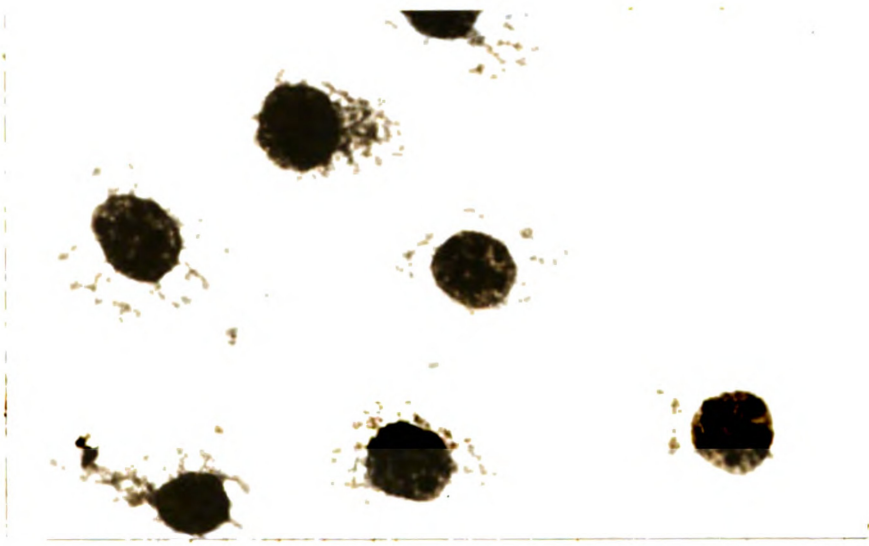
Two causes for the cytopathogenic effects observed were considered. These were (1) the production of toxins by the organism and (2) direct trauma to the cells by the leptospires.

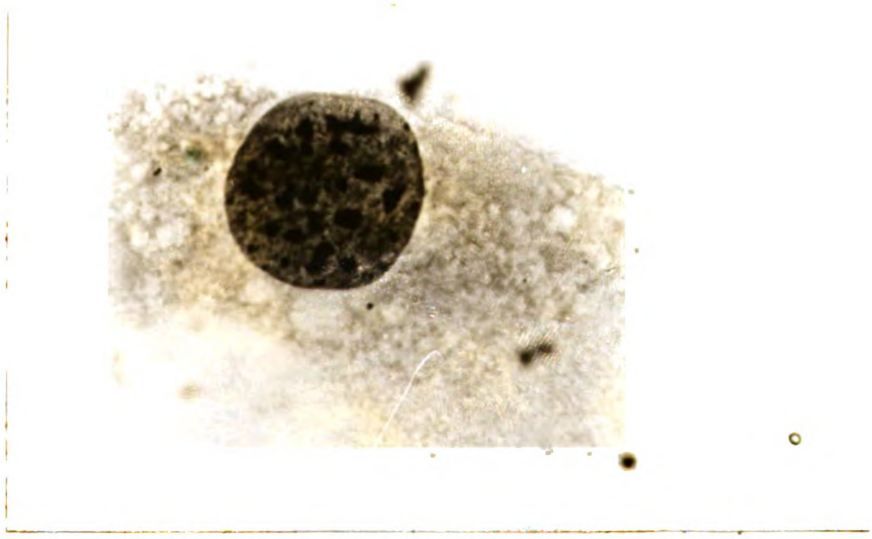
Figure 1. Fibroblast-like cells in bovine fetal kidney culture. Note parallel arrangement of cells (parallelism). May-Grunwald-Giemsa. x 750.

Figure 2. Epithelial-like cells in bovine fetal kidney culture. Note polyhedral shape of cells and round nuclei. May-Grunwald-Giemsa. x 750.

Figure 3. Large epithelial-like cell in bovine fetal kidney culture. Note abundance of cytoplasm and large nucleus. May-Grunwald-Giemsa. x 750.







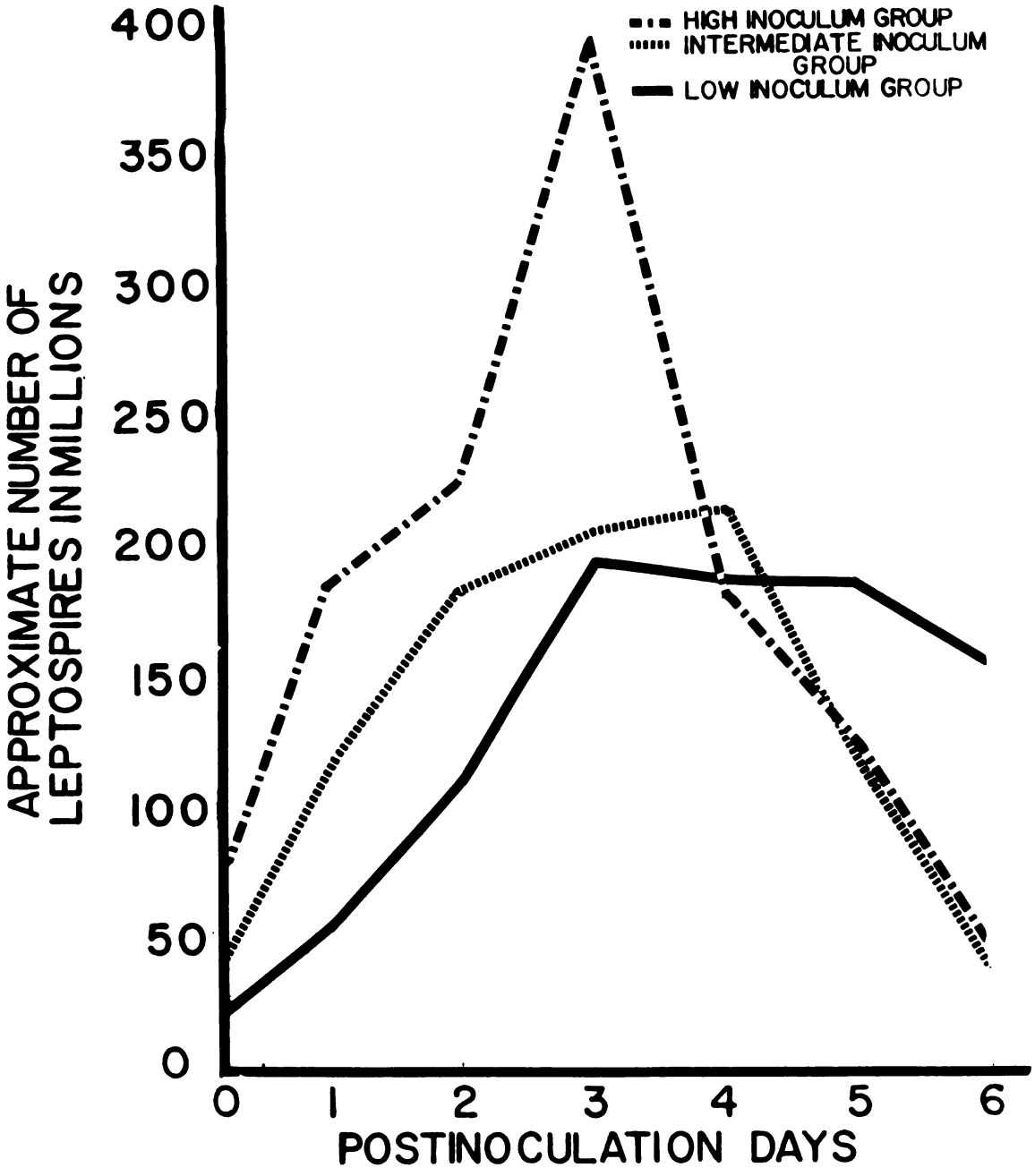


Figure 4. Growth curves of Leptospira pomona at 37° C. in Eagle's Minimum Essential Medium prepared with Hank's Balanced Salt Solution. Each curve was determined by averaging the number of leptospires in three cultures. The approximate number of organisms in millions per ml. is indicated on the ordinate of the graph and the post-inoculation days on the abscissa.

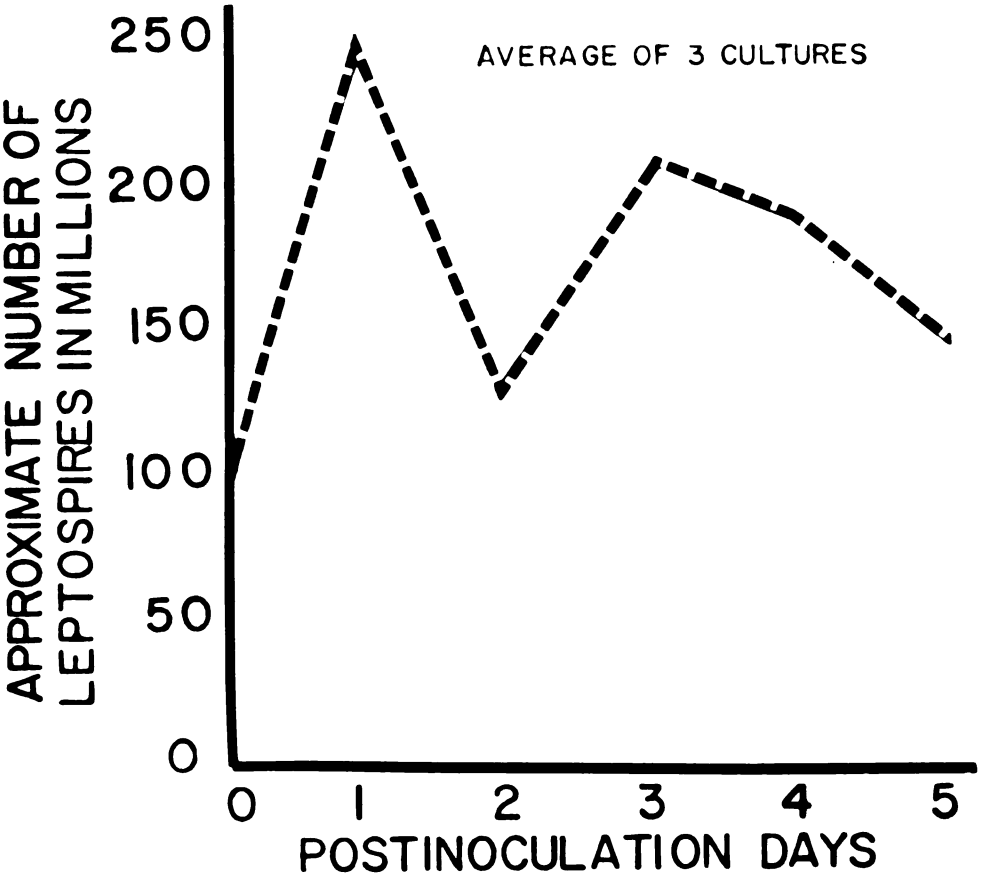


Figure 5. Growth curve of Leptospira pomona at 37° C. in Eagle's Minimum Essential Medium prepared with Earle's Balanced Salt Solution. The approximate number of leptospires in millions per ml. is indicated on the ordinate of the graph and the post-inoculation days on the abscissa.

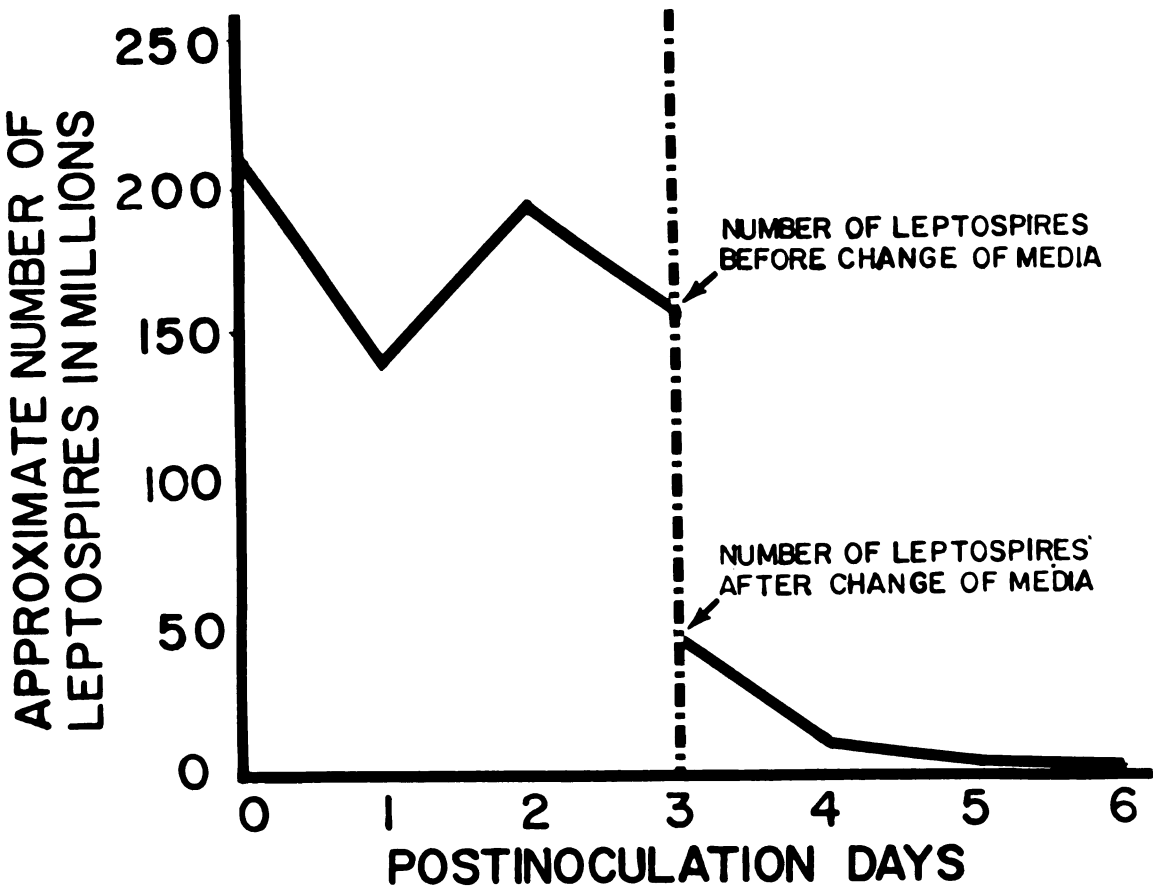


Figure 6. Growth curve of Leptospira pomona at 37° C. in the presence of bovine fetal kidney cells grown in Eagle's Minimum Essential Medium prepared with Earle's Balanced Salt Solution. The curve was determined by averaging the number of leptospires in four cultures. The approximate number of organisms in millions per ml. of medium is indicated on the ordinate of the graph and the post-inoculation days on the abscissa. Three days after inoculation, the medium of the cultures was removed, except for approximately 0.3 ml. which acted as a "seed", and replaced with fresh medium. Two values are therefore given at three-days post-inoculation. The second value represents the number of organisms after dilution of the "seed" inoculum.

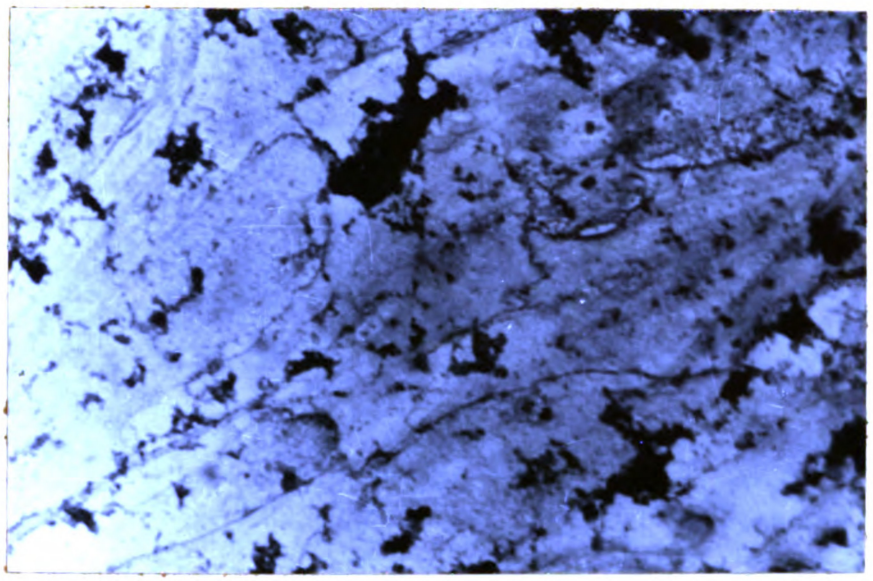


Figure 7. Formalin precipitated medium in bovine fetal kidney culture. Warthin-Starry. x 187.

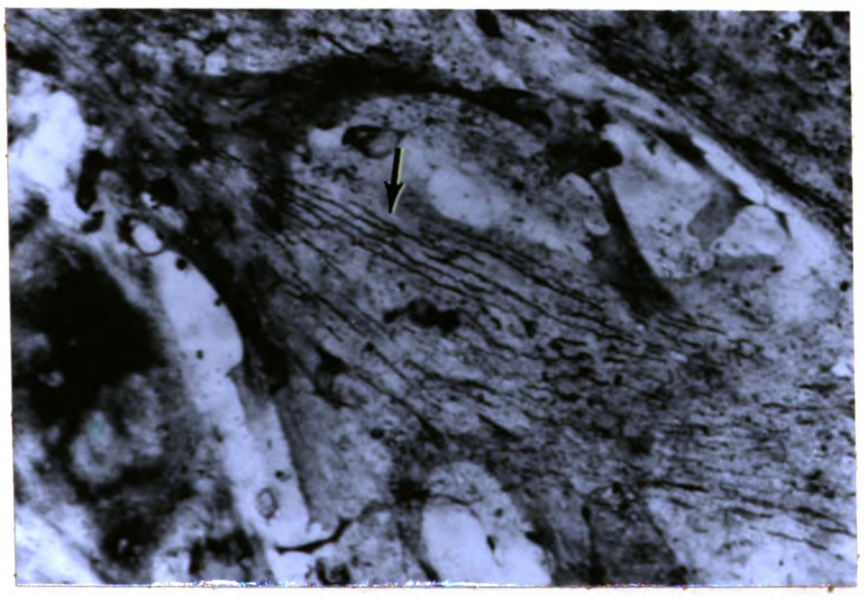


Figure 8. Argyrophilic cytoplasmic filaments in bovine fetal kidney culture. Note filaments (arrow). Warthin-Starry. x 187.

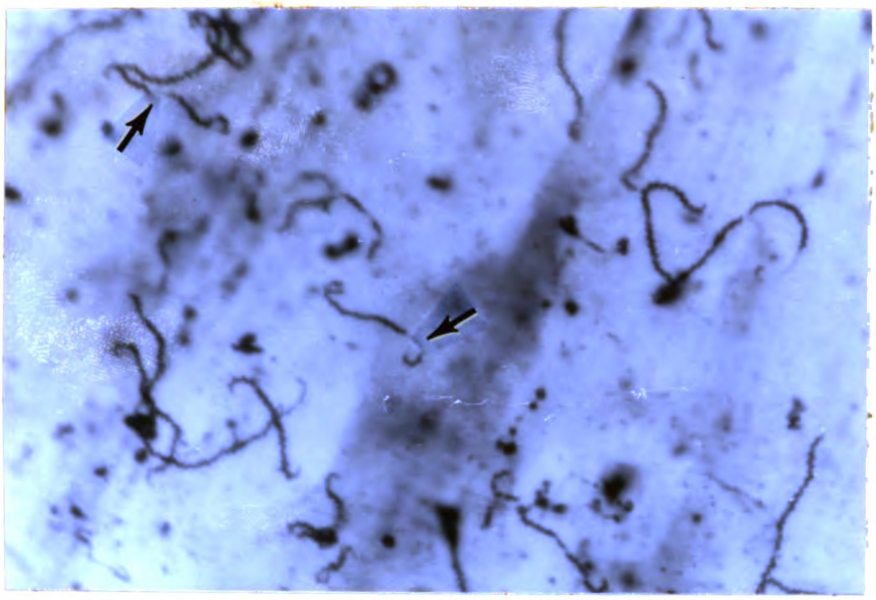


Figure 9. Bovine fetal kidney culture one day after infection with Leptospira pomona. Note axial filaments (arrows) of segmented leptospires. Warthin-Starry. x 1875.

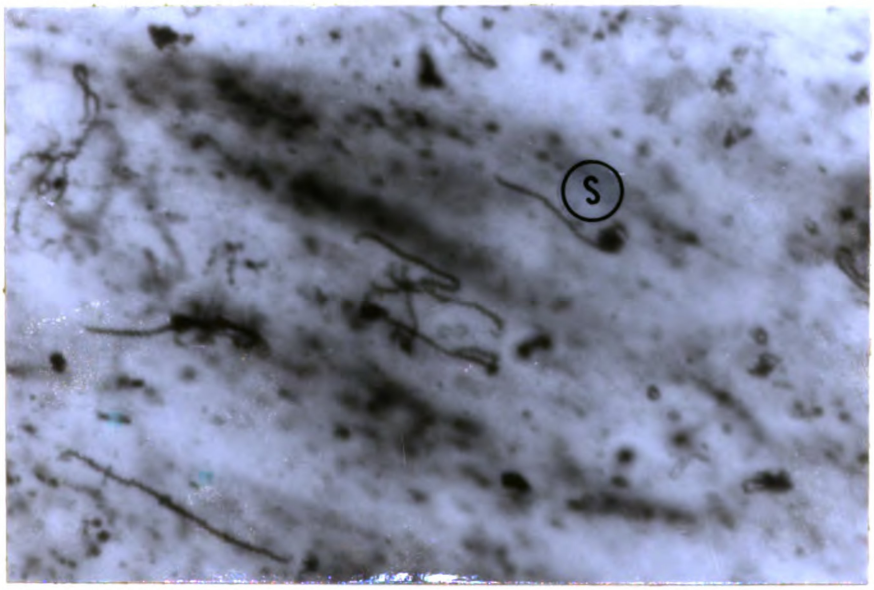


Figure 10. Bovine fetal kidney culture one day after infection with Leptospira pomona. Granule-with-tail form of Leptospira (S). Warthin-Starry. x 1875.

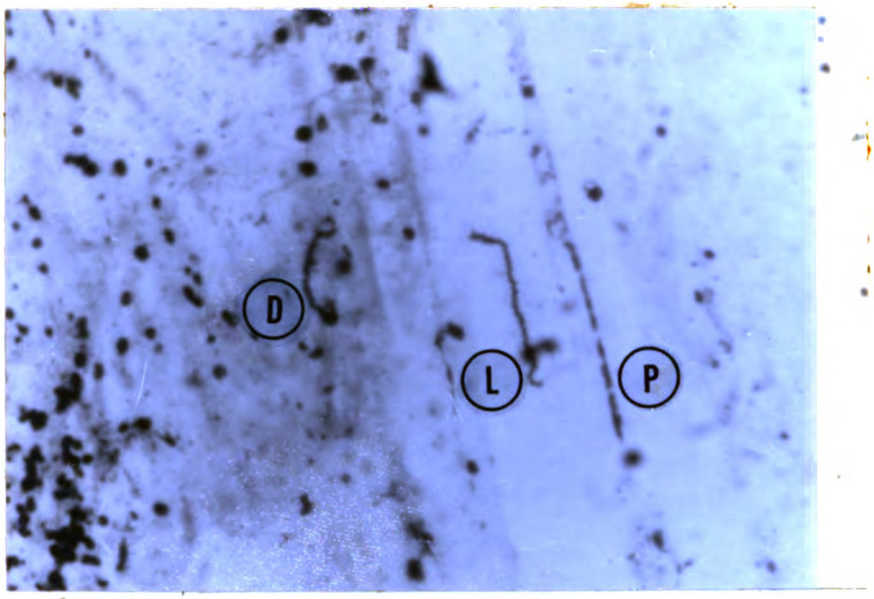


Figure 11. Bovine fetal kidney culture one day after infection with Leptospira pomona. Dumbbell form of Leptospira (D). "Normal" Leptospira (L). Stained cytoplasmic process (P). Warthin-Starry. x 1875.

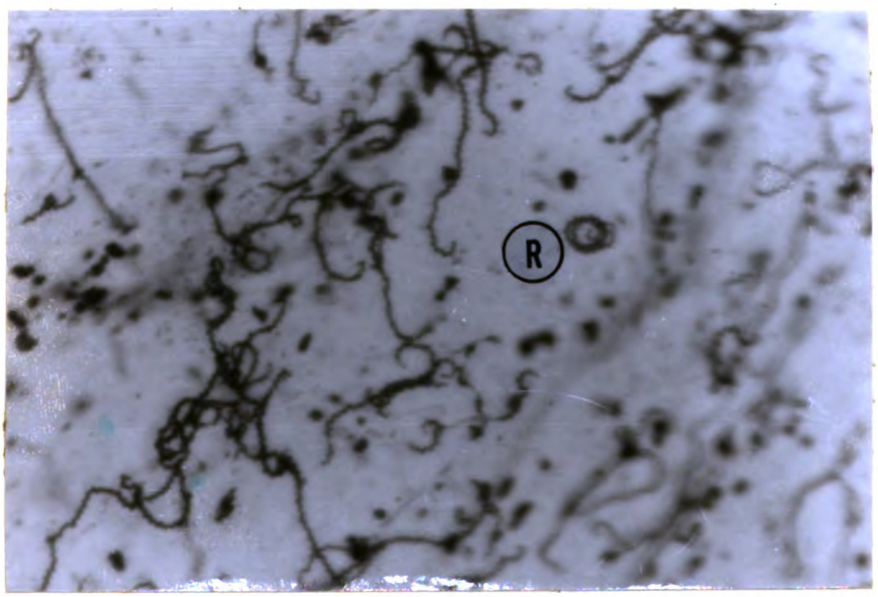


Figure 12. Bovine fetal kidney culture one day after infection with Leptospira pomona. Coiled-ring form of Leptospira (R). Warthin-Starry. x 1875.

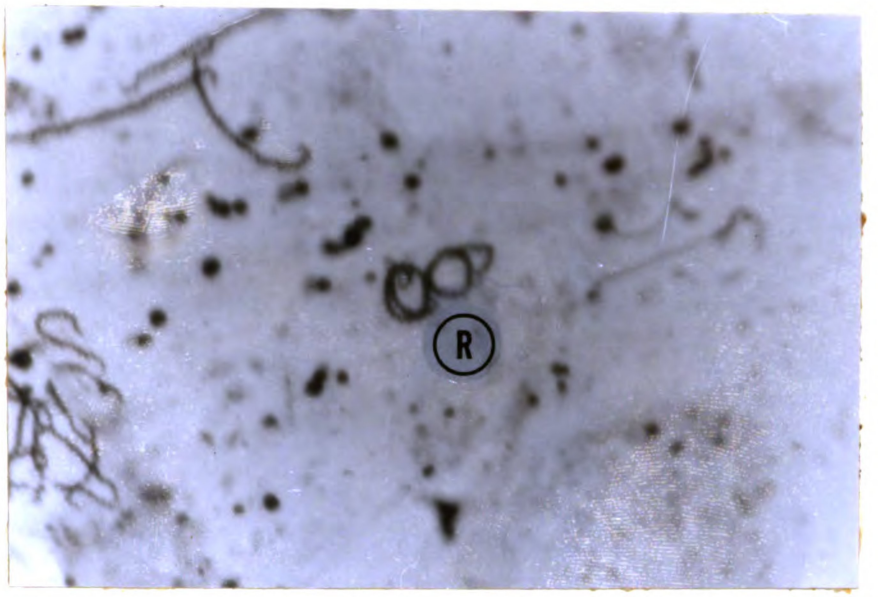


Figure 13. Bovine fetal kidney culture one day after infection with Leptospira pomona. Coiled-ring form of Leptospira (R). Warthin-Starry. x 1875.

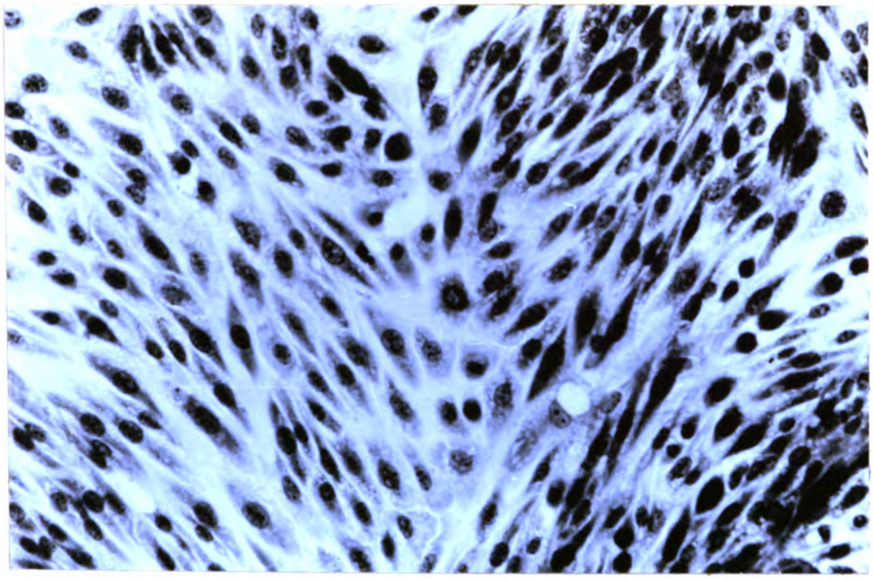


Figure 14. Three-day-control (noninfected) bovine fetal kidney culture. Note the even complete monolayer and compare with infected culture shown in Figure 15. May-Grunwald-Giemsa. x 187.

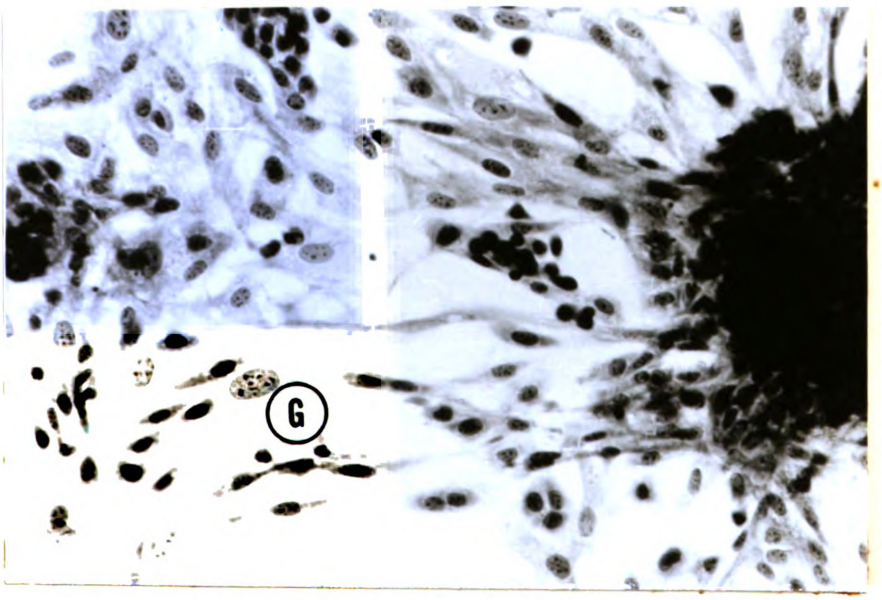


Figure 15. Bovine fetal kidney culture three days after infection with Leptospira pomona. Note large epithelial-like cell (G). May-Grunwald-Giemsa. x 187.

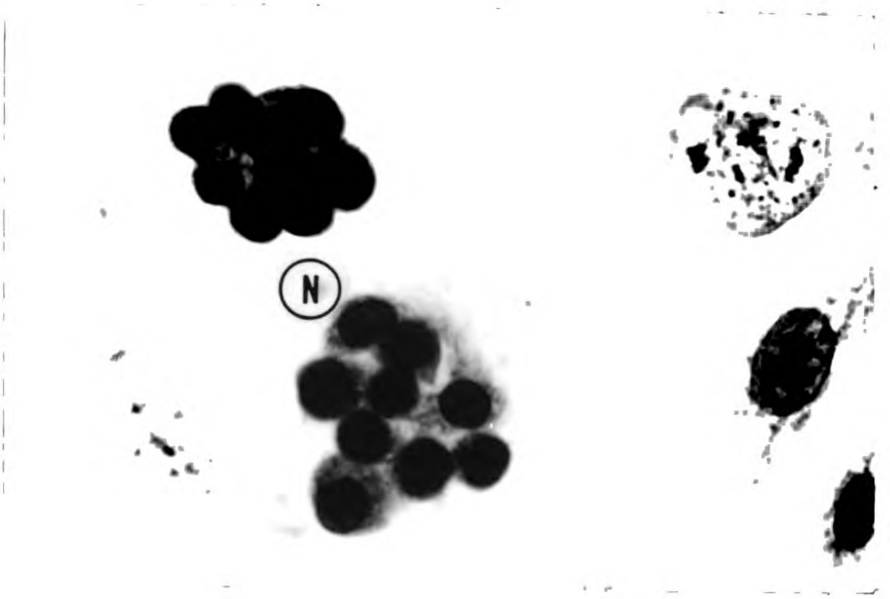


Figure 16. Bovine fetal kidney culture three days after infection with Leptospira pomona. Two cell niduses are present (N). May-Grunwald-Giemsa. x 750.

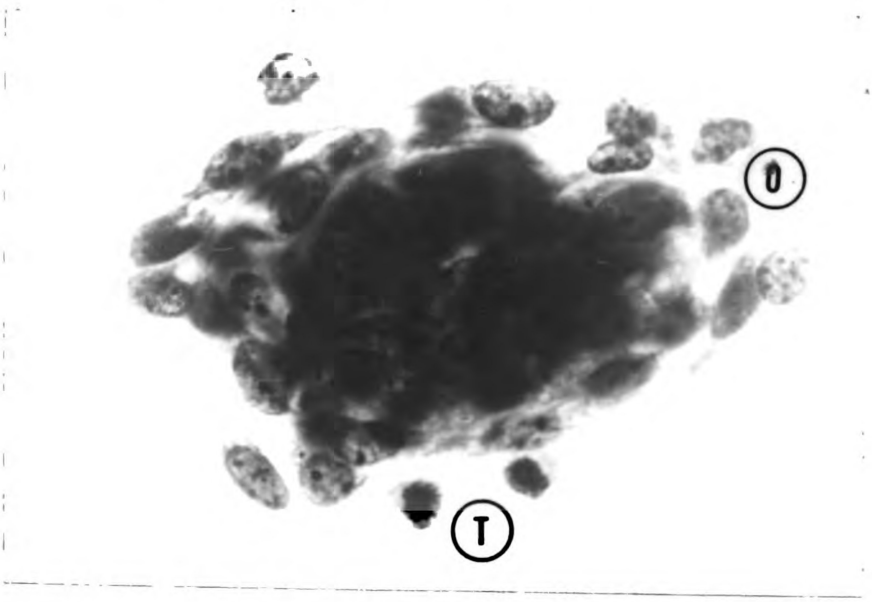


Figure 17. Bovine fetal kidney culture three days after infection with Leptospira pomona. Note outgrowth of cells from cell nidus (O). A cell in telophase stage of division is also present (T). May-Grunwald-Giemsa. x 750.

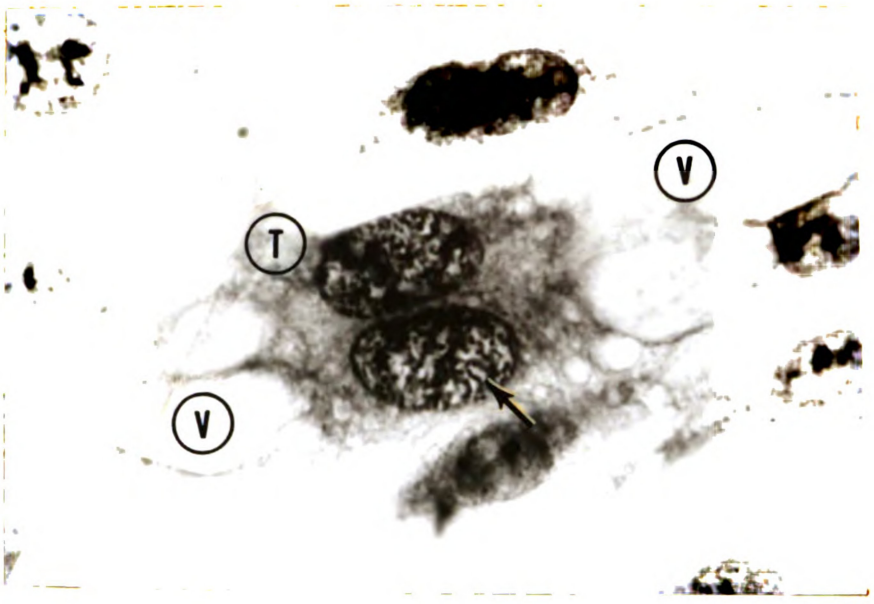


Figure 18. Bovine fetal kidney culture. Note large epithelial-like cell in late telophase stage of division (T), cytoplasmic vacuoles on each side of the nuclei (V) and visible chromosomes in nuclei (arrow). May-Grunwald-Giemsa. x 750.

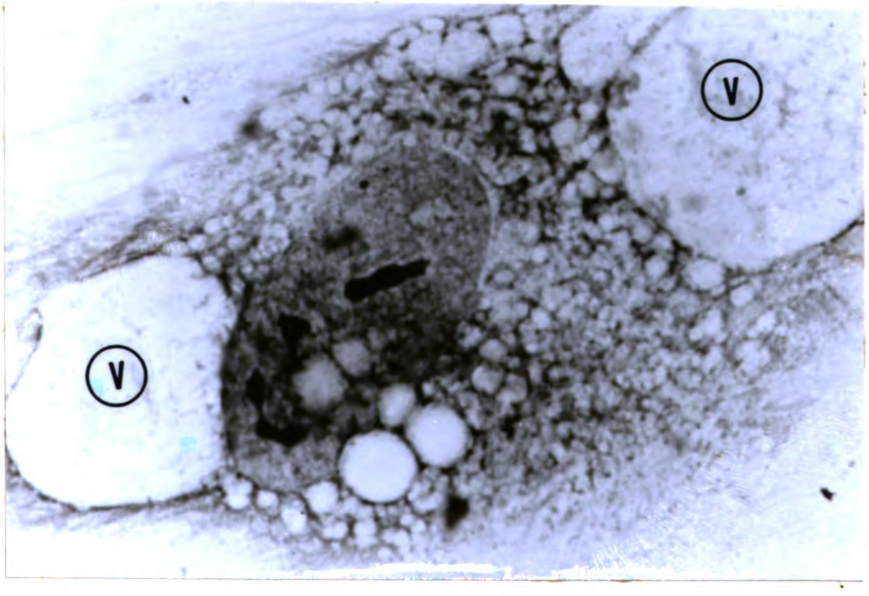


Figure 19. Bovine fetal kidney culture. Large epithelial-like cell with large cytoplasmic vacuoles (V). May-Grunwald-Giemsa. x 750.

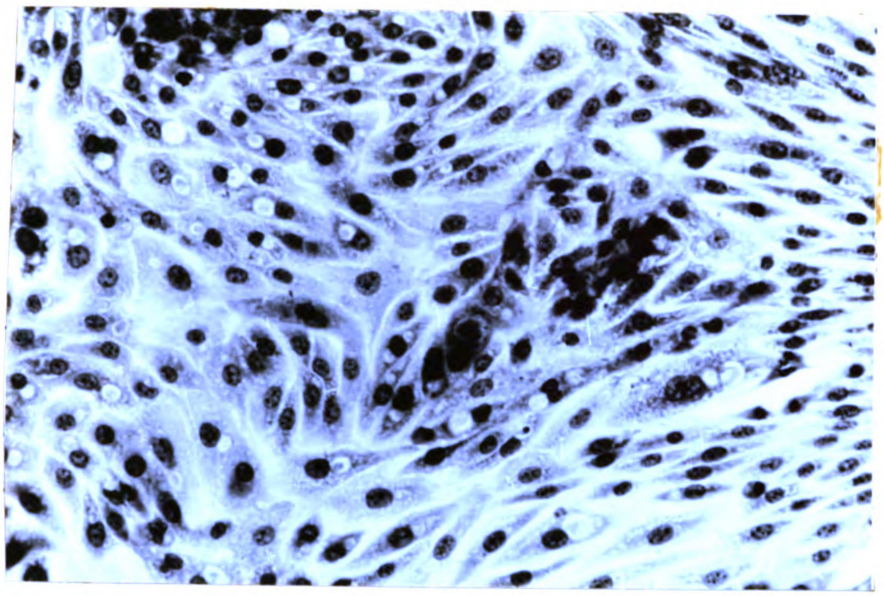


Figure 20. Control (noninfected) bovine fetal kidney culture. Note fibroblast- and epithelial-like cells containing cytoplasmic material surrounded by a halo. May-Grunwald-Giemsa. x 187.

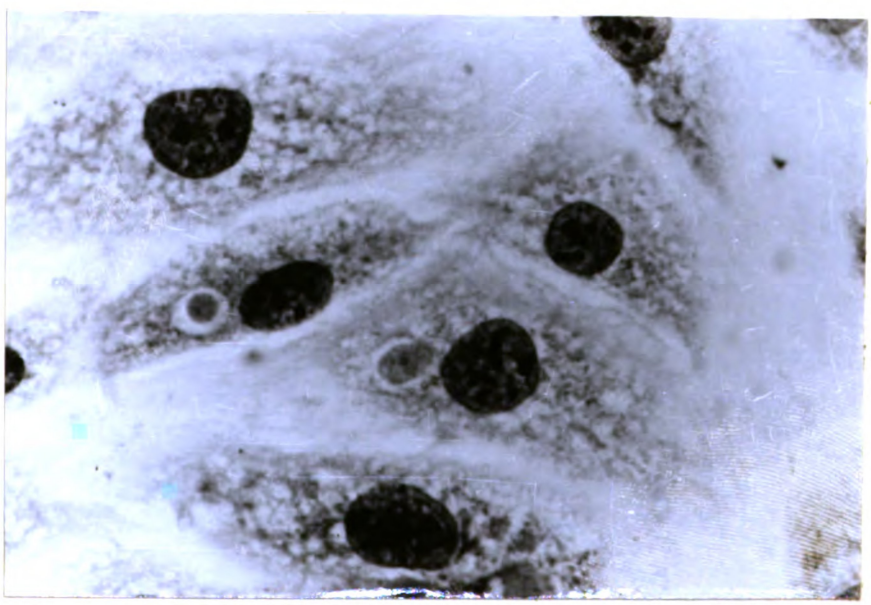


Figure 21. High power view of cells shown in Figure 20. Note cytoplasmic materials surrounded by a halo. May-Grunwald-Giemsa. x 750.

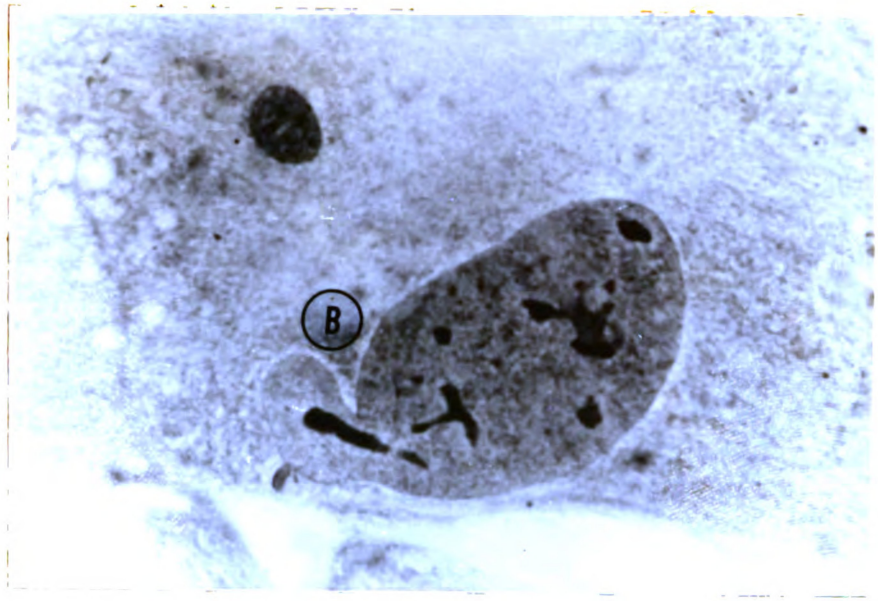


Figure 22. Large epithelial-like cell in bovine fetal kidney culture. Note nuclear bud (B). May-Grunwald-Giemsa. x 750.

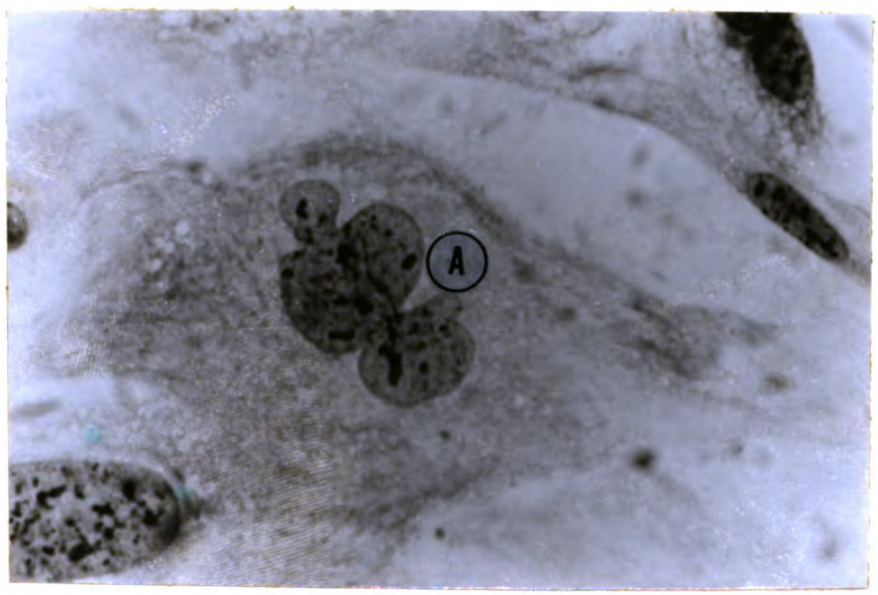


Figure 23. Large epithelial-like cell in bovine fetal kidney culture. Note nuclear aberration (A). May-Grunwald-Giemsa. x 750.

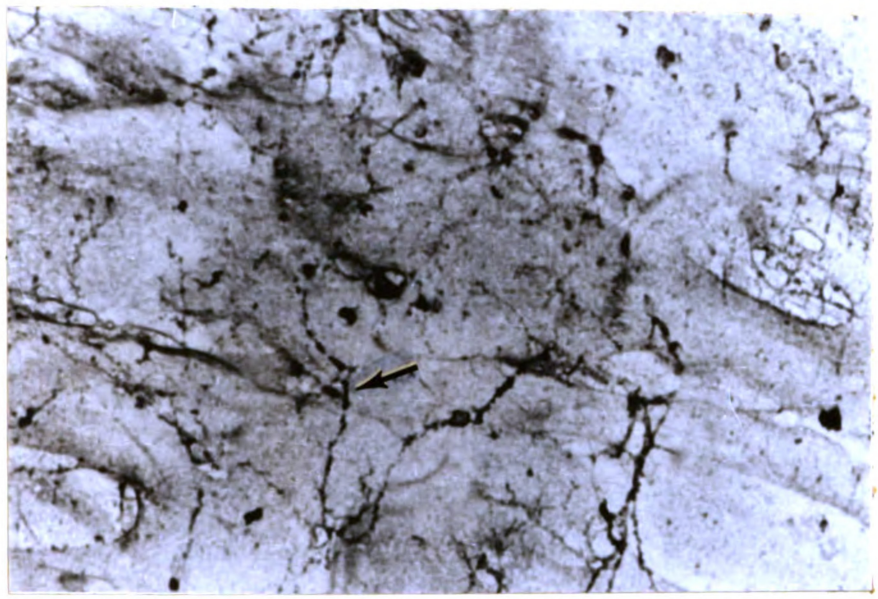


Figure 24. Silver stained control (noninfected) bovine fetal kidney culture. Note stained filaments of precipitated medium (arrow). Warthin-Starry. x 750.

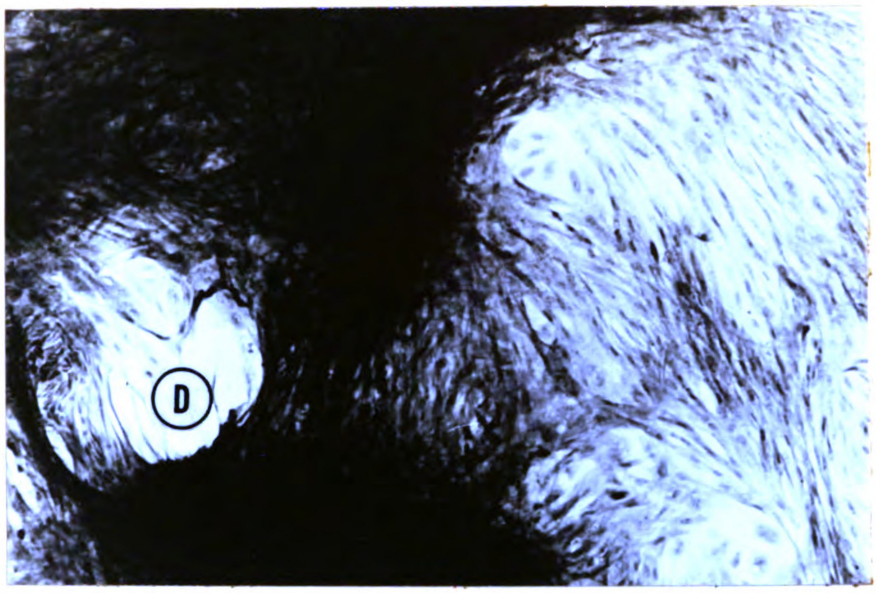


Figure 25. Six-day control (noninfected) bovine fetal kidney culture. Note density of cells and cellular detachment (D). May-Grunwald-Giemsa. x 75.

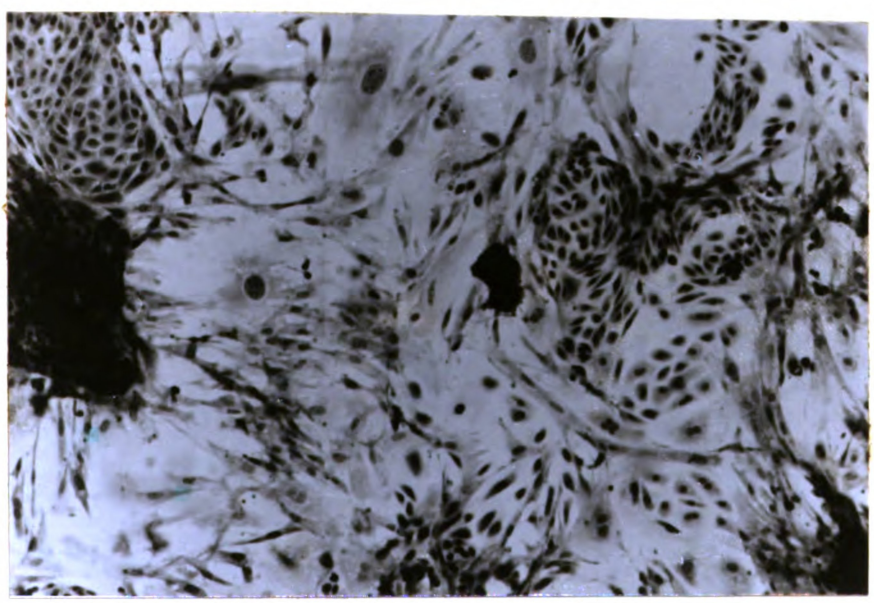


Figure 26. Leptospira pomona infected bovine fetal kidney culture six days after infection. Note "ragged" appearance of monolayered cells as compared to those seen in Figure 25. May-Grunwald-Giemsa. x 187.

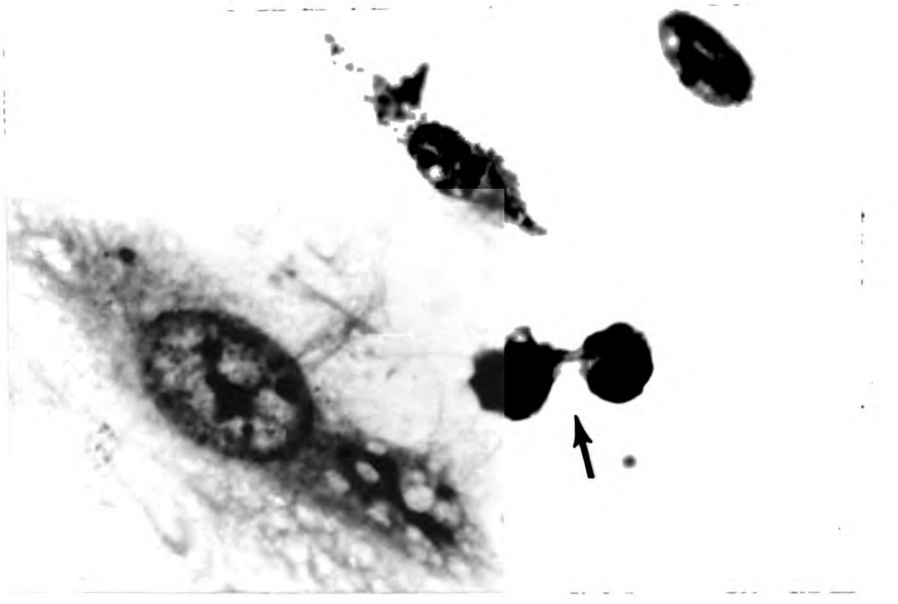


Figure 27. Leptospira pomona infected bovine fetal kidney culture six days after infection. A degenerate cell with contracted and deeply stained cytoplasm in telophase stage of division is shown (arrow). May-Grunwald-Giemsa. x 750.

Figure 28. Bovine fetal kidney culture six days after infection with Leptospira pomona. Note degenerate cell with deeply stained cytoplasm in telophase stage of division (arrow). May-Grunwald-Giemsa. x 750.

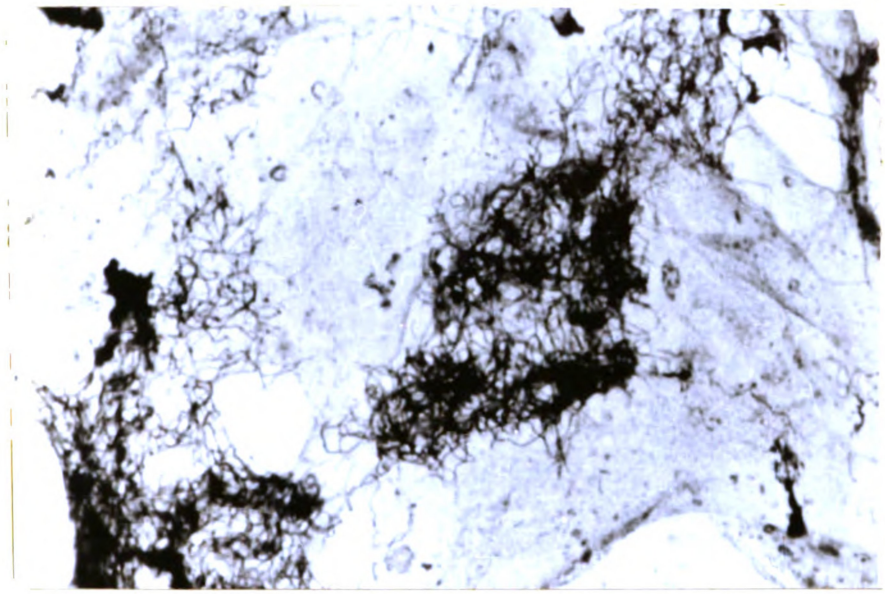


Figure 29. Tangled masses of leptospires in bovine fetal kidney culture six days after infection. Warthin-Starry. x 750.

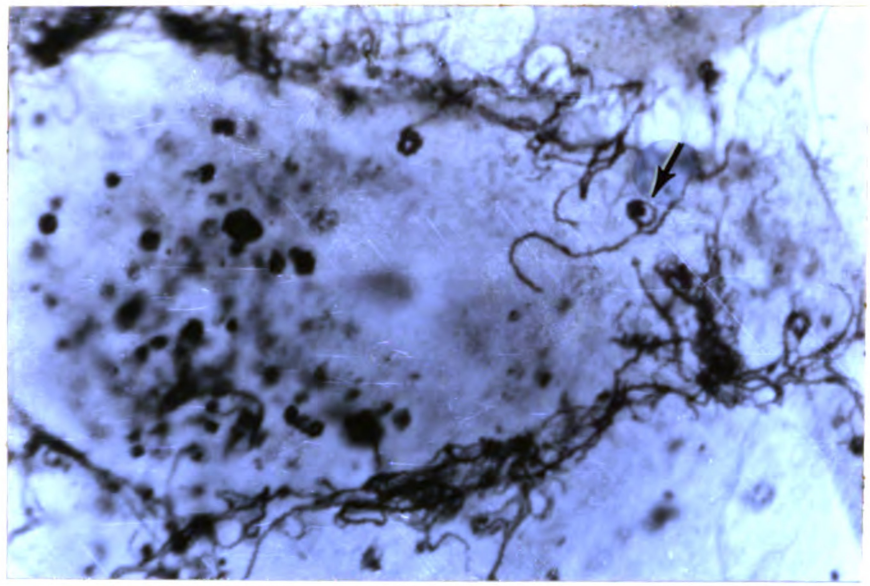


Figure 30. Leptospira pomona infected bovine fetal kidney culture. Note ring structure with an internal granule attached to a Leptospira by a short filament (arrow). Warthin-Starry. x 1875.

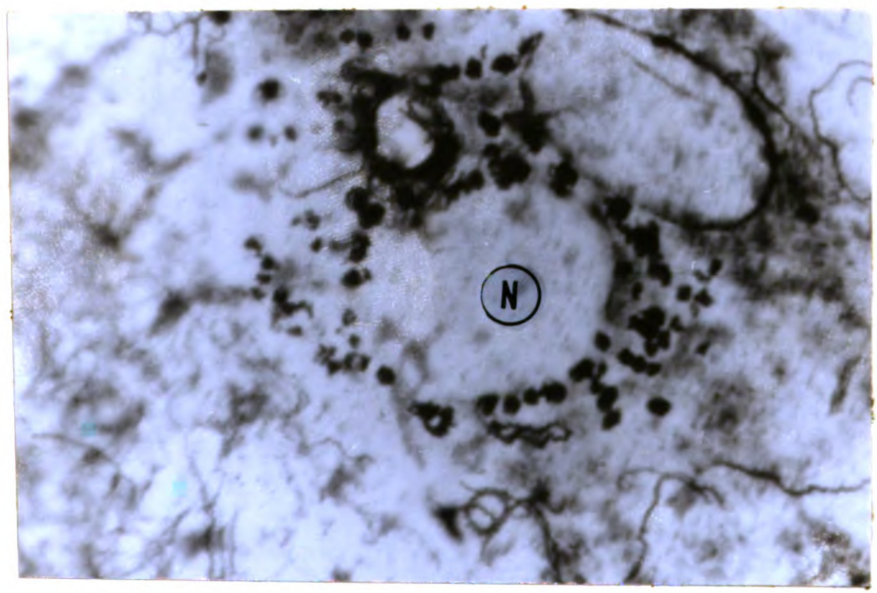


Figure 31. Bovine fetal kidney culture six days after infection with Leptospira pomona. Note granules and coiled forms of leptospires outlining the nucleus (N) of a cell. Warthin-Starry. x 1875.

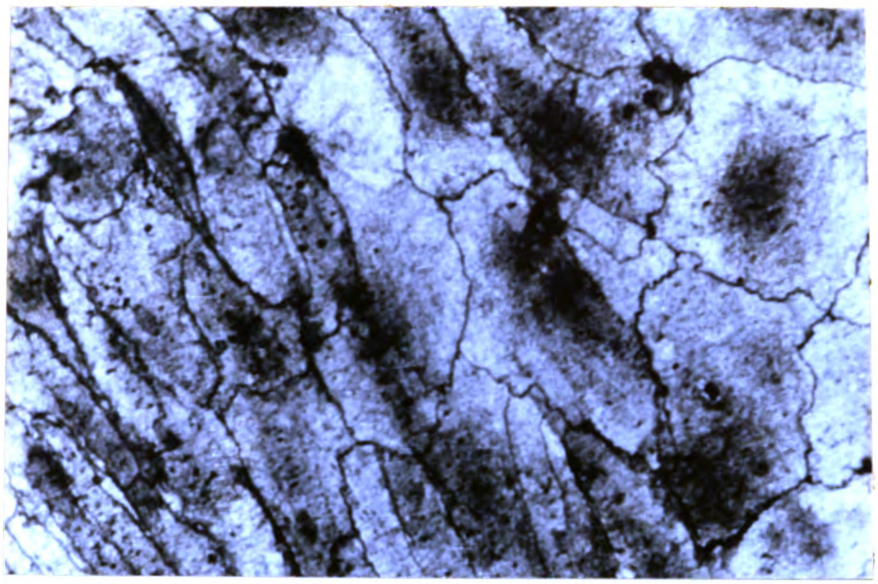


Figure 32. Control (noninfected) bovine fetal kidney culture with silver-stained cytoplasmic junctions. Warthin-Starry. x 750.

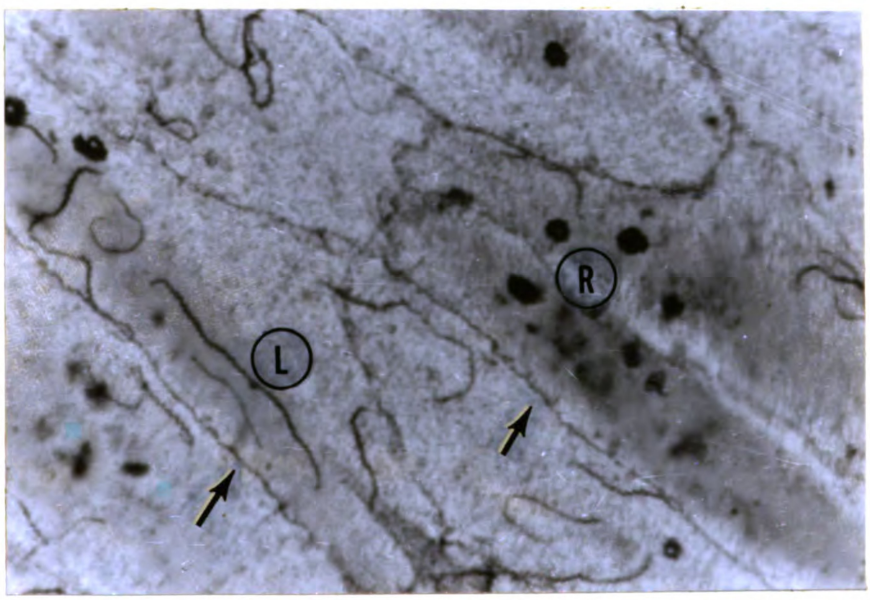


Figure 33. Leptospira pomona infected bovine fetal kidney culture six days after infection. Note silver stained cytoplasmic junctions of cells (arrows), "normal" Leptospira (L) and coiled forms of Leptospira (R). Warthin-Starry. x 1875.

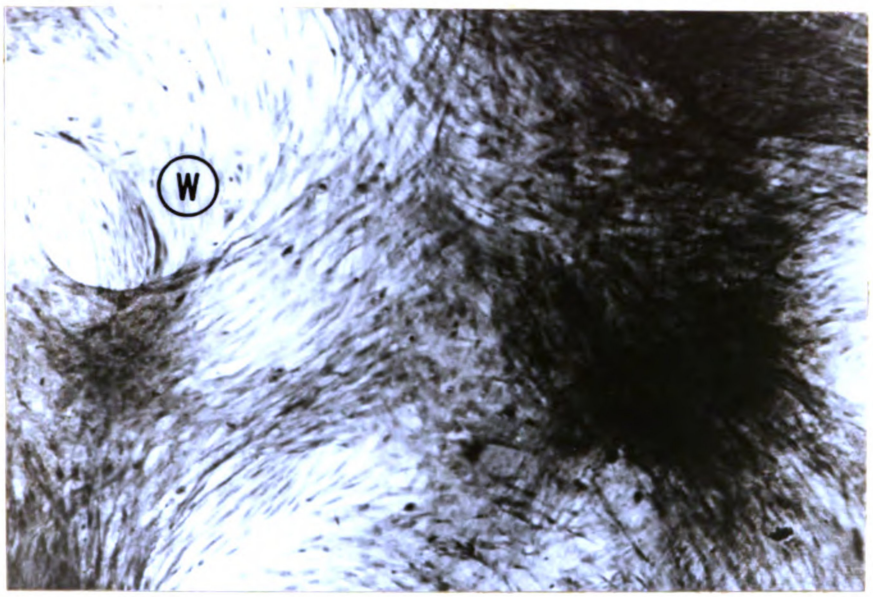
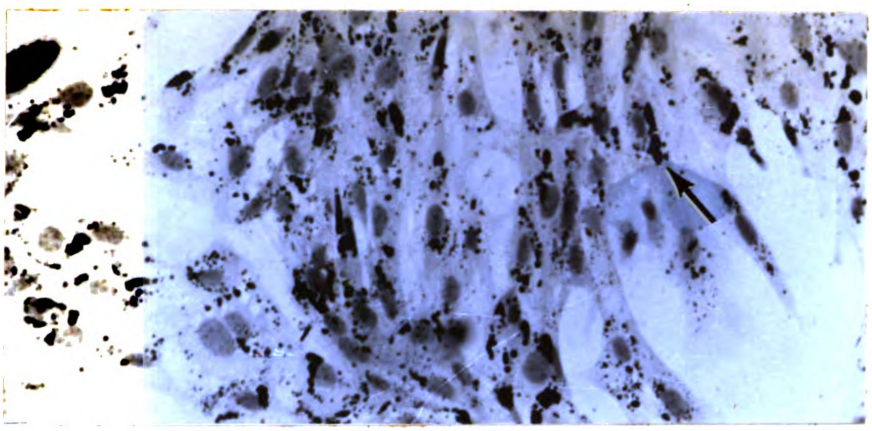


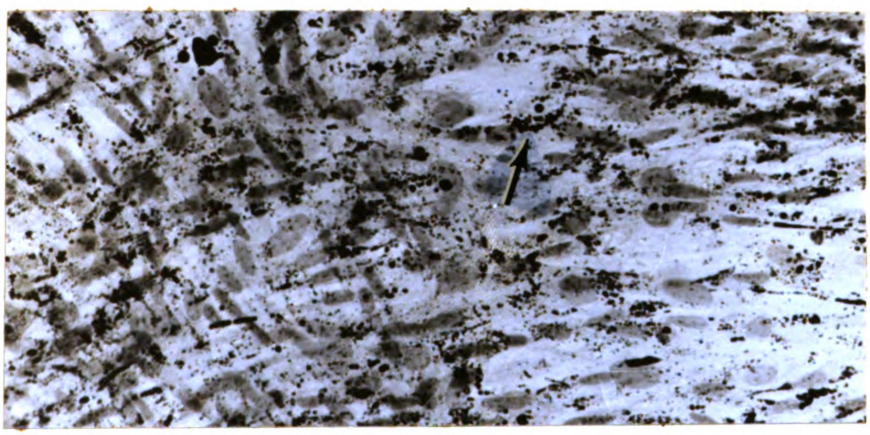
Figure 34. Dense control (noninfected) bovine fetal kidney culture at nine days. Note whorled appearance of the fibroblast-like cells (W). May-Grunwald-Giemsa. x 75.

Figure 35. Three-day-control (noninfected) bovine fetal kidney culture stained for fat. Note abundance of cytoplasmic fat (arrow). Oil Red O. x 187.

Figure 36. Six-day-control (noninfected) bovine fetal kidney culture stained for fat. Note abundance of cytoplasmic fat (arrow). Oil Red O. x 187.

Figure 37. Nine-day-control (noninfected) bovine fetal kidney culture stained for fat. Note abundance of cytoplasmic fat (arrow). Oil Red O. x 187.





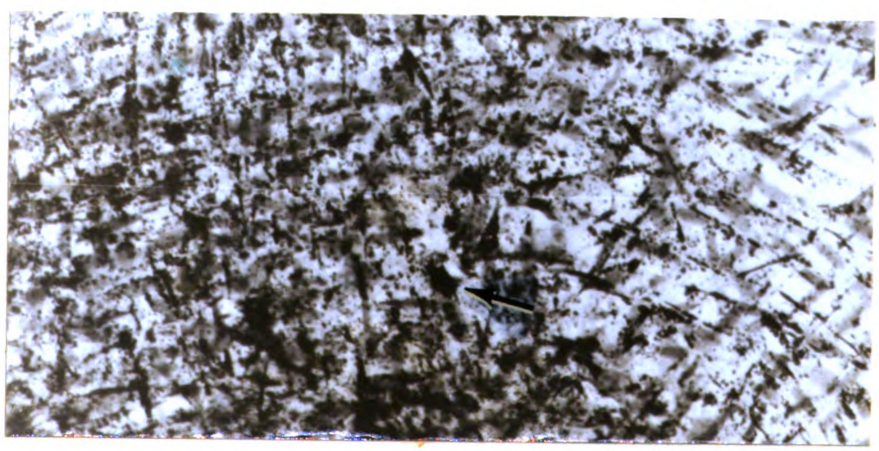
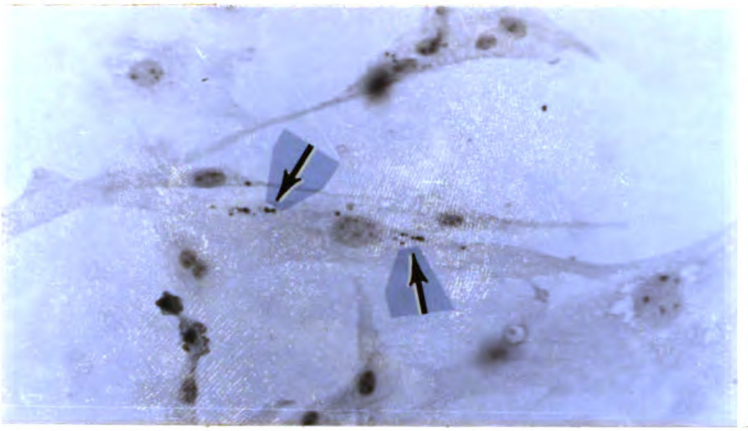
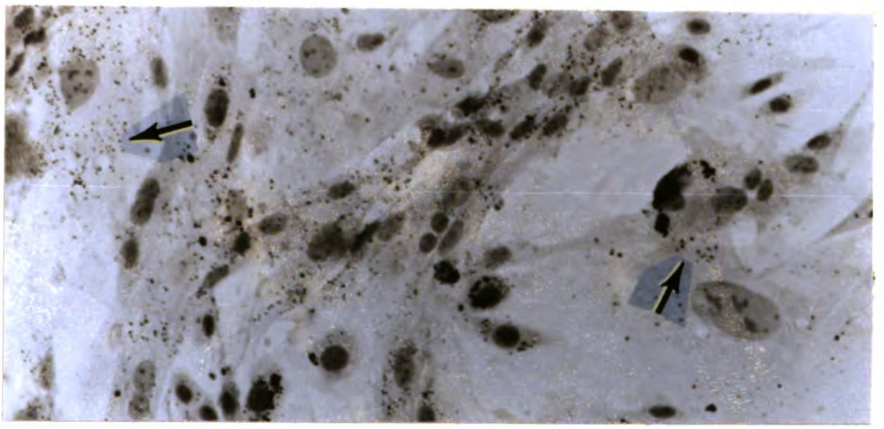


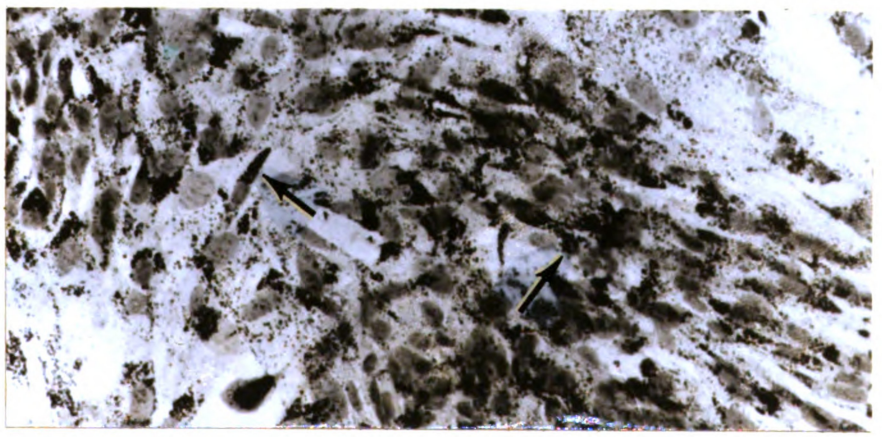
Figure 38. Bovine fetal kidney culture three days after infection with Leptospira pomona. A few cytoplasmic droplets of fat are present (arrows). Oil Red O. x 187.

Figure 39. Bovine fetal kidney culture six days after infection with Leptospira pomona. Note numerous small cytoplasmic droplets of fat (arrows). Oil Red O. x 187.

Figure 40. Bovine fetal kidney culture nine days after infection with Leptospira pomona. Note abundance of cytoplasmic fat (arrows). Oil Red O. x 187.







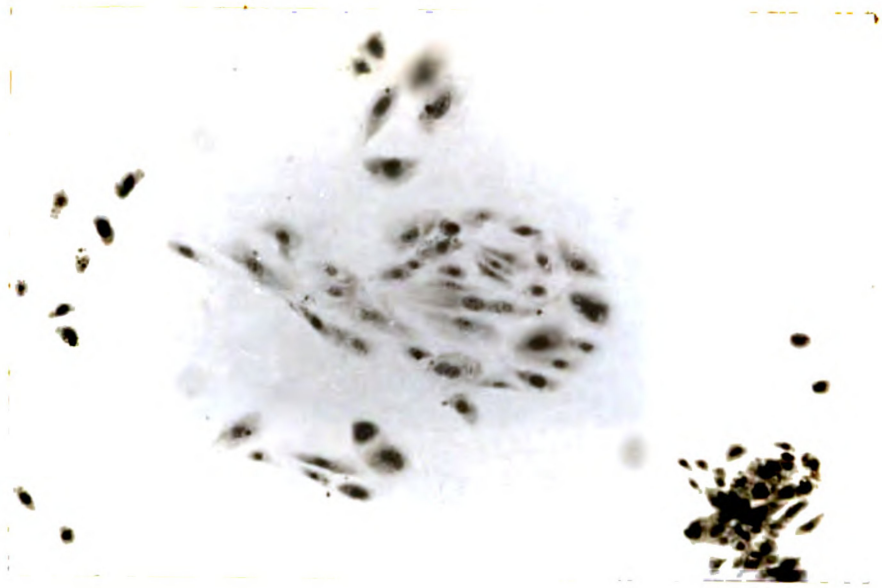


Figure 41. One-day control (noninfected) bovine fetal kidney culture. Compare with Figure 42. May-Grunwald-Giemsa. x 187.

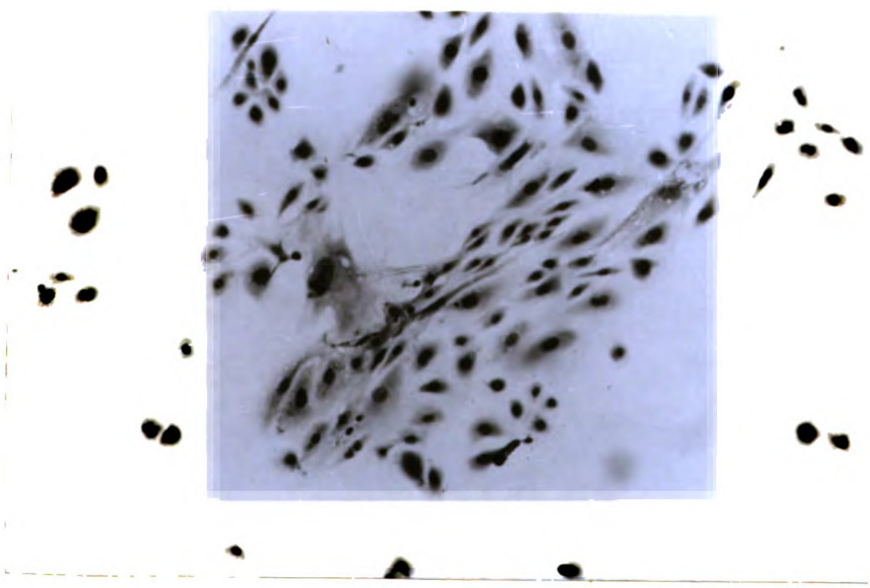


Figure 42. Leptospira pomona infected bovine fetal kidney culture one day after infection. May-Grunwald-Giemsa. x 187.

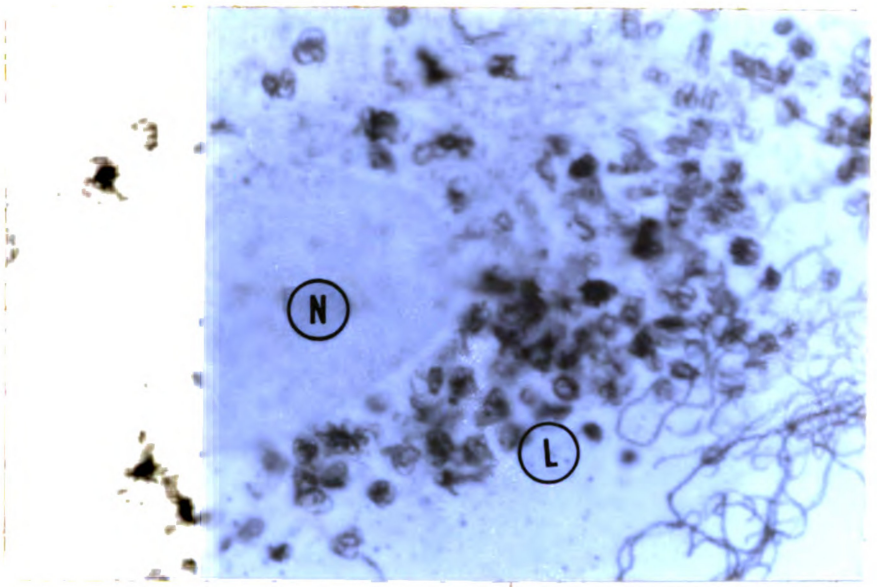


Figure 43. Leptospira pomona infected bovine fetal kidney culture one day after infection. Note coiled leptospires (L) outlining nucleus of cell (N). Warthin-Starry. x 1875.

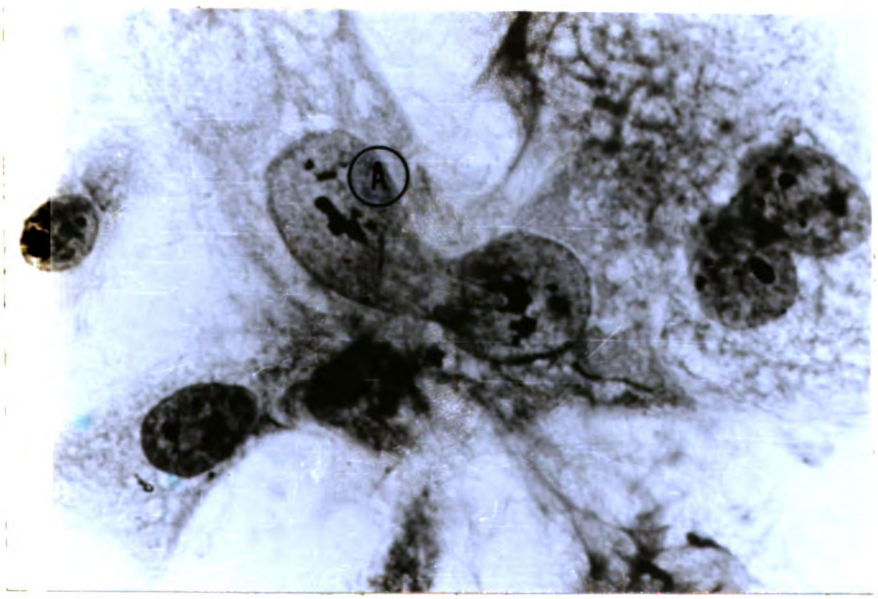


Figure 44. Nuclear aberration of large epithelial-like cell in bovine fetal kidney culture (A). May-Grunwald-Giemsa. x 750.

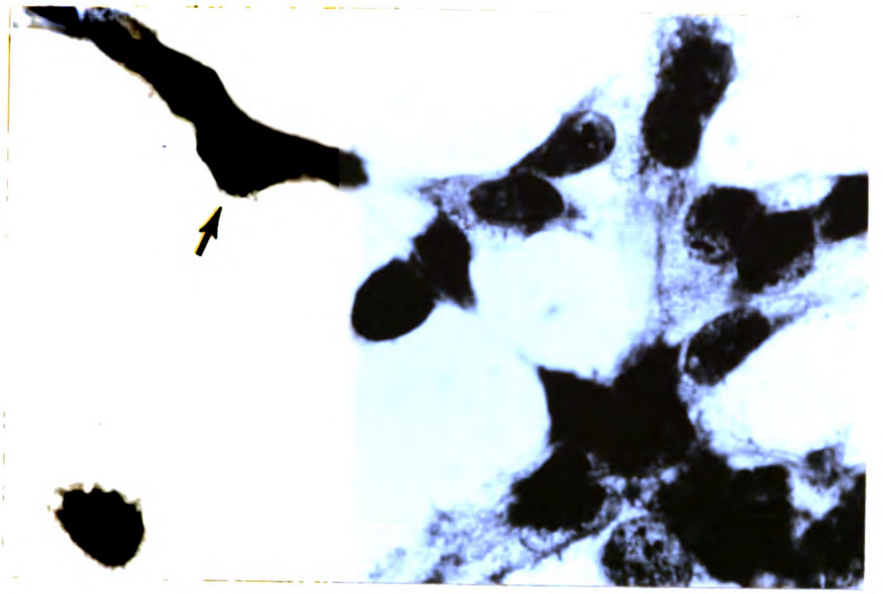


Figure 45. Leptospira pomona infected bovine fetal kidney culture two days after infection. Note contracted and deeply stained cytoplasm of some cells (arrow). May-Grunwald-Giemsa. x 750.

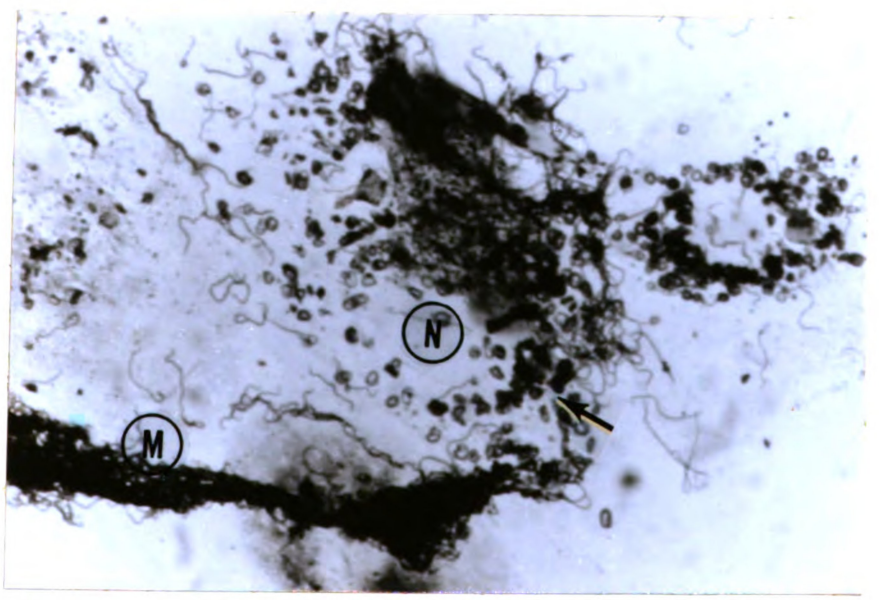


Figure 46. Leptospira pomona infected bovine fetal kidney culture two days after infection. Note mass of leptospires (M), nucleus of cell (N), and granule and ring forms of leptospires (arrow). Warthin-Starry. x 750.

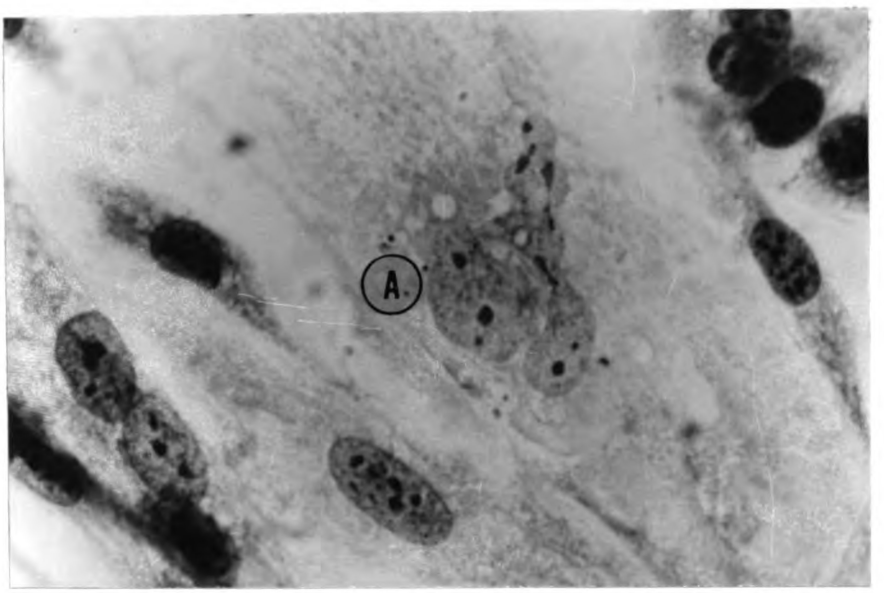


Figure 47. Three-day Leptospira pomona infected bovine fetal kidney culture. Note nuclear aberration of large epithelial-like cell (A). May-Grunwald-Giemsa. x 750.

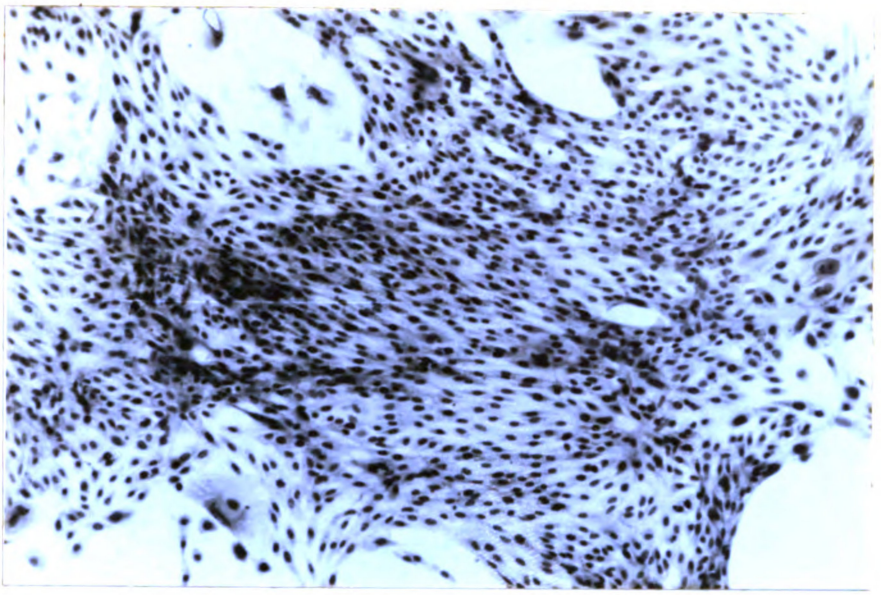


Figure 48. Control (noninfected) bovine fetal kidney culture at four days. Compare monolayer pattern and density with that of Figure 49. May-Grunwald-Giemsa. x 75.

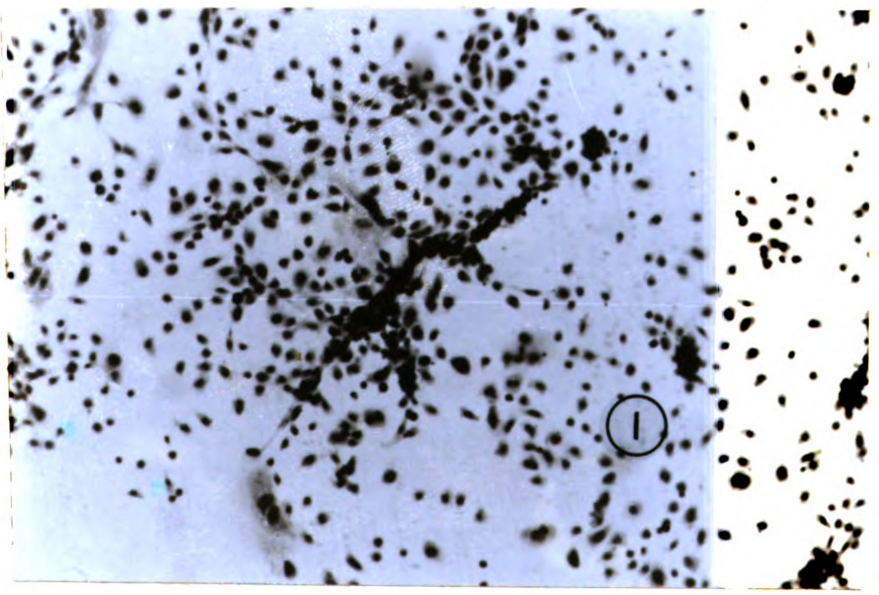


Figure 49. Leptospira pomona infected bovine fetal kidney culture four days after infection. Note "individualization" of cells and cell groups (I). May-Grunwald-Giemsa. x 75.

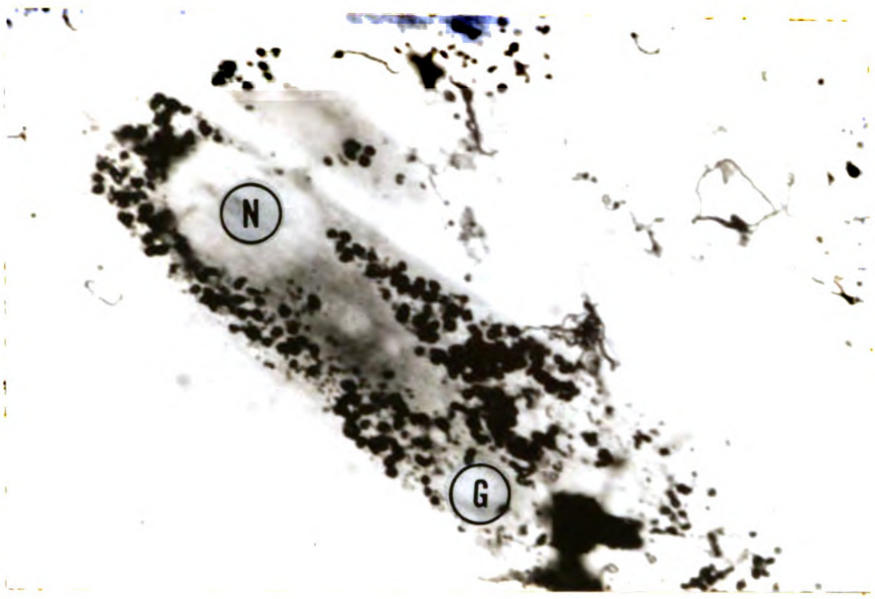


Figure 50. Leptospira pomona infected bovine fetal kidney culture four days after infection. Note outlining of cell nucleus (N) by apparently intracytoplasmic granules (G). Warthin-Starry. x 750.

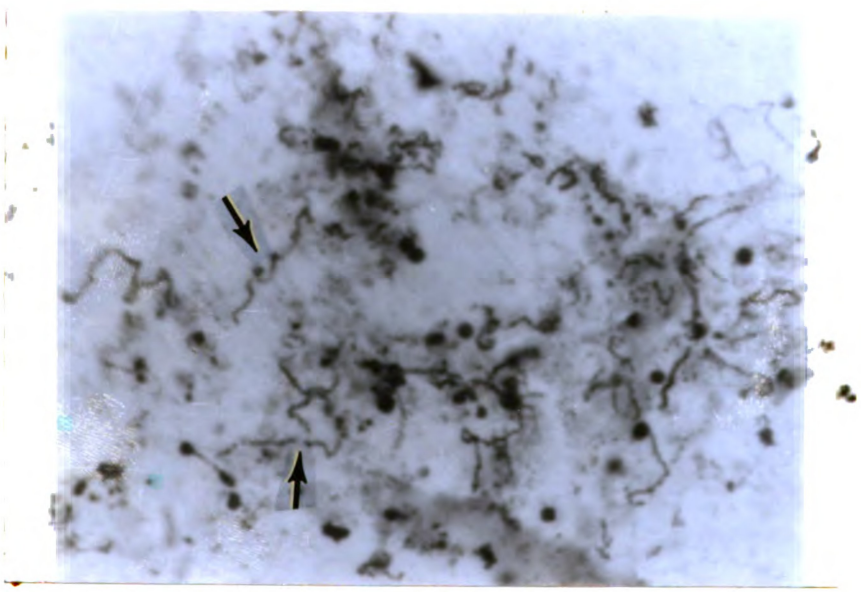


Figure 51. Five-day Leptospira pomona infected bovine fetal kidney culture. Note beaded and fragmented leptospires (arrows). Warthin-Starry. x 1875.

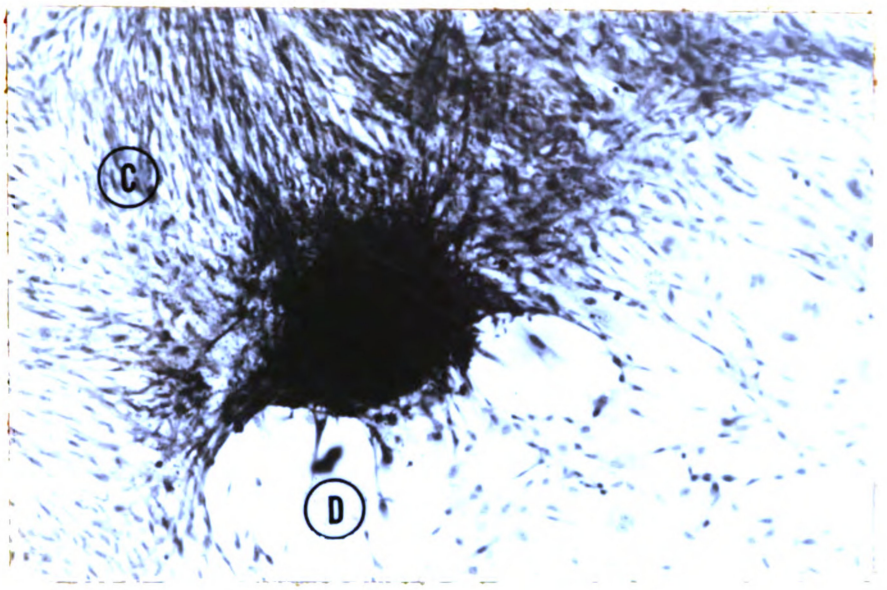


Figure 52. Control (noninfected) bovine fetal kidney culture at six days. Note cellular detachment (D) and dense cell growth (C). May-Grunwald-Giemsa. x 75.

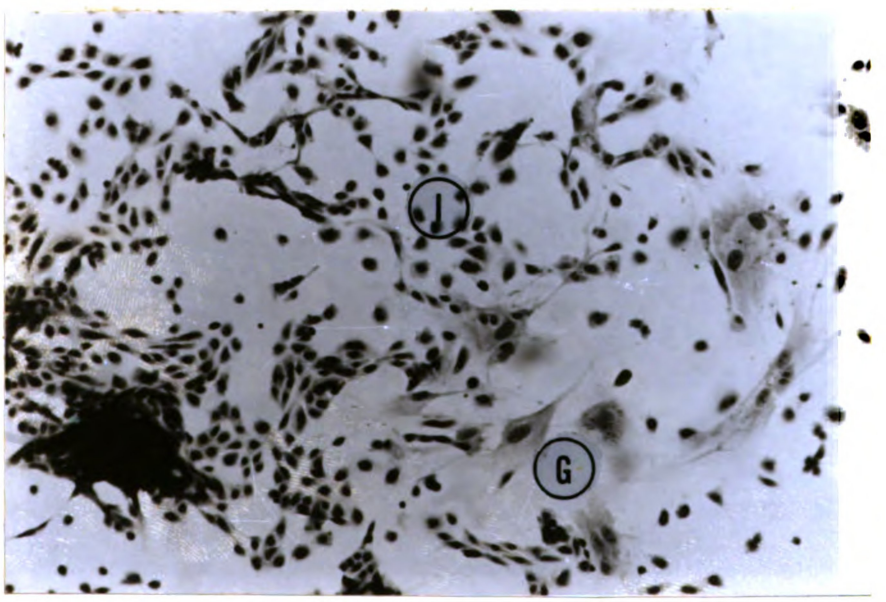


Figure 53. Leptospira pomona infected bovine fetal kidney culture six days after infection. Note large epithelial-like cell (G) and individualized cells (I). May-Grunwald-Giemsa. x 187.

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