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CYTOPATHOLOGY OF VIRUS-INFECTED
KIDNEY CELL CULTURES OF SWINE

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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* * * * * *

The Ohio State University
1960

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INTRODUCTION

The cell is the unit of structure and of function of all living things; consequently, it was inevitable that attempts should be made to cultivate cells from various organs and tissues in order to study their anatomical, physiological, nutritional, and biochemical activities. Since the pioneer work of Harrison (1907), cell cultures have been used for a wide variety of studies, but it was the demonstration that they could be used for the propagation of viruses that led to an intensive and extensive study of cell cultures. Cells from virtually every organ in the animal body have been grown in vitro, and more and more of the highly specialized and fastidious cells are being successfully grown to supply the demand for different types of cells in research.

Cell cultures are now the foremost system for the propagation of virus. In addition to their use for such things as the production of virus for vaccines, for example polio, they provide an efficient system for the isolation of viruses from animals, and literally hundreds of previously unknown viruses have been isolated by this technique. Serving as an indicator system, they make practical the large numbers of neutralization tests necessary to identify and categorize these viruses. It now seems likely that each species of animal is host to a number of viruses.
which produce no demonstrable disease but if introduced into other species might do so.

Because of the practical value of the cell culture technique in virology, most of the present investigations deal with cell culture as a means for propagating viruses. However, there are many possible systems that have not been studied and much detailed information on the nature of the cell-virus relations which needs further elaboration.

Just as much of our knowledge of the function of tissues and organs has come from observations of the abnormal or diseased, so observations of virus infected cells has contributed to and will continue to contribute to the knowledge of the structure and function of normal cells. Conversely, knowledge of the normal is essential for a study of the abnormal, or the changes produced by the infection of virus.

In any scientific procedure, the methods used for observation and measurement determine the limitations of the technique. It is understandable that the classical procedures of histology served as the basis of early studies of cell cultures, since most were directed toward the morphology of the cell. While many significant developments in microscopy, such as phase and electron microscopy, have since occurred, stained preparations still form an
important part of studies on the cell. The experimenter still wants to put the cell under the microscope and see the results of his manipulations whenever possible.

Suitable stained preparations of cells may serve as the primary technique or as a valuable adjunct to studies involving cell cultures. They make possible the critical evaluation of the health of cultured cells and provide one means of following the pathological changes that are the result of a toxic or infectious agent. Histochemical techniques may be utilized to follow biochemical changes in the cell. It is possible by the use of such preparations to detect more subtle types of viral cytopathogenicity which otherwise would go unnoticed and thereby increase the value of a cell type for virological studies.

In cell lines an alteration in the virus susceptibility may be related to an alteration in the genetic integrity of the cells. This may be followed by cytological techniques. The process of mitotic division may be closely followed, the chromosomes studied and counted, and the genetic characteristics of the cells determined.

The development and standardization of staining techniques is a continuous problem. While some of the techniques used today are many years old, many still remain strictly empirical. When staining techniques are used on new types of cells, many trials are necessary to standard-
ize the procedures for reproducible results. Sometimes it is even necessary to standardize the techniques for new batches of stains.

New techniques are being developed, particularly in the area of histochemistry. These make possible a more rational basis for many of the staining techniques or, as the title of one text indicates, it makes possible Biochemical Cytology (Brachet, 1957). These new techniques include not only the usual chemical reactions but also the more sophisticated techniques which make use of fluorescent dyes, with or without antibody coupling, and autoradiography.

This study deals with the standardization and adaptation of staining techniques, both old and new, to swine kidney cell cultures, and the use of these techniques to define the cytopathology of these cells infected with viruses. For convenience, the work is considered in two parts. Part 1 deals with the normal structure and appearance of the cells of this system as stained by the techniques used. Part 2 deals with the cytopathic changes produced by viruses. These include several well-known viruses in addition to a group of enteric viruses of swine isolated in this laboratory and designated Enteric Cytopathogenic Porcine Orphan (ECP0) viruses.
STUDIES ON UNINFECTED SWINE KIDNEY CELL CULTURES

Before attempting to delineate the pathologic changes in virus infected cells, it is obviously necessary to know what to expect in the "normal cells" of the system, or as Cohen (1959) has stated: "A virologist must have cells to practice his discipline and he should be a cellular biologist before approaching problems of virus infection." The "normal" cells can be expected to vary in microscopic appearance depending upon the physical and chemical environment, the various fixation and staining techniques used, and the age of the cell.

The first phase of the experimental study is devoted to establishing a suitable base line by examining cells at various stages of growth and under different environmental conditions and stained by the various techniques. It is emphasized that this portion is not a detailed cytological study of the tissue culture swine kidney cells from the histological standpoint - nor is it intended to be. Its purpose is to define the architecture of the normal cell which may be altered or modified by virus infection and thus serve as an additional control on studies of virus infected cells.
Review of the Literature

Cell Cultures

The universal application of cell culture techniques in most fields of experimental biology has resulted in a voluminous literature. The comprehensive bibliography by Murray and Kopoch (1953) covered the period 1884-1950 and required two volumes. Recently, Swim (1959) has concisely and critically reviewed the "modern or microbiological era" of cell culture. This review covered the period of 1948-1958 and was restricted as much as possible to the cultivation of cells per se although as Swim pointed out, the studies on cell culture have been so intimately integrated with applied aspects, that it is difficult to separate the two.

The literature on the cultivation of swine cells is much less extensive than that on the cells of many of the other animals. Young, Underdahl, and Sabina (1957) have reviewed the reports on the subject published prior to 1956. In the even more limited area of the cultivation of swine kidney cells, the reports are few indeed. The bibliography by Murray and Kopoch (1953) lists only one reference (Smith 1921) and unfortunately this is in error. This work was concerned with the ingestion of melanin pigment by chick embryo cells in culture. The melanin pigment was obtained from the retina of a number of species, including the pig.

Fortunately, the use of swine cells in tissue culture
and more specifically, swine kidney cells has received considerable attention in recent years, mainly for the propagation of viruses (Madin, 1959). In previous reports (Hancock, 1957, Hancock, Bohl, and Birkeland, 1959), the earlier work on the cultivation of swine kidney cultures was reviewed. This included the work of McClain, Madin and Andriese (1954), Millian and Robbins (1956), Fieldsteel and Emery (1954), and Madin, Andriese and Darby (1957) on cultures prepared from fragments either by the plasma clot technique or the dry tube technique. Cultures prepared from trypsinized cells were employed by Lee (1956), Moscovici, Ginevri, and Mazzaracchio (1956), Beran, Nerder, and Jenner (1957), Young, Underdahl, and Sabina (1957), and Guerin and Guerin (1957).

In addition to these reports, other workers have mentioned the use of swine kidney cell cultures with little or no information on their preparation. These include Mckercher, Moulton, Madin, and Kendrick (1957), McClain and Hackett (1958), Moscovici, Ginevri, and Mazzaracchio (1959).

Wesslen and Lannek (1954) grew cells from the lungs and kidneys of pigs "not more than one day old." The cells were cultivated by the chicken plasma clot technique in a medium composed of bovine amnionic fluid. The cultures were used for the isolation of a cytopathogenic agent from pigs with enzootic pneumonia.

Hjarre, Dinter, and Bakos (1954) cultivated cells from lungs and kidneys from "one to several day-old-pigs or pig
embryos" by the plasma clot technique. The cells were destroyed by swine influenza virus. Little information was given on the details of the tissue culture. Bakos (1957) later compared the cytopathogenic effects of an additional strain of swine influenza virus on these cells.

Schwobel and Mayr in a series of articles (Mayr and Schwobel 1956, Schwobel 1957, Mayr and Schwobel 1957, and Mayr 1957) reported on the use of swine kidney cell cultures from year old pigs in the propagation of "Teschen's disease virus (porcine poliomyelitis). The cells were grown in a lactalbumin hydrolysate medium containing bovine serum.

Schwarz, Zirbel, Estela, and York (1958) prepared cultures from the kidneys of one to eight week old pigs by the trypsinization technique. The cells were susceptible to infectious bovine rhinotracheitis virus. No detailed cytological studies were made.

Singh, Bohl, and Birkelard (1959) described the cultivation of cells from adult swine kidneys in prescription bottles and their use in the study of swine arteroviruses by the plaque technique.

The examination of stained preparations of cells grown in vitro has a history as old as cell culture itself. Harrison's original study was designed to prove whether or not the nerve fibers were extensions of the neuron cells. The earlier studies, even those attempts that antedate Harrison's work, were devoted to attempts to more accurately
determine the structure of the cell (White, 1959). As a further example of the interest in cytological methods, the booklet on tissue culture by Buchsbaum (1936) contains a special section on histological methods.

The early studies were hampered by many technical difficulties other than those associated with the cultivation of the cells. The common method of cell culture, the slide culture, lends itself to close observation and subsequent staining, but one of the difficulties is the requirement of a plasma clot which later interferes with the staining procedures. Quite commonly the whole mount is fixed, embedded and cut with a microtome in the same manner as animal tissues. This procedure requires considerable skill if the flat zone of outgrowth is to be suitably prepared.

The technical advances in tissue culture such as the use of antibiotics in culture media, and the cultivation of cells on glass from trypsinized suspensions, made possible the cultivation of uniform sheets of cells which are in ideal position for staining with a minimum of manipulation. However, cells grown on the inside walls of culture tubes are difficult to study at higher magnifications. Techniques developed for removing them to conventional slides are quite involved. The cultivation of sheets of cells directly on coverslips ("flying coverslips") was a significant technological advance in this regard and makes possible more de-
talled studies of the cells. Although there is consider-
able interest in this technique only a few reports have
appeared on cytological studies of swine kidney cells.

Switzer (1959) has recently reported on the tech-
niques employed for a number of swine tissue culture sys-
tems. By his techniques he was able to pass swine kidney
serially through 105, nasal mucosa through 87, lung through
65, and endothelium through 22 passages. The cultures were
prepared by the trypsinization technique and grown in a
lactalbumin hydrolysate medium supplemented with swine or
calf serum and in the case of the passage cells with chick
embryo extract. The susceptibility of these passage cells
to a number of viruses as well as the primary cell culture
was described. Cytological studies by means of Giemsa
Stained preparations of cells grown on coverslips were de-
scribed and photographed.

Larin, Harr, and Orbell (1959) prepared primary cul-
tures, by the fragment technique and primary, secondary,
and tertiary trypsinized cultures from a number of species
including the pig. The cultures were used for a study of
cytological aberrations that might occur in normal cultures.

Betts (1960) prepared trypsinized cultures from the
kidneys of 3-6 week old pigs and used them to isolate a
polio-encephalomyelitis virus from the tonsils of normal
pigs. Only a limited amount of detailed cytological work
was reported. However, Jennings and Kelley (1960), working in the same laboratory, reported in greater detail on the cytopathology of this virus in swine kidney cell culture.
Materials and Methods

Cell Cultures

The basic procedures for the preparation of the cultures have been described (Hancock 1957, Hancock, Bohl, and Birkeland 1959). Monolayer cultures were prepared on 10 by 50 mm. No. 1 coverslips in 16 by 125 mm. Leighton test tubes. The coverslips were cleaned by transferring, with forceps, to Wassermann tubes containing a mixture of equal parts of 95 per cent alcohol and acetone. These tubes were placed in a covered can to prevent evaporation and allowed to remain overnight. The acetone-alcohol was drained off, the tubes inverted and the slios allowed to dry. They were then "poured," without touching, into clean flat tubes. The tubes and coverslips were sterilized by dry heat.

The cultures were implanted with 2 ml. of a 1:200 suspension of trypsinized cells from adult swine kidneys. The complete growth medium consisted of 0.5 per cent lactalbumin hydrolysate (LAHM), 10 per cent porcine serum and 89.5 per cent Hanks' solution (HS). Antibiotics were added to give a final concentration of 200 units of penicillin, 200 mg. of dihydrostreptomycin, and 50 units of nystatin. The pH was adjusted with sodium bicarbonate (4.4%) to 7.2.

The cultures were incubated at 36°C. in conventional

stationary tissue culture racks and the medium changed at 3-4 day intervals. The maintenance medium was similar in composition except the porcine serum was replaced with 3 per cent bovine serum and the pH adjusted to 7.6. The monolayer was usually complete in 7-10 days.

**Cytological Procedures**

A number of fixation and staining techniques were used throughout the study. Since it was necessary to revise and adapt many of the standard techniques to this particular system, the detailed procedures are given in the Appendix. These include May-Grunwald-Giemsa, hematoxylin and eosin, Heiderhains iron hematoxylin, Sajal's trichrome, and Giemsa.

Cytochemical techniques were used to a limited extent. These included the Feulgen reaction for DNA, the methyl-green pyronine stain for RNA, and oil red O stain for lipids. A number of standard references give various procedures for the stains, but none were entirely satisfactory without modification. In the adaptation of these techniques, Dr. M. Y. Andres of the Department of Veterinary Anatomy was a constant guide.

In working out the mechanics of the methods, aluminum carriers (Figure 1) were constructed to hold several cover-

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2 The text by Pearse (1960) is an invaluable reference. Most of the comments on the cytochemical techniques are excerpts from this book.
slips, making possible the simultaneous staining of a number of coverslips which is essential for controlled studies. These also simplified the technique. Ointment jars were used as staining vessels and were more satisfactory than coplin jars since it was possible to store the reagents directly in the jars as the screw caps prevented evaporation. In the few instances where it was impossible to use this system because of the action of the reagent on the aluminum carrier, the coverslips were immersed in the stain in a Wassermann tube.

Photomicrography was carried out with A6 Spencer Microstar triocular microscope equipped with a model 404 built in base illuminator and a model 635 35 mm. camera. The objective lenses used were apochromatic.

A Kodak 82C filter was used to bring the color temperature to the desired 3800° Kelvin. A Kodak 82A filter and/or a Spencer no. 608 Dydimun filter was used as necessary for desired contrast. The film used was Kodachrome Professional Film Type A.
Figure 1. Fabricated aluminum carriers for staining 10 x 40 mm. coverslips. The carrier on the left is used in ointment jars.
Results

Appearance of Normal Cells

In the porcine kidney cell cultures, as in most other types of cell cultures, the cells tend to form two distinct morphological types - epithelial-like and spindle-shaped or fibroblastic (Figure 2). The epithelial cell predominated. Puck, Cieciura, and Fisher (1957) in studies of clonal lines, stated that these morphologic features are true genetic characters since single cells of each type breed true. Puck has since modified his view somewhat. He stated in a personal communication (Puck, 1959) that in recent studies he has been able to convert these lines to different morphological types by manipulation of the culture medium.

Undoubtedly there are different types of cells in cell cultures, cells of different origins and of different viral susceptibility. At this stage we can only recognize this point - not clearly define it. Until such is possible we may only refer to the two broad morphological categories - fibroblastic and epithelial. These two terms are widely used in cell culture work with a definite morphological application. They should have no embryological significance.

The predominant epithelial cell in swine kidney cultures is approximately 25 microns in the greatest diameter in uncrowded cells, although cells twice this size are observed. In areas of crowded cells, the individual cells
Figure 2. Normal swine kidney cells. X 200 May-Grunwald-Giemsa stain. Several areas of fibroblastic cells are visible.
are smaller. The outline is irregularly round or oval, although the cytoplasmic margin is indistinct with no distinct membrane. The cytoplasm is finely granular in healthy cells and contains in some instances phagocytized particles and vacuoles. Most of the nuclei are approximately 10 microns in size, although an occasional nucleus up to 25 microns is seen. The nuclei are usually oval to round, but occasional bizarre forms are seen. Most of the various aberrations described by Larin, Barr, and Orbell (1960) were seen (Figure 3), although they were observed with greater frequency in some cultures than in others. One or two nucleoli were present as well as karyosomes which varied in size from small clumps of chromatin up to structures as large as nucleoli.

The final cell population varied from tube to tube. Counts made on individual tubes by trypsinizing the cells and enumerating in a hemocytometer showed total counts of from 1 x 10⁵ to 2.27 x 10⁵. The coverslips were usually two-thirds to three-fourths covered by the cell sheet although an occasional slip was only covered on the lower one-half and an occasional one would be completely covered. Another cause of variation was in the extent to which cells were "packed" into a given area. Microscopic cell counts ranged from 15 to over 40 cells for a given oil immersion field. This would represent from 2.25 x 10⁵ to 6 x 10⁵ cells for a slip three-fourths covered with cells.
Figure 3. Normal swine kidney. X 900. May-Grunwald-Giemsa stain. Several aberrant nuclei may be seen in this field.
Observations on the Growth Period

In order to study the growth of cultures, a series of preparations were made over the active growth period. Three slips were removed each day, beginning 24 hours after planting. One was stained by the May-Grunwald-Giemsa technique, one fixed in Rossman's solution and stained with Cajal's trichrome stain. The entire series was performed in duplicate, one group of cells grown in medium containing 10 per cent porcine serum and the other group with medium enriched with 10 per cent bovine serum. This made a total of six slides a day. The series was followed for 8 days with media changed on the third and sixth days.

The observations on stained preparations over the period of active growth in porcine serum confirmed, in general, those previously made on unstained cultures. At 24 hours, large numbers of basophilic pynotic cells were fixed to the slips. These were 2.3-2.5 microns or somewhat smaller than normal nuclei in diameter and had the appearance of bare pyknotic nuclei (Figure 4). Characteristically they occurred singly although an occasional cluster was seen. Single or paired cells with a distinct cytoplasm were also observed. These were rounded with uneven edges. Most commonly these healthy cells occurred in three dimensional clusters with no discrete boundaries to the cytoplasm. The nuclei had one or two nucleoli in addition to masses of chromatin. In some of the preparations the fine network of chromatin material
Figure 4. Swine kidney cell cultures two days after implantation. Trichrome stain. X 430. Note the large number of pyknotic "nuclei."
was visible. Very few division forms were seen at this stage and many preparations did not contain any. Some cells occurred singly and had a definite eosinophilic cytoplasm.

At 44 hours, multiplication had accelerated, and the colonies of cells were flattened on the glass surface. The cytoplasm was granular, irregular in outline and without distinct margins. The debris was removed in the immediate area of the colonies and the cytoplasm contained vacuoles and eosinophilic granules, indicating active phagocytosis. The internal matrix of the nucleus was granular and at this stage most contained a single nucleolus although some had two.

The cells grown in bovine serum multiplied at a more rapid rate during this initial phase of growth. This differential was overcome following the first media change, and the two groups reached confluency at about the same time. The only other observable difference between the two groups was the persistence of the basophilic pyknotic cells, along with cellular debris in the bovine serum group. Most of this type of material had disappeared from the porcine group after the first media change. Immediately following the media change, there was a "burst" of mitotic divisions as would be expected. During the remaining period of active growth there were no other unusual cytological features observed.
Numerous mitotic division forms were readily visible in cells stained in the active growth stage (Figure 5). In many of those in metaphase, the chromosomes could be observed in detail and counted. In some instances the method of Tjio and Puck (1957) was employed to further delineate the chromosomes. Dr. Puck observed these and stated that they appeared to be normal with few polyploid cells present. These characteristically showed the diploid number of forty chromosomes (20 pairs). 3

Effects of Physical and Chemical Environment

In order to compare the survival of cells in maintenance medium with and without serum, 32 tube cultures of 1 week old porcine kidney cells were divided into two groups. The media was poured off as completely as possible, the cells were washed once with HS and the media replaced. One half of the tubes received LAHM with 3 per cent bovine serum pH 7.8 and the other one half received an identical medium without serum. No replacement or additions were made to the media during this period of observation. Slips were removed at daily intervals, fixed in Bouin's without a preliminary wash, and stained by H & E (R).

On the second day after the media change there was a

---

3 These preparations are excellent for the instruction of students on mitosis of mammalian cells. A set is currently being used in the School of Veterinary Medicine for this purpose.
Figure 5. Normal swine kidney cell cultures. Trichrome stain. X 1000.
slight difference and in the third day there was a definite
difference in the pH between the groups. The pH of tubes
containing serum was lower around 7.2-7.4, as compared to
approximately 7.4-7.6 in those without serum.

By the third day, dead cells could be detected and
they seemed to be more numerous in the tubes without serum.
They were easily washed off the slip. The degenerating
cells were pyknotic with an eosinophilic cytoplasm. There
were no visible alterations in the internal structure of
the remaining normal cells, and there was no observable
difference between the two groups other than the number of
cells dying.

By the fourth day the death loss was accelerating in
both groups but more so in the group without serum. For
the most part the cell sheet was still confluent although
an occasional hole was observed on the slips without serum.

On the fifth day definite breaks were observed in the
continuity of the cell sheet in the tube without serum.
Also, numerous light staining areas were observed. On
closer observation these proved to be areas in which the
cells had thinned out considerably. It appeared as though
the remaining cells in the area had expanded their cytoplasm
to fill in the areas on the glass left vacant by the dying
cells.

On the seventh day, cultures would be unsatisfactory
as controls for virus infected cultures because definite
holes in the sheet were numerous. From this point onward, the remaining cells on the sheet rapidly degenerated.

By the eighth day, the cells in cultures containing the serum enriched medium were showing the changes characteristic of the other group at five days. The death of the cells followed the pattern described above.

The swine kidney cells will remain viable in culture for a considerable length of time, when proper consideration is given to the maintenance medium. In one series which were held for 4 weeks, during which time the medium was changed regularly, the cells did not show any marked cyto logical changes. The only difference that could be noticed was an increased death rate in individual cells. These cells were continually replaced by mitotic division so that the sheet remained confluent.

This tendency of cells in culture to multiply whenever there is room on the glass can cause difficulties in cyto logical work on viruses capable of inducing latent infection. Newcastle disease virus is one example. Puck and Cieciura (1957) reported on the development of the carrier state in surviving cells exposed to Newcastle virus.

In order to further define the normal degeneration under different physical conditions, a group of cultures were selected, the media replaced with maintenance medium and divided into three groups. Group A was returned to the 37° C. incubator, group B was placed at 25° C. and group C was
stored at 50°C. At varying days thereafter, one slip was removed from each group, fixed and stained.

In the cultures maintained at 37°C., the cell sheet was maintained intact until the fifth day, after which there appeared areas of degeneration. This time interval was somewhat earlier than is usually seen with cells in this system. The rate of degeneration varies with different batches of cells and even from tube to tube within a lot. This could easily account for the discrepancy found in this series and our previous experience with the system.

In the cultures held at 25°C. there were no marked cytological changes until the eighth day. At that time the sheet began to degenerate in the same manner as described for the cultures held at 37°C.

In the remaining group, held at 5°C., the changes were more distinctive (Figure 6). There was a gradual retraction of the cytoplasmic margins of individual cells which was quite obvious by the third day. The nuclei became pyknotic and more chromophilic. These changes remained constant throughout the observation period.
Figure 6. Swine kidney cells held at 5° C. for 7 days. X 1000. Heidenhains iron hematoxylin stain.
Discussion

In a study of normal cells prior to their use in virus studies, attention must be given to aberrations which could mimic viral changes. Kleinfeld and Melnick (1958) emphasized the frequency of cellular aberrations of monkey kidney cells that exist even in primary cultures. Larin, Barr and Orbell (1959) described them in cultures of other species.

Cellular aberrations are certainly not surprising when one considers the circumstances under which the cells are maintained in vitro. The cells are flattened on a glass surface and when they show ameboid movement both the cell membrane and the nuclear membrane will assume irregular shapes. If a large particle of debris is phagocytized, it can easily cause an indentation in the nucleus which will be prominent because of the extreme thinness of the cell.

Abnormal division forms have been observed by several workers although they seem to be rare in the swine kidney cell cultures. Whether or not they are of greater frequency in vitro than in vivo is questionable. Certainly some of the conditions of cell culture could act as mild mutagenic agents, but what is probably of more significance is the fact that the conditions would make any aberrations more prominent, and some would perhaps have a better chance of survival in vitro.

The possibility that a cell inclusion, even an intranuclear inclusion, is due to something other than the virus
must be considered. Kleinfeld and Melnick (1958) introduced the term "nuclear phagocytosis" to denote the engulfing of pyknotic cells by the nuclei of simian cells in vitro. In addition, Larin (1958) described the occurrence of intranuclear inclusions in uninfected cultures of canine kidney cells. In a recent publication Larin, Barr, and Orbell (1959) have studied the nuclear aberrations occurring in "normal" cell cultures of dogs, pigs, ferrets, and chick embryos. They observed several types of intranuclear inclusions: (1) a "cloud like mass," (2) engulfed nuclear chromatin, (3) Cowdry type A inclusions, (4) crystals.

A few degenerating cells were usually observed in normal cultures. These cells showed a clumping of the chromatin and gradual pyknosis of the nucleus. The cytoplasm was condensed as indicated by shrinkage and an increased chromophilia. The number of cells in the late stages of degeneration in both control and virus infected cultures seemed to be governed by the technique of fixation. Most of the techniques described for cells cultivated on "flying coverslips" call for the cells to be washed in saline before fixation. The advantages of this step are said to be the removal of excess debris (which it does) and removal of excess serum protein from the cell (which is doubtful). The procedure has the disadvantage of increasing the possibility of dislodging cells in the late
stages of destruction. In this study, slides were stained both with and without the preliminary washing. When this step was omitted, there was considerable debris, but it was felt that more of the degenerating cells remained. A good slide from the experimental standpoint is not always a "pretty" slide.
STUDIES ON VIRUS INFECTED CULTURES

The virus-cell relationship is the most intimate of the host-parasite relationships. The study of cell cultures infected with a virus offers a convenient and exact means of studying the changes that occur in virus-infected cells. In addition, such a system makes possible a preliminary correlation of specific changes with the release of new virus particles. It is the purpose of this phase of the study to examine the changes that occur in swine kidney cell cultures infected with viruses.

Review of the Literature

Since much of the early work on virus propagation in cell cultures was not directed primarily toward the detection of cellular damage, information regarding specific cytopathology is frequently fragmentary. Many times the observations on tissues infected in vitro were examples of tissue survival rather than cell culture. Lynn and Morgan (1954) reviewed the reports of the investigative work that included detailed examination of cells infected with animal viruses in vitro and in which there was definite visible changes other than mere alteration of internal structure or inclusion body formation. The viruses were grouped as those having marked, moderate, or no cytopathogenic activity.

Enders (1954), in his more extensive review proposed as a broader and more generally accepted definition of
cytopathogenicity "the capacity to induce any demonstrable departure from the normal either in the morphological or functional properties of cells."

In order for cells in culture to attain their ultimate value in virus studies it is necessary that they exhibit some visible manifestation of virus growth. The changes are usually referred to as the cytopathogenic effect (CPE). The classical work of Weller, Anders, Robbins, and Stoddard (1953) on the multiplication and cytopathogenicity of polio virus in cultures of human cells popularized this point and began a new era in virology. When the recognition of virus multiplication can be made directly in vitro, the more expensive and time consuming and sometimes impossible work in experimental animals is unnecessary. This work also stimulated renewed interest in the cytopathology of viruses. Recently a symposium has been devoted to this subject (Love, 1959).

Early attempts at quantitating the effects of a virus employed the number of cells destroyed over a period of time. Fogh (1955) clearly defined the variations that occur depending upon the titre of viruses and more correctly, the factors such as multiplicity of exposure and adsorption.

The methodology of study of virus infected cell cultures has developed considerably over the past few years. The present refinements were made possible by the development of trypsinization and stationary culture techniques.
One of the most significant developments was the use of the "flying coverslip" which was a considerable improvement over the collodion stripping technique.

Various authors have followed the sequential cytological changes occurring in cells in culture following exposure to virus. The human enteric viruses in different cell culture systems, mainly monkey kidney and Hela cells have been studied in the greatest detail. Shaver, Barron, and Karzon (1958) have reviewed the work on these viruses and compared them in further work on monkey kidney cells. Fewer studies have been reported for animal viruses in tissue culture. These include the work of Leader (1958) on infectious canine hepatitis in dog kidney cells and the report of Lanrek and Wesslen (1955) on the changes induced in bovine cells by the swine enzootic pneumonia virus. In the specific area of swine cells Mussey (1957) used porcine kidney cells and studied the effects of Teschen's virus by means of fluorescent antibody.

Pseudorabies virus is pathogenic for most species of animals and its most produces a disease which is characteristic of the name, pseudorabies or "mad itch." In swine the disease is more moderate and rarely produces a high mortality. Hurst (1933) described in detail the pathological lesions induced in a number of animal species. He was unable to demonstrate the intranuclear inclusions in the histopathological study of the swine lesions although they
were apparent in other animals. However, Shahan, Knudson, Seibold, and Dale (1947) demonstrated their presence in experimentally infected pigs and Done (1957) reported their presence in the natural disease.

Pseudorabies virus has been successfully propagated in a number of cell culture systems and in most instances produces inclusion bodies and destruction of the cells, Traub (1933), in his original work, reported acidophilic intranuclear inclusions in 60 per cent of the cultures of minced rabbit testicle suspended in rabbit serum and Tyrode's solution. Although he demonstrated the multiplication of the virus in cultures of chick embryo and guinea pig tissues, the cultures were not examined histologically.

Scherer (1953) studied the type A intranuclear inclusion produced in the L Strain of mammalian fibroblasts, and Scherer and Syverton (1954) reported similar cytopathogenicity for the HeLa cell.

Takuman (1956) reported on the cytopathogenic pattern produced by pseudorabies on monkey kidney cells. By selective passage at limiting dilutions, he was able to separate two strains of virus from his sample, one of which produced rounding and granulation as described by Scherer and Syvertont in HeLa cells, and one which produced primarily giant cells. Intranuclear inclusions were observed in both types.

Christensen (1955) reported on the general CPE of pseudorabies on swine cells in culture and recently Switzer
(1959) reported on the changes observed following infection of various swine cell cultures with this virus and stained with Giemsa. He reported that the cells were greatly enlarged and the nuclei swollen. No mention was made of internal changes in the cell. He observed giant cells with 4-12 nuclei. Beran (1958), in his report on a field outbreak of the disease, described the cytopathology in swine kidney cell cultures. In the infected cells stained with H and E, he demonstrated large chromophobic, irregular, intranuclear inclusions.

Infectious canine hepatitis is a virus disease of dogs. In the uncomplicated clinical form, it is characterized by a high morbidity and low mortality. Specific intranuclear inclusions are formed and serve as a diagnostic aid. Although the virus has a limited host range, it has been propagated in dog kidney cell cultures by Cabasso, Stebbins, Norton, and Cox (1954), Fieldsteel and Emery (1954) and Muller and Thordal-Christensen (1954). Other workers have propagated the virus in swine kidney cells (Hancock, 1957, Emery and York, 1957).

Recently, the specific cytopathology for cell cultures has been more fully described. Leader (1958) infected coverslip cultures of dog kidney cells and removed the slips at intervals for routine staining. He reported the typical basophilic intranuclear inclusion similar to that seen in the natural disease. Estimates of virus production showed
that virus release increased as the percentage of infected cells increased.

Carmichael (1959) studied a similar system by means of cytochemical methods. He found that the nuclear changes were accompanied by quantitative changes in the nucleic acids which closely parallels the in vivo response described by other investigators. Maximum virus concentration is obtained at the time of the appearance of mature inclusions. This effect would be barely discernable on unstained cells.

The pox viruses are a related group which cause similar diseases in many species of animals. Swine appear to be susceptible to two strains of pox virus. One is the vaccinia virus, and the other is an immunologically distinct virus infectious only for swine. Both viruses have been isolated from clinical cases in swine.

Many of the pox viruses have been extensively studied in cell cultures, but prior to the work of Kasza, Bohl, and Jones (1960), the true swine pox virus had not been propagated in cell culture. These workers isolated a virus from a field case of swine pox, identified it as true swine pox, and reproduced the disease in susceptible pigs by inoculation of the culture fluid from infected swine kidney cells. Preliminary observations were made on the specific cytopathogenic effects of the virus revealed intranuclear "vacuoles" as the outstanding cytological feature.

The wide-spread use of cell cultures in virological
research as resulted in the discovery of many previously unrecognized viruses. The intestinal tract of man has yielded many viruses which are not as yet associated with any disease syndrome. These have been designated enteric cytopathogenic human orphan (ECHO). Similar "orphan" enteroviruses have been recovered from other species (Kalter, 1960). Moscovici, Ginevri, and Mazzarachio (1956), Beran, Nerder, and Wanner (1957), and Hancock (1957) have reported the isolation of enteroviruses from swine. The swine enteroviruses isolated in our laboratory have been tentatively designated as enteric cytopathogenic porcine orphan (ECPO), and five immunologically distinct isolates were considered prototype strains with numbers assigned in sequence following the precedent established for the ECHO viruses (Bohl, Sirgh, Hancock, and Kasza, 1960).

Pneumonia is one of the most important conditions affecting young swine. In some cases, it assumes a serious form resulting in high death losses. Many times, however, it is present in a more insidious form, resulting in a chronic debilitating condition which leads to unthrifty pigs commonly referred to as "lungy" pigs. It is possible that this form results in a greater economic loss than the more dramatic fatal form. Also, in times of stress, secondary invaders can manifest their activity and lead to severe losses in herds chronically infected with pneumonia.

The etiology of swine pneumonias presents a confusing
picture. There are many agents which produce this syndrome acting alone or, more commonly, in combination. The classification of respiratory diseases of swine proposed by Whittlestone (1957) in his excellent review is of value in clarifying the overall picture. It is as follows:

I. Primary diseases of known etiology
   a. swine influenza
   b. lungworm infection
   c. aspiration pneumonia
   d. acute bacterial pneumonia

II. Primary diseases of uncertain etiology
   a. virus pneumonia
   b. rhinitis

III. Diseases secondarily affecting the respiratory system
   a. hog cholera
   b. Ascaris lumbricoides infection

It is apparent from a review of this field that there are primary pneumonias of swine caused by viruses other than the swine influenza virus.

In summary, the situation regarding viral pneumonias of swine is as follows: influenza virus causes a specific type of pneumonia. Separate and distinct from this condition, there is a clinical syndrome of high morbidity and low mortality which is referred to by a number of names and which is caused by a virus or viruses. This condition is of
considerable economic importance in many countries.

In the confusing picture which is presented, terminology becomes of paramount importance. Virus pneumonias are referred to by many different terms. In most instances, these syndromes have been differentiated from swine influenza at least on clinical grounds. Fett's (1952) used the term "Virus Pneumonia in Pigs" (VPP) for the disease described by Gulrajani and Beveridge (1951). This is the term generally applied to the condition.

The information regarding the cell culture propagation of the virus of VPP is contradictory. Wassslin and Lannek (1954) reported the isolation of a cytopathogenic agent from six of eight clinical cases in cultures of porcine lung and kidney from day old pigs. The agent also produced a similar CPE in embryonic human lung tissue and embryonic skin and lung tissue. In a subsequent paper, Lannek and Wassslin (1955), reported on the histological examination of bovine embryonic skin cells infected with one of the agents. On the other hand Hjarre, Dinter and Bakos (1954) were unable to confirm these findings.

Switzer (1959) was not successful in producing a CPE in his cultures following inoculation with a known agent which produces virus pneumonia.
**Experimental**

**Pseudorabies**

The pseudorabies virus was originally obtained from the American Type Culture Collection. Cell culture fluid from the 16th to 18th passage in swine kidney cells was used as inoculum. Prior to inoculation, the media was removed from the tubes and the cells were washed two times with Hanks' solution. The inoculum varied slightly in infectivity in the several series, but in each instance 1 ml was added to each tube. As Ressig, Howes, and Melnick (1959) have pointed out, in such a study it is desirable to infect as many cells as possible (ideally 100 per cent) in as short a period of time as possible. Therefore, the titre was maintained at a high level in the inoculum. An equal number of control cultures received only Hanks' solution during which time they were gently agitation several times. The tubes were stoppered and set one hour at room temperature. The fluid was then poured off, the cells washed, and fresh maintenance medium was added.

At varying periods of time thereafter, usually one hour intervals, virus infected slips and control slips were removed and transferred to fixative. The fluids were frozen and saved for virus assay. Titrations were performed in tubes in the standard manner or in bottles by the plaque technique as described by Singh, Bohl, and Birkeland (1959).

It was the practice in this study to include a con-
trol slip with each virus infected slip. They were carried through the staining procedures together and in most instances mounted side by side on a single microscopic slide.

Pseudorabies virus produces a dramatic and rapid destruction of the tissue culture swine kidney cells (Figures 7, 8, 9). Because of this rapid destruction and since the virus produces intranuclear inclusions in vivo and in vitro, a detailed sequential study of the cytological changes in tissue culture was attempted. Such a study would also serve as a control for other viruses in porcine kidney cells.

A number of experiments were run in which a group of cultures were inoculated with pseudorabies virus and examined at various times afterwards. For purposes of discussion the changes that occur in the cell have been divided into five phases. It is emphasized that there is not a clear cut distinction between each phase.

Phase 1. The first observable change was a gradual clumping of the chromatin. Some cells showed an extensive vacuolization of the cytoplasm. Irregular eosinophilic masses slightly larger than the nucleoli were observed in a few cells.

Phase 2. This stage was characterized by a swelling of the nucleus with clumped chromatin along fine strands of Feulgen positive material. This gave the chromatin a stringy, beaded appearance. In many of these cells, there was a dense, slightly eosinophilic paranuclear mass.
Phase 3. The nuclei of these cells showed many varied and bizarre shapes with a large eosinophilic Feulgen negative mass almost completely filling the nucleus and following the very irregular outline of the nuclear membrane resulting in the margination of the chromatin. The cells still retained the paranuclear mass and in many instances the nucleus was indented in this area.

Phase 4. The nuclei were reduced to approximately normal size and again assumed a rounded appearance. Large inclusions were still present in the nuclei with the resulting margination of the chromatin material.

Phase 5. The nuclei were more pyknotic and more chromophilic. The chromatin was marginated by a central mass. Very little cytoplasm remained and it was very irregular in outline with the majority in the paranuclear eosinophilic mass.

The pseudorabies virus caused very rapid destruction of the swine kidney cells. The exact time varied with the titre of the inoculum. When a maximum number of infective particles were used, the cells may be completely destroyed in approximately 10 hours. In one series inoculated with $6 \times 10^6$ pfu per tube, the process was as follows: As early as 2 hours after inoculation cells showing some of the changes described for phase 1 were present. By 3 hours, a large portion of the cells had progressed to phase 1 and some of the cells were showing the swelling characteristic
Figure 7. Pseudorabies virus-infected cells. X 1000. H and E stain. Note the paranuclear mass (a) and the intranuclear inclusion (b).
Figure 8. Pseudorabies virus-infected cells in early phase of degeneration. X 1000. H and E stain.
Figure 9. Pseudorabies virus-infected cells. X 1000. H and E stain. The late phase of degeneration shows the clumping of the cells with intranuclear inclusions.
of phase 2... An occasional cell would show the bizarre nucleus seen in phase 3 degeneration. After 4 hours, some cells had progressed to the later phases and there were indications that some had fallen from the glass surface. There was corresponding increases in the numbers of cells in the other phases of destruction.

By 5 hours, few normal cells remained on the glass surface. The remainder of the cells were showing varying degrees of degeneration, with the majority showing the changes characteristic of phases 3 and 4. The changes continued to develop until most of the cells were detached from the glass at 8-9 hours.

Titrations of the culture fluid (Figure 10) showed that there was an increase in free virus as early as the first hour after washing. The concentration remained relatively stable throughout the period of observed degeneration. Since the titre during this period is considerably lower than expected, it must be assumed that many of the cells dropped from the glass surface before complete destruction and liberation of virus.

In outlining the changes that occur, no mention has been made of giant cell formation. In many instances, nuclei in the latter stages of virus infection have been clumped together and at this stage they have little distinct cytoplasm; however, it was impossible to say conclusively whether these were giant, multinucleated cells. They did not appear to be.
Figure 10. Titrations of fluids from coverslip cultures infected with pseudorabies virus.

(1) Original inoculum. (2) Inoculum after adsorption period.
(3) HS from third wash. (4) HS from sixth wash.
(5) Medium from infected cultures.
In the work of Takumaru (1956), it appeared that he was able to demonstrate two strains of virus which showed varying types of cytopathogenicity. One strain was characterized by giant cell formation. However, the variation was one of degree since he stated that it was not possible to separate them "sufficiently cleanly to test them for genotypic characteristics." Switzer (1959) has also described giant cells in his Giemsa stains of pseudorabies infected cells. In the preparations examined in this study, it was impossible to demonstrate definite giant cell formation and there seemed to be only a single type of cytopathogenicity. It is logical to assume that the author was working with a "pure strain" of virus since some of the previous passages had been made at limiting dilutions of the virus and other passages were made by "picking" a plaque from an agar overlay bottle. Therefore, it appears that this strain produced few if any giant cells.

The vacuoles observed cannot be attributed directly to the virus since they were observed in many of the control cells, but it is possible that the vacuoles are a transitory effect as Reissig, Howes, and Melnick (1956) have reported for monkey kidney cells infected with polio virus. Lwoff, Dulbecco, Vogt, and Lwoff (1955) suggested that these vacuoles play an important role in the release of polio virus.

At times in the pseudorabies virus infected slips, as
well as those infected with other viruses, it was possible to observe cells in mitotic division adjacent to dead and dying virus infected cells. It seems improbable that they could have escaped infection. Evidently this is not an unusual situation, since Coffin (1959) has described cells with typical canine distemper inclusion bodies undergoing division. Bang (1959) has taken the other view. He points out that the effects of virus infection on the cellular changes occurring during mitosis are almost unknown because the virus kills the cell before mitosis occurs.

The in _vitro_ changes described for pseudorabies are not typical of the natural disease. The cytopathology of a virus in tissue culture may be similar to that found in the natural host and again it may not. As Cheatham (1959) has pointed out, if they are similar, this may provide evidence that a given disease is caused by the agent isolated, and may explain in part the pathology and pathogenesis of the disease in the host. The disimilarities may, in the future, prove of greater value in basic virology. These may provide the clues and evidence for the fundamental relationship between virus and cell.

**Infectious Canine Hepatitis**

The infectious canine hepatitis (ICH) virus was a cell culture adapted strain which had been carried through sixteen passages in dog kidney cells and twelve passages in
the swine kidney cells (Hancock, Bohl, and Birkeland, 1959). In general, the same experimental procedure as employed for pseudorabies was followed. Virus titrations were not preformed and less attention was given to employing a high concentration of virus particles for inoculation. The slips were removed at approximately four-hour intervals, and the total time required was usually about 36 hours in the several series.

The outstanding characteristics of the cytopathology were the nuclear changes (Figure 11-14). Early in the degenerative process, multiple intranuclear inclusions, as many as eight to ten, were observed. In cells stained by H and E, these appeared as eosinophilic bodies, two or three micron in size, with a lighter staining center. In preparations stained by other methods, there seemed to be a definite limiting membrane to these bodies, and when stained by the Feulgen technique, the surface of the inclusion gave a positive reaction for DNA.

In other cells, which were possibly showing more advanced changes, the chromatin had formed a fine network linking the inclusion together and to the nuclear membrane. Enmeshed in this network of chromatin and inclusions, many cells contained large numbers of vacuoles which did not stain by any of the techniques employed, including the fat stain.

The degenerative process seemed to follow a pattern
Figure 11. ICH virus-infected cells. X 1000. H and E (R) stain. Immature inclusions (a) and vacuoles (b) may be seen in many of the cells.
Figure 12. ICH virus-infected cells. X 1000. Giemsa stain.
Figure 13. ICH virus-infected cells. X 1000. Heidenhains iron hematoxylin stain. The light staining immature intranuclear inclusions and vacuoles may be seen in many cells. Several mature inclusions are present.
Figure 14. ICH virus-infected cells. X 1000. Feulgen stain.
of gradual fusion of the small inclusions into only a few larger bodies. The internal detail of these structures could be observed by iron hematoxylin stain and it appeared that the original small inclusions had decreased in size but retained their light staining center thus giving the structure a foamy appearance. It is at approximately this stage that the cytoplasm degenerates.

The maturation of the inclusion seems to be brought about by the migration of the vacuoles to the periphery of the nucleus. The mature inclusion is seen in only a few cells and is a single large basophilic body completely surrounded by vacuoles which separate it from a heavy nuclear membrane. The nucleoli still remain in some of the cells at this stage and are adjacent to the nuclear membrane. The cells then become more pyknotic and soon fall from the glass surface.

In general, these changes are similar to the cytopathology described and illustrated for the dog kidney cells infected with ICH. However, the percentage of cells containing mature inclusions is much less in the swine kidney cells. This could be due to cells falling from the glass surface before complete degeneration. The mature inclusion is almost identical with the characteristic inclusion which is seen in vivo and which serves as a means of diagnosis.
Swine Pox

The swine pox virus employed was the strain isolated by Kasza, Bohl, and Jones (1960). Tissue culture fluid from the sixth passage in swine kidney cells was used as a source of inoculum. The inoculation procedure was similar to that used for pseudorabies virus. The cytopathogenic effect was much slower with this virus, and the coverslips were removed at 12 to 24 hour intervals.

Significant changes were not observed in the cells until 24-48 hours after inoculation. The nuclear changes began with the formation of one or more non-staining vacuoles (Figure 15). These seemed to enlarge gradually and coalesce until there was only a single vacuole filling the nucleolus and forcing the chromatin and nucleoli to the margin. At this point, the vacuoles seemed to disappear and many nuclei were observed with a punctured, collapsed, or "deflated" appearance. The chromatin was organized into numerous small round masses. The nucleoli were still present. As the nuclear membrane contracted, the chromatin formed into fewer and larger masses until finally there was only the typical basophilic, amorphous nuclei remaining.

The cytoplasm begins to degenerate at about the time the vacuoles disappear. It becomes condensed and more eosinophilic. In the later stages, one or more round homogenous, eosinophilic inclusion may be seen. These are
Figure 15. Swine Pox virus-infected cells. X 1000. H and E stain. Cells show the non-staining intranuclear vacuoles.
approximately 2 microns in size with a definite halo, a light staining center, and Feulgen negative.

The *in vitro* cytopathology of swine pox virus cannot be compared with the natural disease since there are no good descriptions of the specific cellular lesions available. The typical cytoplasmic inclusion (Guarnieri or Bollinger body) which is observed in most pox lesions was not seen in any of the cells examined in this study.

**Enteroviruses of Swine**

A study was undertaken to compare the cytopathology of the Enteric Cytopathogenic Porcine Orphan (ECPO) viruses isolated in this laboratory to further characterize these agents. The origins, isolation, and characteristics of the five prototype strains have recently been summarized (Bohl, Singh, Hancock, and Kasza, 1960).

Throughout the earlier work on the effect of these viruses on swine kidney cells, stained preparations of infected cells on coverslips were examined at infrequent intervals. For the most part, these were stained with the May-Grunwald-Geimsa technique. This stain is excellent for the observation of healthy cells, but cells in the later stages of destruction and degeneration are too heavily stained, making detailed studies of internal structure difficult, if not impossible. The changes observed were not specific enough to enable one to make an identification on this basis alone. However, it was thought that in
some instances changes in the cells could be visualized by other stains that might serve to further characterize the various viruses. In addition, this type of information is of value in studying the virus-host cell relationship.

In general, the same methods as employed for the study of other viruses were used for the ECP0 viruses. In each instance an attempt was made to infect a majority of the cells at one time. The timing for the removal of the virus infected slips obviously had to be adjusted for the various agents. In all instances, control slips were fixed and stained along with the virus infected slips.

ECP0-1, formerly designated 6-6, produced changes which were characteristic of several of the enteric viruses studied. A single basophilic inclusion was present in the nucleus of degenerating cells throughout the process of degeneration. This structure was slightly larger than a nucleolus or approximately three microns in size and irregular in outline. There was no distinct halo although the nuclear matrix appeared less dense adjacent to the periphery of the body. It is possible that this structure is the remains of nucleoli since it is similar in staining characteristics, is Feulgen negative and since there are no nucleoli present in the cells at this stage. If so, there was a definite alteration and fusion since there were never more than one such inclusion observed in a cell where-
as the normal cells usually contained more than one nucleolus. As the cytoplasm degenerated and the cell became more and more pyknotic, the nuclear membrane became heavier and wrinkled with the margination of the chromatin. In the last stages of degeneration, the nucleus is quite small, about two to three microns. The chromatin is formed into several smooth rounded clumps surrounding the inclusion, which by this time is only faintly stained. It is still Feulgen negative.

There are two main features to the cytoplasmic changes. After becoming slightly denser, the cytoplasm seems to break off of the cell in clumps or balls. It gives the cell the appearance of "bubbling" (Figure 16). At times these balls are observed attached by a thin stalk. Feulgen positive material is sometimes present intermixed with the clumps of cytoplasm. A thin layer of cytoplasm remains around the nucleus.

The overall size of the cell then appears to remain constant even though the nucleus continues to shrink. It is at this stage that the second feature appears—a round, homogenous, faintly eosinophilic inclusion with a definite halo. In many cells, it is only discernable as a lighter area of the cytoplasm, but in other cells, particularly those stained by the Reissig modification of H and E stain, it is a definite red.
Figure 16. BCP-1 virus-infected cells. X 1000. May-Grupwald-Giemsa stain. Note the "bubbling" cytoplasm in the degenerating cell.
The cytopathology of iCPO-2, formerly designated 11-56b, differed markedly from iCPO-1 (Figure 17). The earlier nuclear changes were a gradual margination of the chromatin material and the formation of an eosinophilic, Feulgen negative inclusion which persisted throughout the degeneration. In some cells, there appeared to be several smaller inclusions but they evidently coalesced since cells in the later stages of degeneration never contained more than one inclusion. Concurrently with the formation of the inclusion, the nucleoli disappeared, and the nuclear membrane appeared heavier due to the increasing margination of the chromatin. In some cells, the nucleus appeared empty of all material except the inclusion. The nucleus became more and more pyknotic. Evidently this was a gradual process since the nuclear membrane was smooth and unwrinkled. As the membrane contracted it took on a beaded appearance from the enlarging clumps of Feulgen positive material. The final appearance was of three to five clumps of DNA packed around the Feulgen negative inclusion.

The cytoplasmic changes were characterized by a gradual contraction as indicated by the reduction in size and increase in density. During the later stages, an inclusion identical with that observed in iCPO-1 was present in the cytoplasm. This was only distinct in H and E preparations although a faintly stained area could be detected in the other stains. The inclusion was Feulgen negative.
Figure 17. ECPO-2 virus-infected cells. X 1000. 
H and E stain. The intranuclear inclusion may be seen in the small degenerating cell in the center of the field.
The cytopathic changes caused by ECPO-3 virus, formerly designated number 7, closely resembled the changes caused by ECPO-2 and differed mainly in degree (Figure 18). The nucleus seemed to shrink more in the earlier stages of degeneration so that the nuclei containing the inclusions were already reduced to about one-third normal size or about eight microns, and the large "empty" nuclei containing the inclusion were not seen. The degeneration seemed to progress further and the nuclear clumps of DNA continued to fuse until some cells were observed which contained only a single clump of DNA which entirely filled the nucleus. The cytoplasmic eosinophilic inclusion was more prominent in this series than in the cells infected with ECPO-2 and it appeared to be slightly larger or about three microns.

The cytopathology of ECPO-4 virus varied in several respects from the other viruses studied (Figure 19). The first observable nuclear change was a clumping of the chromatin material into many fine bodies smaller than those described for the other viruses. There was a margination of many of these giving the nuclear membrane a heavy, finely, beaded, wrinkled, appearance. The nucleoli were still present at this stage. Many nuclei were showing abnormal shapes and some appeared to be swollen. The nucleus then became progressively pyknotic and there was a fusion of the chromatin material into larger bodies. In some cells,
Figure 18. ECPO-3 virus-infected cells. X 1000. H and E (R) stain. The eosinophilic cytoplasmic inclusion typical of the ECPO viruses may be seen in this field.
Figure 19. ECPO-4 virus-infected cells. X 1000. May-Grünwald-Giemsa stain.
a large number of bodies two microns in size were scattered throughout while in other cells, only three or four were present. In addition there was a central, slightly basophilic inclusion body present in both types of cells. The nucleus eventually became a small mass (about three microns) of closely packed DNA bodies.

The cytoplasmic changes began with an increase eosinophilia. In many of the cells with distorted nuclei, a particularly dense paranuclear mass was observed. As the nuclei contracted, the eosinophilic inclusion appeared as with the other LCPO viruses and persisted throughout. During this time the cytoplasm was fragmenting and contracting. Particles appeared to break off although not as dramatically as for LCPO-1. Narrow cytoplasmic processes and strands protruded from many cells and sometimes were joined to adjacent cells.

The cytopathology of LCPO-5 virus was almost identical to LCPO-1. The cytoplasm rapidly degenerated and became granular and lobulated. Because of this early degeneration, the cell rapidly lost its attachment to the glass surface. A heavy basophilic body was observed in one portion of the cell. This could have been the nucleoli coated with chromatin. In a majority of these cells, there was also a round homogenous eosinophilic inclusion body (2.3 u) present in the cytoplasm.
On comparing the five prototype strains of the ECPO viruses, it was observed that ECPO-1 and ECPO-5 produced identical cytopathology. It is interesting to note that these viruses produce an identical type of plaque and are antigenically related (Bohl, Singh, Hancock, and Kasza, 1960). The remaining antigenically distinct viruses produced distinguishable cytopathic changes.

One of the advantages of detailed microscopic studies of suitable preparations is the detection of viral-induced changes which are not visible by the usual methods of observation. In some cases this would provide a means of studying the virus. An illustration of this would be the case of the changes detected by phase microscopy of cells infected with hog cholera virus. It was felt that the techniques as utilized in this work should be tested on a virus system which does not give a distinct CPE.

Attempts were made to detect changes in swine kidney cells following exposure to TGE virus since this has been reported to grow in tissue cultures of swine kidney cells without a distinct CPE (Lee, 1956). No changes were observed. Further studies in this laboratory by Kasza and Bohl (1959) failed to confirm the findings of Lee. Fortunately, an unknown agent was isolated from a pneumatic pig lung which seemed to fulfill the requirements.

In the early summer of 1957, a lung was obtained from a pig which exhibited lesions typical of virus pneumonia.
The pig was from a herd of swine which were experiencing respiratory trouble, which, in the opinion of one of the ambulatory clinicians of the College of Veterinary Medicine, was not swine influenza. Bacteriological studies of the tissue failed to reveal any bacteria of significance.

The lung was ground in a mortar and pestle to a 10 per cent suspension in Hanks' solution with the aid of sterile sard. The resulting suspension was treated with antibiotics (penicillin, streptomycin, mycostatin) and centrifuged. The supernatant was inoculated into tubes of swine kidney cells.

The first passage of the lung material showed considerable toxicity. The tissue culture fluid was re inoculated into new tissue cultures. After 4 to 5 days of incubation, a rather indefinite CPE was observed. The agent was repeatedly passed in tissue cultures with variable results. At times, there would be an almost complete destruction of the cells and at other times, there would be virtually no effect. Other series were initiated from the original lung with identical results. The variation appeared to be due entirely to the particular lot of cells rather than a variation in titre of the different passages. This was borne out by the simultaneous inoculation of a given lot of cells with several passages of the virus. Serum was eliminated from the media to rule out the possibility of antibody neutralization. Obviously, any detailed
work with the virus in such a system was impossible.

After repeated attempts to produce a dramatic and consistent CPE in tissue cultures, attempts were made to propagate the agent in the chick embryo. Six day embryos were inoculated by injecting 0.25 ml. of tissue culture fluid into the yolk sac. Four of six eggs were dead at four days, and the remainder died on the sixth post-inoculation day. The agent was passed by means of a 20 per cent yolk sac suspension in Hanks' solution. It consistently produced death in 5-7 day embryos. The transfer from cell cultures to eggs has been repeated. No gross abnormalities were observed other than a thickening of the chorioallantoic membrane in an occasional embryo. No consistent deaths were observed in eggs inoculated by other routes. Attempts to demonstrate PPLO by Glemsa stain and inclusion bodies by Macchiavello's method were unsuccessful. Routine cultural procedures for anaerobic and aerobic bacteria and PPLO failed to show growth.

Hemagglutination tests with washed chicken erythrocytes were negative. Infant mice, one day old, inoculated intracerebrally, subcutaneously, or intraperitoneally showed no clinical signs. Attempts at producing plaques on swine kidney monolayers under agar were unsuccessful.

A group of coverslip cultures were inoculated in the usual fashion with culture fluid from the eighth tissue
culture passage of the agent. Slips were removed at five and eight days for staining. The original inoculum and the culture fluid from the coverslips removed on the eighth day were inoculated into six day embryos to confirm the presence of the agent. These slides were then used to confirm several previous series of slips that had been stained.

It was hoped that some specific change could be observed that would serve as a definite indication of virus' multiplication but such was not the case. The inoculated cells were destroyed at a more rapid rate that the controls but the changes observed were not specific. The cells showed a clumping of the chromatin and a gradual condensation of the cytoplasm resulting in a gradual pyknosis of the cell (Figure 20). The eosinophilic cytoplasmic inclusion observed in the studies of the ECO viruses was present in many of the cells, but was not nearly as prominent.
Figure 20. Cells inoculated with the virus-like agent from pig lung. X 1000. H and E stain.
Discussion

Detailed study of the cytopathology of virus infected cell cultures may have three objectives. First, to further characterize and identify a virus; second, to provide a means of recognizing virus multiplication when no CPE is observed on unstained cultures; and third, to study the virus-cell relationship.

For the positive identification of a virus, it is necessary to use neutralization tests with specific antisera. Since such tests are expensive and time consuming, it is more practical to obtain some clue to the identity of the virus and then run only a few such tests for confirmation. The original source, resistance to physical and chemical agents, and the experimental host range including cell cultures are of value in the preliminary identification. Additional information may be obtained if the symptomatology of the experimental host or the cytopathology of the cell cultures is characteristic. In the present study, a number of viruses cytopathogenic for swine kidney cell cultures produced distinctive changes that are of value in this regard. The intranuclear inclusions produced by ICH, pseudorabies, and swine pox viruses are sufficiently characteristic to provide presumptive identification and to differentiate them from the ECPO viruses.
The cytopathology of the prototype strains of the individual ECPO viruses was more subtle and less characteristic. ECPO-1 and ECPO-5 produced, for practical purposes, an identical pattern of changes. ECPO-2 and ECPO-3 produced changes which were so similar that it would be difficult to separate these two but they could be distinguished from the previous two agents. Similarly, it was felt that the cytopathology of ECPO-4 was distinctive. Obviously, it will be necessary to examine other isolates of these agents before definite conclusions may be reached.

An attempt was made in this study to demonstrate the value of cytological techniques for detecting viral changes which are not visible in the unstained cell. It was unsuccessful. The agent chosen was an unknown agent from a pneumonic swine lung. Studies indicate that the agent is a virus although the possibility that it is a PPLO must still be considered. These organisms are at times difficult to culture and our techniques may have been inadequate.

Cell cultures provide an excellent system in which to study the cell-virus relationship. The general advantages include a reproducibility of results, quantitation of the "reactants," the virus and the cells, and the control of the physical and chemical environment. One of the main advantages is the number of different experimental techniques that may be used to examine the system. The classical histological procedures as used in this study serve to de-
lineate the gross changes occurring in the virus infected cell.

The most characteristic and dramatic reaction of cells to virus invasion is the formation of an inclusion body. These structures have given virologists some recognizable manifestations of viruses since the birth of this science. Some of the inclusions were known before the discovery of viruses. The molluscum body discovered in 1841 and the Bollinger body of fowlpox discovered in 1873 were long considered to be protozoa or fungi. In spite of the long continued interest in these structures, inclusions have been observed in only about one half of the known viral infections of man and lower animals (Pinkerton, 1959).

A feature of inclusion bodies which does not receive the proper emphasis is their dynamic state. In general, one may assume that they are the result of an altered metabolism of the cell. As a result they are not formed instantaneously but are laid down over a variable period. During this process, they may exhibit variations in morphology and staining characteristics. In view of the fact that these properties may vary with the fixatives and staining techniques used, it is surprising that some have been characteristic enough to provide the basis of histopathological diagnosis of diseases.

The question as to whether or not a given inclusion contains the active virus has intrigued many investigators.
Yet with only a few viruses do we have what approaches a complete answer. Liu (1959) has reviewed this point from the standpoint of canine distemper, infectious canine hepatitis, herpes, and rabies viruses and pointed out the value of the fluorescent antibody technique in determining the viral nature of inclusions. It is interesting to compare the variation shown by these agents. With ICH virus, the intranuclear inclusions contain a high concentration of the viral antigen. In distemper, the antigen is found in the eosinophilic inclusions visible by Seller's stain (faint grey-blue in Shorr's stain) but not in the reddish bodies of the cells stained with Shorr's stain. In herpes, the antigen is present during the early stage of development, is released, and leaves behind the eosinophilic inclusion as a cytological scar.

An inclusion body may be loosely defined as any discrete abnormal structure within a cell. It may be that this general definition is the most meaningful one. Certainly it should be the aim of quantitative virology to specifically define such structures in terms of their metabolic synthesis and so relate them to the virus replication. This, however, remains a goal and not an accomplishment.

Although the techniques employed in this study lay a necessary foundation and understanding of the cellular changes, they do not materially contribute to the finer
understanding of the events in the process of virus replication. Newer techniques offer much more promise in this regard.

After examining a system by means of polychrome stains, one may proceed to more elaborate procedures such as cytochemical stains. The essential principle of all cytochemical methods is the application of a specific chemical test to the cell. The localization of the reaction product is ascertained under the microscope. Prachet (1957) has pointed out the two absolute prerequisites for a valuable cytochemical technique. First, the reaction must be specific and second, the localization of the reaction product must remain unchanged. Two of the most widely used are the Feulgen reaction for DNA and the methyl green-pyronine method for RNA. These fulfill the requirements but they are not without their disadvantages for virus studies. The latter method is difficult to standardize, and in both, the reacting particles may be so small as to be invisible if they are dispersed rather than concentrated.

The use of the fluorescence microscope offers considerable promise in the study of virus infected cells. The image is formed by the emission of light from the specimen itself and detection depends upon the intensity of light emitted. Thus an object, stained with a fluorescent dye, at the limits of, or even beyond, resolution with transmitted light may be detected. One of the most valuable of
the fluorochromes is 2.8 bis-dimethylaminoacridine, or acridine orange. This dye may be used as a vital stain and it produces a differential polychromatic fluorescence with different tissue elements, particularly the nucleic acids. A recent review by Anderson, Armstrong, and Niven (1959) summarizes the progress in this field.

Fluorescent techniques employing antibody coupling can yield much information on the location of viral antigens within the cell. Problems of resolution will most probably limit the usefulness in following the replication process. It seems more likely that their greatest value will be in the diagnostic field, where all that is necessary is to locate a particular homologous antigen in a cell. A considerable body of developmental work must be completed in each instance to adequately control the preparations.

One other technique in the broad field of cytochemistry is autoradiography. Isotopes such as tritium in DNA precursors such as thymidine may be added to the culture medium. After a suitable period of time an autoradiograph will show the specific areas of radiation due to the newly formed labeled DNA. An additional advantage is that the cells may be subjected to other staining procedures without interfering with the results.

The examination of ultrathin sections of virus in-
fected cell cultures offers a means of extending our limits of resolution and visualizing the development of virus particles. This is not without its disadvantages since hundreds of sections may be required for the examination of a single cell. And of course, to follow the replication process adequately, it is necessary to examine hundreds of cells.

Two major aims of quantitative virology are the definition of specific intracellular lesions from a morphological and functional standpoint, and concurrently, the elucidation of the replication process in physical and chemical terms. The use of cell virus systems as described in this work and studied by the many and varied techniques available will be of significant value in the attainment of these goals.
SUMMARY

Trypsinized swine kidney cells were cultivated on 10 x 50 mm coverslips in Leighton test tubes. Standard histological and histochemical staining procedures were adapted and standardized for use on these coverslip cultures. Microscopic studies were made of these cells throughout the growth and maintenance periods and when subjected to various physical and chemical conditions likely to be deleterious to the cells. Major consideration was given to structures likely to be altered by virus infection.

The cytopathology produced by several viruses was studied. Attempts were made to follow the changes produced by removing the coverslip cultures at intervals after virus infection. Pseudorabies virus was studied most intensively and the cytopathic changes were followed and correlated with release of newly formed infectious particles. The cytopathology appears to be unique for swine kidney cell cultures and different from that reported for other types of cell cultures and from that seen in vivo.

A recently isolated strain of swine pox virus was found to produce a cytopathic change characterized by a large, chromophobic, intranuclear vacuole. The cytoplasmic inclusion typical of many pox viruses was not seen.

Infectious canine hepatitis virus produced changes
primarily in the nucleus of the cells which were very similar to those reported for dog kidney cells in culture. The characteristic intranuclear inclusion seen in vivo was observed as the mature inclusion in the swine kidney cells.

The cytopathology of a group of swine enteric viruses isolated in our laboratory and designated as Enteric Cytopathogenic Porcine Orphan (ECPO) viruses was studied from the standpoint of further characterizing these agents. Although these viruses produce a similar cytopathology, there were significant differences between some of the serologically distinct strains. The differences could be correlated with the serological pattern as well as with the size and appearance of the plaque formed under agar overlay. These changes could aid in the preliminary identification and characterization of new viruses of this group.

An unknown agent isolated from the respiratory tract of a pig with pneumonia was studied in the hope that a specific cytopathic lesion could be found since this agent could not be detected consistently in unstained cultures. Although the death rate of the cells was increased in virus-infected cultures, no specific pathognomonic lesions could be observed.
APPENDIX

Cytological Techniques for Cell Culture

I. Fixatives

A. Neutral formalin (10%)

Formula:

Formalin A.R. 1 part
Distilled water 9 parts

Add a small amount of sodium carbonate to adjust to pH 7.

Procedure:

Coverslips are usually allowed to remain in fixative overnight then washed 5 minutes in running water; however, fixation times as short as 30 minutes have been used with success.

B. Zenker's

Formula:

Potassium dichromate 2.5 gms
Mercuric chloride 5.0 gms
Distilled water 100 ml

Salts dissolved with gentle heat. Add 5% glacial acetic acid immediately before use.

Procedure:

Zenker's fixative 30-60 min.
Wash in running water several hours (overnight)
Gram's iodine 5 min.
Wash in water 1-2 min.
Sodium thiosulfate few seconds
Wash in running water 10 min.
C. Rossman's

Formula:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute ethanol saturated with</td>
<td>9 parts</td>
</tr>
<tr>
<td>picric acid</td>
<td></td>
</tr>
<tr>
<td>Neutral formalin 10%</td>
<td>1 part</td>
</tr>
</tbody>
</table>

Procedure:

Coverslips remain in fixative overnight at 5°C, then are washed in several changes 70% ethanol and finally running distilled water for 5-10 minutes.

D. Bouin's

Formula:

<table>
<thead>
<tr>
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<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin A.R.</td>
<td>1 part</td>
</tr>
<tr>
<td>Saturated solution picric acid</td>
<td>3 parts</td>
</tr>
</tbody>
</table>

Procedure:

Coverslips remain in fixative 30-60 min. and then are washed in several changes of 70% ethanol.

E. Osmium tetraoxide

Formula:

<table>
<thead>
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<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbital buffer</td>
<td>10 ml</td>
</tr>
<tr>
<td>0.974 sodium acetate (.3H₂O)</td>
<td></td>
</tr>
<tr>
<td>1.4714 sodium barbital</td>
<td></td>
</tr>
<tr>
<td>50 ml dist. water</td>
<td></td>
</tr>
<tr>
<td>HCl N/10</td>
<td>10 ml</td>
</tr>
<tr>
<td>Osmium terraoxide 2%</td>
<td>25 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Procedure:

OsO₄ vapors at room temperature 2 hrs.
Invert coverslip on two glass rods in dish. Add 0.25 ml. 1% buffered $O_3O_4$ to petri dish. Cover dish. Caution: do not inhale vapors!

Distilled water 5 min.
Ethanol 35% 5 min.
Ethanol 50% 5 min.
Ethanol 70% 5 min.

II. Staining Techniques

A. May-Grunwald-Giemsa Stain

Fixation:
1. Hanks' solution at room temp. 15 min.
2. Absolute methyl alcohol 5 min.

Staining Procedure:
1. May-Grunwald stain (sat. sol. in absolute methyl alcohol) 5 min.
2. Giemsa (stock Solution diluted 1:10 with de-ionized water just before use) 7-9 min.
3. Acetone few seconds
4. Acetone few seconds
5. Acetone-xylene (equal parts) few seconds
6. Acetone-xylene (equal parts) few seconds
7. Xylene 5 min.

Coverslips are mounted on clean slides with picolyte.

Results:
This technique stains the Feulgen positive deoxy-
ribonucleoproteins red-purple and the ribonucleoprotein of the cytoplasm blue. The cytoplasm is retracted. This is an excellent stain for normal cells but degenerating cells are overstained to the point of obscuring all interval detail. This problem is partially resolved by reducing the staining time to the times given above. References: Jacobson (1951), Christensen (1955)

B. Hematoxylin - Eosin Stain

Fixation: Zenker's, Bouin's, formalin

Staining Procedure:

1. After fixation, coverslips are transferred to a dish and washed in running distilled water 5 min.
2. Harris hematoxylin 20 min.
3. Water wash - dip to blue nuclei
4. Acid-alcohol - dip to red color (1% HCl in 35% ethanol)
5. Tap water rinse to blue color
6. Ethanol 35% 30 sec.
7. Ethanol 50% 30 sec.
8. Ethanol 70% 30 sec.
9. Ethanol - 95% 30 sec.
10. Eosin (5% in 95% ethanol) 1 min.
11. Ethanol 95% dip
12. Ethanol - absolute 1 min.
13. Xylol 5 min.
Mount in picolyte
Results: cytoplasm light red, nuclei blue
References: Dept. of Veterinary Anatomy, The Ohio State University.

C. Hematoxylin and Eosin Stain - (Reissig modification)

Fixative: Zenker's

Staining Procedure:
1. Harris hematoxylin (5% acetic acid added just before use 20 min.
2. Water wash dip
3. Acid-alcohol dip
4. Distilled water (pH 7 with lithium carbonate) 10-20 min.
5. Water wash 30 min.
6. Eosin Y 0.5% aqueous solution 2 min.
7. Eosin Y 0.5% in 95% etharol 2 min.
8. Ethanol 95% 1 min.
9. Ethanol absolute 1 min.
10. Xylol 5 min.
Mount in picolyte
Results: Certain intranuclear inclusions are more distinct with this stain.
Reference: Reissig, Howes, and Melnick (1958)
D. Heidenhain's Iron-Hematoxylin

Fixation: Zenker's, Bouin's

Staining Procedure:
1. Iron-alum. solution 6 hrs.
2. Wash in running water 10 min.
3. Hematoxylin 0.5% solution overnight
4. Wash in running water 5 min.
5. Iron-alum. 30 min.
6. Wash in running water 1 hr.
7. Saturated solution orange G 2 hrs.
8. Ethanol 95% 30 sec.
9. Ethanol absolute 2 min.
10. Xylol 5 min.

Mount in picolyte

Results: Cytoplasm light orange-brown while nuclear structures are blue-black. Excellent stain for fine nuclear detail.

Reference: Gayer (1953)

E. Trichrome Stain of Cajal for Connective Tissue (Castreviejo Modification):

Fixation: Rossman's, formalin

Staining Procedure:
1. Slides are washed in running distilled water 5 min.
2. Acetic fuchs in formalin 5 min.
3. Distilled water 2 sec.
4. Carmine stain 10 min.
5. Distilled water 2 sec.
6. Ethanol 70% 2 sec.
8. Absolute ethanol 30 sec.
9. Carboxylol 3 min.
10. Xylol 5 min.

Mount in picolyte

Formula:

- Ziehl-Neelsen carbolfuchsin 3 cc
- Glacial acetic acid 6 drops
- Formalin 6 drops
- Water 30 cc
- Indigo carmine 0.25 gm
- Sat. Sol. pierie acid 100 cc
- Glacial acetic acid 5 cc

Results:

Nuclei stain deep red violet; cartilage, mucin and most cells, intense blue violet; cytoplasm, green or yellowish green; connective tissue, intense blue.

Reference: Krajian and Gradwohl (1952)

G. Feulgen Stain

Fixation:

Coverslips are washed in Hanks' solution at room temperature for 10 minutes and then transferred to 10% neutral formalin and held at 5°C overnight.

Staining Procedure:

1. Wash in running water 5 min.
2. N HCl room temperature  1 min.
3. N HCl 60°C  5 min.
4. N HCl Cold  1 min.
5. Schiff’s reagent  2 hrs.
6. Acid Sulfite  10 min.
7. Acid Sulfite  10 min.
8. Acid Sulfite  10 min.
9. Wash in running water  5 min.
10. Rinse in dist. H2O
11. Counterstain in 0.5% alcoholic fast green FCF  5 min.
12. Ethanol 95%  few sec.
13. Ethanol Absolute  2 min.
14. Xylol  5 min.

Mount in picolyte

Formula:

Acid sulfite
Potassium metabisulfite (or sodium bisulfite) 10%  6 ml.
N HCl  6 ml.
Distilled water  120 ml.

Results:

DNA stains pink to red. The nuclear chromatin a deep purple. Pearse (1960) prefers Cornoy, and objects to formalin for nuclei acid because it blocks a number of reactive groups; although it is permissible to use the Feulgen reaction as a qualitative procedure after almost any fixative.
Reference:
Lillie (1954)

F. Giemsa Stain

Fixative:
1. Rinse 0.85% NaCl few seconds
2. Bouin's fixative 30 min.

Staining Procedure:
1. Ethanol 70% 10 min.
2. Ethanol 70% 10 min.
4. Ethanol 70% (destain) few seconds
5. Ethanol 95% few seconds
6. Ethanol absolute few seconds
7. Xylol 10 min.

Mount in picolyte

Results: Nuclei stain blue and the cytoplasm pink in normal cells

Reference: Switzer (1959)

H. Oil Red O Stain

Fixation:
Formalin 30 min.

Wash in water 5-10 min.

Staining Procedure:
1. Absolute propylene glycol 2 min.
2. Oil Red O in propylene glycol 20-30 min.
3. Differentiate in 85% propylene glycol
4. Wash in 2 changes of distilled water
5. Harris hematoxylin
6. Wash in 2 changes of distilled water
Mount in glycerin jelly

Results:
The fat droplets are red and the nuclei are counterstained blue.

Reference:
Armed Forces Institute of Pathology Manual (1957)
BIBLIOGRAPHY


I, Billy Bertram Hancock, was born in Dublin, Texas, February 17, 1927. I received my secondary school education in the public schools of Dublin, Texas, and my pre-professional training at Tarleton State College at Stephenville, Texas. I was admitted to the School of Veterinary Medicine, Agricultural and Mechanical College of Texas, in 1946 and received the Doctor of Veterinary Medicine Degree in 1951.

After a period of employment by Fort Dodge Laboratories, Inc. as Assistant Director of the Biological Laboratory, I received an appointment as an Instructor in the Department of Bacteriology, Ohio State University, in January, 1956. I held this position while completing the requirements of the Master of Science and Doctor of Philosophy degrees.