A Study on the Pas-Positive Reacting Substances in the Hamster Egg and Early Embryos

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A STUDY ON THE PAS-POSITIVE REACTING SUBSTANCES IN
THE HAMSTER EGG AND EARLY EMBRYOS

by

Hal D. McReynolds

A Thesis Submitted to the faculty of the Graduate
School of Loyola University in Partial Ful-
fillment of the Requirements for the
Degree of Master of Science

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BIOGRAPHY

Hal D. McReynolds was born in Bloomington, Illinois, on January 5, 1941.

He attended McLean High School in McLean, Illinois, from where he graduated in May of 1959. After attending the University of Notre Dame, Notre Dame, Indiana, he entered the University of Illinois, Urbana. He majored in Zoology and graduated with a Bachelor of Science degree in June, 1965.

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I wish to express my sincere appreciation to my advisor and Chairman of the examination committee, Dr. Robert Hadek, Associate Professor of Anatomy, for his enthusiastic support, professional guidance, and constructive criticism during this investigation.

I am also indebted to Dr. Leslie A. Emmert, Assistant Professor of Anatomy, and to Dr. Martin B. Williamson, Professor of Biochemistry, for donating their time and assistance to be members of the examination committee.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. BRIEF HISTORY OF THE MAMMALIAN EGG</td>
<td>1</td>
</tr>
<tr>
<td>B. FORMATION OF THE EGG IN THE OVARY</td>
<td>1</td>
</tr>
<tr>
<td>C. FERTILIZATION AND CLEAVAGE</td>
<td>3</td>
</tr>
<tr>
<td>D. IMPLANTATION</td>
<td>5</td>
</tr>
<tr>
<td>THE PROBLEM</td>
<td>6</td>
</tr>
<tr>
<td>A. ENERGY SOURCES FOR PREIMPLANTATION DEVELOPMENT</td>
<td>6</td>
</tr>
<tr>
<td>B. PURPOSE OF THE INVESTIGATION</td>
<td>8</td>
</tr>
<tr>
<td>REVIEW OF THE LITERATURE</td>
<td>9</td>
</tr>
<tr>
<td>A. THE NUCLEUS</td>
<td>9</td>
</tr>
<tr>
<td>B. THE CYTOPLASM</td>
<td>9</td>
</tr>
<tr>
<td>C. THE ZONA PELLUCIDA</td>
<td>11</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>12</td>
</tr>
<tr>
<td>A. DESCRIPTION OF THE ANIMAL SUBJECT</td>
<td>12</td>
</tr>
<tr>
<td>B. CARE OF THE ANIMALS</td>
<td>12</td>
</tr>
<tr>
<td>C. MATING THE ANIMALS</td>
<td>13</td>
</tr>
<tr>
<td>D. COLLECTION OF THE EGGS</td>
<td>13</td>
</tr>
<tr>
<td>E. FIXATION</td>
<td>14</td>
</tr>
<tr>
<td>F. STAINING METHODS</td>
<td>17</td>
</tr>
<tr>
<td>EXPERIMENTAL RESULTS</td>
<td>25</td>
</tr>
<tr>
<td>A. THE PAS REACTION</td>
<td>25</td>
</tr>
</tbody>
</table>
INTRODUCTION

A. Brief History of the Mammalian Egg

Although Cruickshank (1797) observed the rabbit ovum in the Fallopian tube, Von Baer (1827) was the first investigator to recognize the ovary as the source of the mammalian egg. Since Von Baer's discovery, numerous studies have been made on the mammalian egg and developing embryos.

By the middle of the 19th century, the ovarian egg was known to be a single-cell structure, composed of a cytoplasmic mass or vitellus, containing a large centrally located nucleus, and surrounded by a thick elastic membrane, the zona pellucida.

The first description of fertilization (union of the egg and sperm pronuclei) was that of Van Beneden (1875) followed by Sobotta's (1895) study of fertilization and cleavage in the mouse egg. Schenk (1878), one of the earliest experimental investigators, was able to maintain eggs in vitro. However, in vitro fertilization was not accomplished until the middle of the 20th century (Smith, 1951; Chang, 1959).

B. Formation of the Egg in the Ovary

Discussion of ovum development begins with the primordial germ cells. These cells appear early in mammalian development
(third week in humans) and are located in the wall of the yolk sac in the caudal end of the embryo. From this location, they migrate to the developing female gonad where they rapidly divide to give rise to the oogonia (the most primitive female germ cells). Later, the oogonia (without further division) differentiate into the larger primary oocytes which subsequently commence the prophase of the first meiotic division. A primary oocyte, together with its surrounding layer of flat epithelial (follicular) cells is termed a primary follicle.

At birth, the primary oocytes have completed the zygotene stage of the prophase of the first meiotic division and have entered the dictyotene (resting) stage in which they remain until sexual maturity is reached.

After puberty, the follicular cells become cuboidal and divide to form a thick covering around the oocyte (multilaminar primary follicle). In addition, the follicle cells secrete an amorphous intercellular fluid that solidifies around the oocyte and is known as the zona pellucida.

The zona is formed as a discrete and discontinuous structure which increases in thickness by accumulation of material on its external surface. The zona material is elaborated in small molecules as a monomer. The zona pellucida, itself, is a polymer and it may be the presence of the ovum which brings about the polymerization.

Small extensions between the follicular cells and the oocyte
may be significant in the transport of materials from the follicular cells to the oocyte during its growth.

As development continues, irregular fluid-filled spaces appear between the follicular cells. Later, they coalesce to form the follicular antrum which becomes filled with follicular fluid. As the antrum enlarges (maturing follicle), the oocyte becomes eccentrically located on a mound of follicular cells (the cumulus oophorous) and those cells massed around the oocyte itself are known collectively as the corona radiata.

At the time of ovulation, the primary oocyte completes its first meiotic division to form two daughter cells, each still containing the diploid number of chromosomes.

One cell, the secondary oocyte, receives most of the cytoplasm. The smaller cell, the first polar body, lies between the zona pellucida and the cell membrane of the secondary oocyte. The nucleus of the secondary oocyte begins the second maturation division (if sperm penetration occurs) which results in a mature ovum and second polar body.

C. Fertilization and Cleavage

During estrous the egg is extruded from the follicle in a process called ovulation. As the egg travels the fallopian tube towards the uterus, it mixes with the sperm that have been deposited by the male during coitus. In the hamster, coitus occurs during estrous (usually several hours prior to ovulation). Upon
reaching the ovum (in the ampullary region of the Fallopian tube),
the spermatozoa cluster over its surface. Although several sperm
may penetrate the zona pellucida, only one sperm will pass through
the vitelline membrane. Following access to the vitellus, the sperm
loses its tail, the sperm head forms the male pronucleus
(which unites with the female pronucleus in a process called syn-
gamy) to complete fertilization.

The main results of fertilization are: (1) determination of
the sex of the zygote; (2) restoration of the diploid number
of chromosomes; (3) initiation of a series of mitotic divisions
known as cleavage divisions (Langman, 1963).

The fertilized egg (zygote) begins dividing and passes
through 2-cell, 4-cell, etc., stages. With each cleavage divi-
sion, the individual cells (blastomeres) become smaller and the
total size of the early embryos remains the same (100 to 120 u).
By the 32-cell stage, the embryo has entered the uterus and is
termed morula. It is in the form of a solid sphere composed of
tightly packed blastomeres.

The cells of the morula continue to divide and fluid ac-
cumulates in the intercellular spaces. Finally these spaces be-
come confluent and form a large cavity, the blastocoele. The new
structure, the blastocyst, is composed of an inner cell mass
(embryoblast) located at one pole, and an outer cell mass (tropho-
blast) which forms the wall of the blastocoele.
D. **Implantation**

At this time the zona pellucida is shed, and the cells of the embryoblast begin penetration between the epithelial cells of the uterine mucosa. The time of implantation varies among different mammals. The hamster blastocyst usually implants on the fifth day after fertilization (120 hours post coitum).
THE PROBLEM

A. Energy Sources for Preimplantation Development

The mammalian zygote is a more or less free-living organism as it advances in the Fallopian tube towards the uterus. It requires energy for metabolism, growth, and the cleavage divisions which occur prior to its implantation in the uterine wall. Two possible energy sources, one exogenous and one endogenous, are believed available to the developing embryo.

The possible role of carbohydrate complexes in the energy metabolism of preimplantation embryos has been investigated recently by a number of workers. Although cleavage of the fertilized single-cell mouse embryo does not occur in vitro (Biggers, Gwatkin, and Brinster, 1962), the fertilized one-cell rabbit ovum will develop into a morula in a medium containing lactate as the sole energy source (Brinster, 1965a). Further, Brinster (1965b) reported that 2-cell mouse embryos will also develop into blastocysts in vitro when pyruvate, phosphoenolpyruvate, oxaloacetate, or lactate are employed alone in the incubating medium. However, when such compounds as glucose, fructose, glucose-6-phosphate, ribose, fructose-1,6-diphosphate, and other Kreb's cycle intermediates were employed alone in the medium, no cleavage occurred in the eggs.
The importance of pyruvate and lactate for the development of the early cleavage stages of the mouse ovum demonstrate that metabolism of the Fallopian tube and the ova within the tube may be intimately related. Bishop (1957), and later, Mastroianni and Wallach (1961) found an increasingly high concentration of lactic acid in rabbit oviduct secretions during the first three days after ovulation. Brinster (1965a) discovered that lactic acid dehydrogenase activity in the one-cell mouse ovum was 6 times that of the adult mouse skeletal muscle! However, there was a sharp (seven-fold) decrease in the enzyme activity in the 2 day period from the 8-cell stage to the blastocyst stage.

Other workers (Hammond, 1949; Whitten, 1956) reported that 8-cell mouse ova will continue growth in a medium consisting of glucose alone. In the rabbit egg, Fridhandler (1961) presented evidence that before blastocyst formation, glucose is oxidized mainly by the hexose monophosphate pathway, whereas, after blastocyst formation, glucose is metabolized via the Embden-Meyerof pathway and the tricarboxylic acid cycle.

The above evidence seems to indicate a change in the metabolic capability of the embryo during the cleavage stages. The change may be due to a decrease in the activity of certain enzymes (lactate dehydrogenase) and a simultaneous increase in the activity of other enzymes (phosphorylase, glucose-6-phosphatase, etc.) which become more important for embryonic development. Such changes in metabolic capability may be associated with the
change in the environment of the embryo from the Fallopian tube to the uterus.

B. Purpose of the Investigation

In a previous investigation of the hamster egg, a histochemical attempt to localize lactic acid dehydrogenase was unsuccessful. Although the method employed (Fahimi and Amarasingham, 1964) evinced lactate dehydrogenase (LDH) localization in skeletal muscle controls, no evidence of the enzyme was found in the early cleavage stages of the hamster egg. Therefore, the Periodic acid-Schiff (PAS) reaction was initiated for the following reasons:

1. Thus far glycogen has not been reported in the hamster egg and early embryos.
2. There may be an endogenous energy store of glycogen in the hamster egg.
3. If glycogen presence could be shown, some of the questions concerning the In vivo metabolic requirements of the hamster egg could be more easily understood.
4. By employing the PAS reaction, in conjunction with diverse controls and supplementary staining procedures, it will be possible to localize and classify any other carbohydrate complexes which may be present in the egg.
REVIEW OF THE LITERATURE

Since the first histochemical studies by Raspail (1830), there have been numerous experiments and histochemical studies on the mammalian egg and early embryos. Because experiments employing the Periodic acid-Schiff (PAS) reaction (and supplementary staining techniques) are the most relevant to this study, this review of the literature will concentrate on those reports which are related to this topic.

A. The Nucleus

Harter (1948) reported that no coloration developed in the nucleus of the rat ovarian egg when treated with the Periodic acid Schiff reagent. Toluidine blue staining causes the nuclei to appear purple in the metestrus follicles of the rat ovary (Deane, 1952). However, the result is suspect, since the acid fixative which was employed may have been responsible for the coloration. Nevertheless, Dalcq (1955) observed pale central metachromatic droplets of fluid in the nucleoli of the rat ovum and embryos. He reported that the nucleoli may grow, remaining simple or becoming complex by production of the inner metachromatic fluid.

B. The Cytoplasm

Loewenstein and Cohen (1964) discovered that 16.5 per cent of
the zona-free mouse ovum is carbohydrate. Goldman (1912), employing Best's Carmine (Pearse, 1961), observed a large amount of glycogen in the ovarian as well as the segmenting mouse egg. A somewhat similar observation was recorded by Thomson and Brinster (1966) who claimed that the preimplantation embryos of the mouse contain large amounts of PAS-positive material which can be removed by malt diastase, thus assuming that it is glycogen.

With regard to the rat, there is a difference of opinion concerning the glycogen content of the egg. For example, Wislocki, et al. (1947) observed only a small amount of glycogen in the cytoplasm, while Harter (1948) perceived a sizeable amount of glycogen as well as glycoprotein in the rat egg cytoplasm. Dalcq and Mulnard (1955) repeated the study on the rat egg and described the glycogen to be distributed in a diffuse manner in the pre-implantation embryo up to and including the 16-cell stage. No glycogen was observed in the egg of the sow (Wislocki, et al., 1947).

Cytoplasmic metachromasia varies among the different mammals. Deane (1952) observed metachromasia only in the follicular fluid of the rat, whereas in vivo toluidine blue staining in the rabbit egg shows reddish colored, concentric stratification of the albuminous coat without the zona pellucida (Bascich and Hamilton, 1956). There was no metachromasia observed in the cytoplasm of the rabbit egg. Toluidine blue, when used as a vital stain, causes localized metachromasia in the rat egg indicating the
presence of weak acid mucopolysaccharides (Dalcq, 1955).

C. The Zona Pellucida

The earliest study by Goldman (1912) has indicated that the zona pellucida of the mouse contains a small amount of glycogen. Subsequently, Deane (1952), Braden (1952), and Harter (1948) have shown very strong PAS reactions in the zona of the rat and the rabbit. Notwithstanding Konecny's (1959) demonstration that the zona of the rat contains lipoproteins, Loewenstein and Cohen claim that it is composed of seventy per cent of carbohydrates.

Metachromasia has been observed in the zonae of numerous mammalian eggs. Although staining has been shown in the sow (Wislocki, et al., 1947), the rabbit (Braden, 1952), the cat (Konecny, 1959), and the rat (Dalcq, 1955); other workers (Deane, et al., 1952) did not observe the reaction in the rat egg. Furthermore, metachromasia in the cat zona was found to be hyaluronidase labile (Konecny, 1959) while treatment with the enzyme did not effect the reaction in the sow zona. Stary (1959) suggested that either neutral mucopolysaccharides or highly polymerized hyaluronic acid were responsible for the absence of metachromatic staining from the rat zona pellucida. Although Loewenstein and Cohen were not able to remove the mouse zona at neutral pH, digestion did occur at pH 4.6. However, they were uncertain whether the enzyme or the increased hydrogen ion concentration was responsible for the removal.
MATERIALS AND METHODS

A. Description of the Animal Subject

The golden hamster (Cricetus auratus), a native of Syria, was the animal chosen for this investigation. It is a small rodent which breeds readily in captivity and has a gestation period of 15 to 16 days (Hamilton and Samuel, 1956). The normal mating season is from February to September, but Deanesley (1938) reported that in laboratory conditions the females are capable of reproduction throughout the year. The length of the estrous cycle (4 days) has been described by Kupperman, Greenblatt and Hair (1944), Kent and Smith (1945), Ward (1956), and Hamilton and Samuel (1956).

Female hamsters usually come into heat between 6 P.M. and 9 P.M. on the day of estrous. Ovulation occurs spontaneously about 8 hours after the onset of heat (Harvey, Yanagimachi, and Chang, 1961). When the females are mated, copulation usually precedes ovulation (Hamilton and Samuel, 1956).

Accurate timing is necessary to assure finding the eggs at the desired stage of development. Table 1 summarizes the stages of development of the ova in the female genital tract.

B. Care of the Animals

Male and female hamsters were kept separately in the same room
(68 to 75° F.) until the time of mating. They were fed fresh lettuce daily, in addition to a standard hamster diet (Teklad) and water. The lighting system was regulated to give 8 hours of darkness beginning at 10 P.M. each evening.

C. Mating the Animals

During late afternoon on the day of mating, 1 male hamster was placed together with 3 female hamsters in wire or plastic cages. Vaginal smears were taken the following morning between 8 A.M. and 11 A.M. to determine whether the animals had copulated. The technique involved the deposition and subsequent withdrawal of a small amount of saline with a glass Pasteur pipette from the vagina. This lavage was placed on a glass slide and observed with a compound microscope. Those females which had not mated were returned to the same cage and examined again the following morning. Females found to be sperm-positive were appropriately marked with an earpunch and separated.

D. Collection of the Eggs

Ovulation was considered to have occurred between 2 A.M. and 5 A.M. (Yanagimachi and Chang, 1963), on the morning the animals were found to be sperm-positive. The ages of the zygotes were judged from the time of coitus and representative specimen were collected from 12 to 85 hours post coitum.
The inseminated females were subjected to terminal ether anesthesia at the desired time. With the aid of the dissecting microscope, the genital tract was dissected, the excess tissue removed, and the coiled Fallopian tubes were straightened. With a 30 gauge needle and syringe, the ova were flushed into a small watchglass containing physiological saline.

E. Fixation

Following examination under the microscope to determine whether the eggs were normal or atretic, they were placed into the proper fixatives. According to Humason (1962), fixing solutions should meet the following criteria:

1. "Penetrate rapidly to prevent postmortem changes.
2. Coagulate cell materials into soluble substances.
3. Protect tissue against shrinkage and distortion during dehydration, embedding and sectioning.
4. Allow cellular parts to become selectively and clearly visible by means of dyes and improved refractive indices."

Table 2 lists the fixatives employed in this investigation and outlines their chemical composition.

1. **Bouin's Fixative** (Humason, 1962)

Bouin's fluid was employed as a general fixative in this investigation. Fixation time was not important since tissues can be left in this solution for several weeks without causing damage.
The picric acid used in Bouin's fluid is advantageous because it does not harden the tissue and it is a good fixative for glycogen and proteins. However, since it causes shrinkage of the tissues, acetic acid is added to counteract this undesired characteristic.

Formalin, the remaining component of Bouin's solution, fixes proteins so that glycogen is held by them due to precipitation as a protein complex (Pearse, 1961).

(2) **Rossman's Fixative** (Humason, 1962)

This fixative, a picro-alcohol-formalin mixture, was recommended by Wislocki, et al. (1947) for glycogen demonstration. The solution prevents "polarization" or the streaming of glycogen granules to one pole of the cell (Pearse, 1961). However, due to the small size of the egg and its high water content (85 per cent) the cytoplasm of the ovum was completely disrupted when placed in this highly alcoholic solution.

3. **Tellyesniczky's Fixative** (Thomson and Brinster, 1966)

Thomson and Brinster (1966) recommended this fixative for preserving glycogen in whole mount preparations. The solution contains formalin and alcohol; both recommended by Pearse (1961) for glycogen preservation. The acetic acid component of the solution dissolves the zona pellucida of the eggs and causes them to attach to the slide. However, since acetic acid causes the blastomeres to swell and separate, this fixative cannot be used for
specimen which are to be embedded in paraffin.

4. **Susa's Fixative** *(Humason, 1962)*

A metachromatic reaction is enhanced when tissues are fixed with Susa's solution *(Bacsich and Hamilton, 1954)*. The mercury component of the fixative *(HgCl$_2$)* combines with acidic groups of proteins and is selective for sulfhydryl (-SH) groups. Mercuric chloride penetrates well, shrinks less than other protein coagulants, and distorts cellular structure very little. According to Humason *(1962)*, one of the disadvantages of mercuric chloride is that it deposits in the cell precipitates of mercurous chloride *(needle shaped)* and metallic mercury *(amorphous, irregular clumps)*. The precipitate is removed, prior to staining, by soaking the section in a solution of iodine saturated in 70 per cent alcohol.

\[
2 \text{HgCl} + \text{I}_2 \rightarrow \text{HgCl}_2 + \text{HgI}_2
\]

5. **Corrosive Formol Fixative** *(Gray, 1954)*

This solution is similar to Susa's fixative in its effect on tissues. Trichloroacetic acid and acetic acid are not employed in this fixative. Like Susa's fixative, this fluid is recommended for metachromatic staining.

6. **Calcium Formol Fixative** *(Humason, 1962)*

Baker *(1944)* originally designed this fixative for the
preservation of phospholipids. It is assumed that phospholipids (phosphatides) are prevented from diffusing into the fixing solution by the calcium and cobalt, but that they are not "fixed" in the usual sense and that they can still be removed by lipid solvents. The actions of calcium and cobalt are presumed to be due to their influence on the formation of complex coacervates of phospholipids with proteins and mucopolysaccharides. According to Pearse (1961), coacervates are "structures resulting from the aggregation of molecules, held together by intermolecular forces, in the form of a mosaic or lattice of lipid and protein molecules."

Due to the size of the hamster egg, the fixation time in the various fluids was relatively short. In most cases, one hour was sufficient, excepting Susa's fluid and the Corrosive formol solution in which over 30 minutes fixation time resulted in cellular disruption.

F. Staining Methods

1. Periodic acid-Schiff (PAS) Reaction (Pearse, 1961)

In order to observe the presence of glycogen in the hamster egg, the Periodic acid-Schiff (PAS) reaction was applied to whole mounts and sectioned specimen. Although this technique was first used by McManus (1946) for the localization of mucin, at present the reaction is considered a favorable histochemical method for demonstrating polysaccharides (Hotchkiss, 1948). According to
Pearse (1961), the "naturally-occurring animal substances which are capable of giving a positive PAS reaction are polysaccharides (glycogen), neutral mucopolysaccharides, mucoproteins, glycoproteins, glycolipids, and phosphatides (phospholipids)."

The PAS technique involves two chemical reactions:

1. The formation of aldehydes due to oxidation by the Periodic acid of 1,2 glycols and/or the equivalent amino or alkylamino derivatives, or the oxidation products, and

2. The reaction of the resulting aldehydes with Schiff's reagent to form a red color.

The PAS Technique

1. All sections and whole mount preparations were hydrated through an alcohol series (absolute, 95%, 80%, 70%, 50%, 35%) to water at 2 minute intervals. Those sections which were fixed in solutions containing HgCl₂ were left in 70 per cent ethyl alcohol for 10 minutes. This procedure removes the mercury precipitates.

2. After washing the slides in distilled water (2 minutes), the sections were oxidized in 0.6 per cent aqueous periodic acid (HIO₄) for 10 minutes.

3. The slides were washed for 5 minutes in running tap water to remove the excess oxidizing agent.

4. The sections were treated with Schiff's reagent for 15 minutes at room temperature. During this time, the aldehyde groups react with the Schiff's reagent to
produce a red dye.

(5) The slides were placed in running tap water for 5 minutes to remove the excess Schiff's reagent.

(6) The sections were placed in sulfite baths (0.5% Na$_2$S$_2$O$_5$) in 3 changes for 2 minutes each. This procedure has a bleaching effect and enhances the final coloration.

(7) The slides were washed quickly through the same alcohol series (1 minute per stage), passed through a 1:1 mixture of absolute alcohol and xylene, xylene (1 minute), and mounted in a synthetic resin (H.S.R.).

The whole mount preparations (fixed in Tellyesniczky's fluid) were stained immediately after fixation without embedding in paraffin.

In order to classify the positive-reacting substances which were demonstrated by the PAS method, various controls were employed.

(I) Malt diastase (Nutritional Biochemicals Corporation, Cleveland) was used to remove glycogen from the hamster eggs.

The Malt Diastase Technique

Prior to periodic acid oxidation, alternate sections and whole mounts were incubated in 1.0 per cent malt diastase in physiological saline for 2 hours at 37$^\circ$ C. This substance, first advocated by Lillie and Greco (1947), contains a-amylase, which has a glycogenolytic
effect. Although malt diastase may be contaminated with other enzymes, Pearse (1961) states that this does not influence the results.

(Ia) Controls were incubated for the same length of time in physiological saline.

(II) To establish the nature of the PAS-positive material remaining in the sections following diastase treatment, pyridine extraction was employed. Edgar and Donker (1957) reported that in "formalin fixed sections, 80 to 90 per cent of the total glycolipids are removed by 48 hours cold pyridine treatment.

The Pyridine Technique

Diastase treated sections were extracted by pyridine (48 hours) and then stained by PAS. The results were compared with diastase-PAS treated sections.

2. Sudan Black B Reactions (Pearse, 1961)

Sudan Black B staining was also employed in an attempt to demonstrate the presence of compound lipids (glycolipids and phospholipids) in calcium formol fixed tissue. Pyridine extraction was applied to alternate sections to remove any glycolipids present.

The Sudan Black B Technique

(1) Sections were deparaffinized and hydrated to 70 per cent ethyl alcohol.
(2) Generally, sections were stained for 30 minutes at room temperature in saturated solutions of Sudan Black B in 70 per cent ethyl alcohol. In addition, representative sections were stained for 16 hours at 60° C. to assure sufficient reaction time.

(3) The excess dye was removed by rinsing the sections in 70 per cent ethyl alcohol (10 dips).

(4) The sections were rinsed in running water (30 seconds or less).

(5) Subsequently, the sections were mounted in glycerine jelly.

3. **Toluidine Blue Staining** (Pearse, 1961)

In order to demonstrate metachromatic staining mucopolysaccharides, eggs fixed in Susa's and corrosive formal solution were stained with Toluidine Blue. Pearse (1961) defines metachromasia as the "staining of a tissue component so that the absorption spectrum of the resulting tissue-dye complex differs sufficiently from that of the original dye and from its ordinary tissue complexes, to give a marked contrast in color." Barka and Anderson claim that hyaluronic acid, chondroitin sulphate A and chondroitin sulphate C are capable of developing metachromasia when stained with toluidine blue.

Hyaluronidase (Bovine testes, Sigma Chemical Company,
St. Louis), which attacks the glucosaminidic bond of hyaluronic acid and the chondroitin sulphates, was used as a control for the metachromatic staining of toluidine blue. Ribonuclease (Nutritional Biochemicals Corporation, Cleveland) was applied to representative sections of the preimplantation stages to exhaust RNA and thus to enhance the metachromatic staining (Wislocki, et al., 1947).

**The Toluidine Blue Technique**

(1) Sections were hydrated to water and the mercury precipitate was removed when necessary.

(2) The sections were organized into four groups and processed in the following manner:

a. **Toluidine Blue stained slides** (slides to be used as controls for the hyaluronidase treated sections)

   These sections were incubated in 0.85 per cent physiological saline at 37° C. for 3 hours.

b. **Toluidine Blue stained slides** (slides to be used as controls for the RNAse treated sections)

   These sections were incubated in 0.85 per cent physiological saline at 37° C. for 1 hour.

c. **Hyaluronidase Treated Slides**

   Sections were incubated for 3 hours at 37° C. in hyaluronidase (1 mg/ml in 0.85 per cent
physiological saline).

d. Ribonuclease (RNAse) Treated Slides

These sections were treated for 1 hour at 37° C. in a solution of ribonuclease (1 mg/ml in 0.85 per cent saline).

(3) All sections were subsequently rinsed in distilled water (1 minute).

(4) All sections were stained for 20 minutes in 0.1 per cent toluidine blue (suspended in 30 per cent ethyl alcohol).

(5) Briefly the sections were rinsed in distilled water (3 dips).

(6) Followed by a rinse in 95 per cent ethanol (10 dips), the sections were dehydrated in absolute alcohol (2 minutes) and cleared in xylene (2 minutes).

(7) The sections were blotted dry and mounted in a synthetic resin (H.S.R.).

4. Alcian Blue Staining (Barka and Anderson, 1963)

Alcian Blue staining, first described by Steedman (1950), was employed as a selective stain for acid mucopolysaccharides. Although chemical forces probably play an important role in binding the dye to the substrate, the exact mechanism of the staining reaction is not known (Barka and Anderson, 1963).
The Alcian Blue Technique

(1) Sections fixed in formalin containing solutions were hydrated.

(2) Subsequently, the sections were stained in 0.1 per cent Alcian Blue in 0.01 N HCl for 10 minutes.

(3) The sections were rinsed briefly (30 seconds) in 3 changes of distilled water, followed by a brief rinse (30 seconds) in 2 per cent acetic acid.

(4) The sections were rinsed again in distilled water (1 minute).

(5) The sections were dehydrated through an ethyl alcohol series and mounted in a synthetic resin (H.S.R.)

A more complete analysis of the staining mechanisms of the dyes employed in this investigation will follow in the discussion.
EXPERIMENTAL RESULTS

The selection of appropriate fixatives is important in histochemical investigations. In many instances, the results of the staining procedures were closely related to the method of fixation employed. Therefore, the experimental results will be described in conjunction with the method of preservation.

A. The PAS Reaction

The Periodic acid-Schiff (PAS) reaction was demonstrated in whole mount preparations preserved with Tellyesniczyk's fixative. All stages, from unfertilized to the blastocyst, revealed heavy, diffuse PAS-positive material throughout the cytoplasm of the egg. However, there are disadvantages to the use of this fixative. Due to its relatively high acetic acid content, the zonae are removed and swelling causes the blastomeres to separate. Therefore, this fixative can only be applied successfully to whole mount preparations.

Two fixatives, Bouin's and Corrosive formol, evinced PAS staining when applied to sectioned material. In each case, the zona pellucida stained intensely red while the cytoplasm showed a diffuse, moderately intense reaction. Susa's fixative did not preserve the zona pellucida and the cytoplasmic reaction was much weaker than that following Bouin's fixation. In the sectioned
ova, PAS-positive material was present in all phases of development and there was no observable variation in the intensity of the reaction. No PAS reaction was observed in the nucleus of any of the specimen.

Malt diastase was used to remove glycogen from the sections. In each cleavage stage, there was a decrease in the amount of PAS-positive material, regardless of the type of fixative employed. More specifically, all whole mount preparations (except the 2-cell stage) were completely devoid of coloration. The above mentioned embryo showed a faint trace of stain following enzyme digestion. Sectioned eggs, fixed in Bouin's and Susa's solutions, demonstrated light, magneta coloration both in the zonae and the cytoplasm. Furthermore, a somewhat heavier reaction was discernible in Corrosive formol fixed ova. The zonae, usually destroyed by Susa fixation, retained a minimal amount of PAS coloration when preserved by Bouin's and Corrosive formol techniques.

Although it is generally agreed that mucopolysaccharides do not react with Schiff's reagent following periodic acid oxidation, a number of specimen were treated with 0.01 per cent hyaluronidase prior to staining with PAS. Regardless of the type of fixation, there was no observable removal of the store of PAS reacting substances either from the zona pellucida or from the cytoplasm. On the contrary, in some cases, the enzyme treatment appeared to enhance the strength of the reaction. This effect has been observed by other histochemists (Pearse, 1961; Barka and Anderson, 1963).
Following diastase digestion, pyridine extraction was applied to representative sections of all the preimplantation stages of the hamster egg. After the pyridine treatment, the sections were stained by the PAS reaction and compared with diastase-PAS treated sections. Regardless of the type of preliminary treatment, there was no significant difference in the amount of PAS-positive material remaining in any of the sections, indicating that glycolipids are not present in the hamster egg.

B. The Sudan Black B Reaction

Baker's Calcium Formol fixative was used to preserve any compound lipids present in the hamster egg and embryos. Sudan Black B staining did not evince the blue-black reaction which is characteristic of lipid staining. Likewise, control sections, treated with cold pyridine (48 hours) were devoid of staining.

C. The Toluidine Blue Reaction

Metachromasia was observed, after fixation with Susa's and Corrosive formol solutions, in sections stained with 0.01 per cent toluidine blue. Mucopolysaccharides such as hyaluronic acid, chondroitin sulphate A, and chondroitin sulphate C do not react with Schiff's reagent, but they are considered demonstrable with toluidine blue metachromatic staining.

Prior to staining, slides representative of each stage were
exposed to hyaluronidase. RNAase was applied to the sections to
determine whether the metachromasia was the result of ribonucleo-
protein presence. This procedure allows one to distinguish be-
tween ribonucleoproteins and other basophilic substances such as
mucopolysaccharides (Wislocki, et al., 1947).

Susa's fixative and Corrosive formol were far superior to
Bouin's solutions in demonstrating metachromatic staining in early
hamster embryos. There was a gradual decrease in the intensity of
the metachromasia that paralleled the advancement of development of
the embryo. The zonae of Corrosive formol and Susa fixed ova
(when not removed during fixation) showed a purple (beta) meta-
chromasia. The vitellus and peripheral cytoplasm of all stages
showed a metachromatic ring only when fixed in Susa's solutions.
However, regardless of the fixation, no metachromasia was detect-
ed in the central cytoplasmic region of any of the eggs. All nu-
cleoli and nuclear membranes stained orthochromatically (blue)
with toluidine blue. A faint purple (metachromatic) coloration
was observed in the nucleoli of one Susa preserved ovum.

Sections treated with hyaluronidase generally stained ortho-
Chromatically, although there were some sections in which the en-
zyme digestion did not completely remove the metachromasia.

Decreased cytoplasmic basophilia greatly enhanced the meta-
chromatic reaction in sections incubated with ribonuclease. The
greenish-blue color which appeared in the cytoplasm contrasted
well with the purple zona pellucida and perivitelline ring.
D. **Alcian Blue Staining**

Alcian Blue staining for acid mucopolysaccharides was applied to all stages of hamster egg development. Those sections fixed with Bouin's solution revealed moderate staining in the zona pellucida. Although a somewhat less dense staining was present in the vicinity of the cell membranes, no coloration was found in the nucleus or the inner cytoplasm of any cell.
DISCUSSION

Before one can interpret experimental results obtained from a histochemical reaction, it is necessary to discuss the staining procedures employed and define the substances involved in the reactions.

According to Pearse, "the whole of the modern histochemistry of the polysaccharides, mucopolysaccharides, and mucoproteins is bound up with the periodic acid–Schiff (PAS) reaction." Therefore, it is necessary to examine this reaction and the principles on which it is based.

A. The PAS Reaction

Periodic acid (HIO₄) is an oxidizing agent which attacks carbon-carbon (−C−C−) bonds in the following compounds:

\[
\begin{align*}
\text{CHOH} & \quad \text{CHOH} \\
\text{CHOH} & \quad \text{CHNH}_2 \\
\text{1,2 glycols} & \quad \text{1-hydroxy-2-amino}
\end{align*}
\]
After cleavage of the carbon bonds, the attacked groups are converted into dialdehydes (CHO.CHO). Periodic acid is considered superior to other oxidizing agents because it does not further oxidize the resulting aldehydes, thus permitting their observation with Schiff's reagent.

In acid solution and with an excess of SO₂ present, basic fuchsin (phenyl methane dye component of Schiff's reagent) is reduced to form fuchsin sulphurous acid (a colorless N-sulfinilic acid). Although the exact mechanism of the reaction is not known, it is believed to occur as follows:

\[
\begin{align*}
\text{Fuchsin-sulphurous acid} & \quad \text{Aldehyde} \\
\end{align*}
\]
Molecular rearrangement takes place to give:
The reaction involves an addition followed by a condensation with the final compound exhibiting color due to its quinonoid grouping.

The total reaction, including oxidation of the substrate can be viewed as follows:

```
+ H | O_4
- O
- C
```

According to Hothkiss (1948), any compound which fulfills the following criteria will stain with the PAS reaction:

1. the PAS reacting substances must be preserved during fixation, embedding, etc.

2. the substances must contain the 1:2 glycol grouping, or the equivalent amino or alkylamino derivative, or the oxidation product.
(3) the compounds formed during the periodic acid oxidation must be non-diffusible.

(4) the substances must be sufficiently concentrated to give a final detectable color.

Barka and Anderson (1963) claim that the following compounds are capable of demonstrating a PAS-positive reaction:

(1) polysaccharides (glycogen)
(2) neutral mucopolysaccharides
(3) mucoproteins and glycoproteins
(4) glycolipids
(5) phosphatides (phospholipids)

Glycogen is the only polysaccharide present in higher animals in sufficient quantity to give a PAS-positive reaction after aqueous fixation and paraffin embedding. This material is composed of chains of α-glucopyranose (D-glucose) units linked at α-1,4 positions (α-1,6 at branching points).

The glycogen repeating unit
Various fixatives have been recommended (Carnoy, Zenker, Bouin, Rossman, Gendre, etc.) but Baker (1945) claimed that formalin fixation binds glycogen to protein in sufficient manner to prevent dissolution in aqueous fluids.

Sectioned eggs and whole mounts (representing each implantation stage) were subjected to the PAS-reaction. PAS-positive material was found in the zonae (in the sectioned eggs) and in the cytoplasm of all cells of the preimplantation hamster eggs.

In order to determine whether the PAS-positive material was glycogen, malt diastase was employed to selectively remove the polysaccharide from the sections and the whole mount preparations. Although malt diastase may contain impurities, the preponderant enzyme (a-amylase) hydrolizes glycogen to disaccharide (maltose) units. Since the action of the enzyme is limited to the a-1,4-glucosidic linkage of glycogen, the a-1,6-branched points are not hydrolized. Pearse (1961) states that there is no doubt that glycogen in tissues is always entirely removed by diastase treatment.

The sectioned eggs displayed moderately heavy concentrations of PAS-positive material in the cytoplasm while a much more intense coloration was observed in the zona pellucida. Furthermore, all sectioned eggs revealed remaining stores of PAS-positive material after diastase treatment.

One of the most interesting findings of this investigation was the diastase removal of all PAS-positive material from the whole mount preparations. Apparently Telleyesniczky's fixative
does not preserve PAS-positive material other than glycogen. Only the 2-cell egg showed any coloration following diastase digestion. Since the staining was very faint, it was not considered significant.

Thomson and Brinster, observing similar results in mouse embryos, claimed that since the control slides were devoid of color, the PAS-positive, diastase labile material is glycogen. Therefore, it can be stated that: (1) the PAS-positive, diastase removable material is glycogen, and (2) there is non-glycogen, PAS-positive material in the hamster egg.

Carbohydrate-containing complexes other than glycogen or lipid substances of the phosphatide (phospholipid) class could be the source of the non-glycogen PAS-positive material. However, as reported earlier (experimental results, pages 27 and 28), there was no evidence that pyridine extraction (to remove glycolipids) reduced the total store of PAS-positive material. Likewise, an attempt to demonstrate compound lipids (phosphatides and/or glycolipids) by the Sudan Black B method was unsuccessful.

Furthermore, other workers have been unsuccessful in demonstrating lipids in the rat ovum. Deane (1952), employing Sudan Black B to stain frozen sections of the rat ovary, did not detect Sudanophilia in the ovum. Harter (1948), in order to rule out glycolipids, incubated control sections of the rat egg in equal parts of chloroform and methanol. Following this extraction procedure, the sections were stained with the PAS reaction. Harter
concluded that no appreciable amount of glycolipid was present in the sections and that the stained substances were either free carbohydrates or those bound to protein.

Although neutral mucopolysaccharides are capable of giving a PAS-positive reaction, they usually are not found in higher animals (Pearse, 1961; Barka and Anderson, 1963). Meyer claimed that when neutral mucopolysaccharides do occur they are always firmly bound to proteins. Furthermore, their histochemical reactions are so similar to mucoproteins and glycoproteins that they cannot be distinguished from each other (Pearse, 1961; Barka and Anderson, 1963).

In view of the above discussion and the experimental results, it is likely that the non-glycogen, PAS-positive material is a carbohydrate-protein complex. Mucoproteins (mucoids) are hexosamine containing polysaccharides which are found in firm chemical union with a peptide, the hexosamine content of the whole being greater than 4 per cent. (Meyer, 1938). The usual components of a mucoprotein are hexose and hexosamine structures, although uronic acid may be present. Arbitrarily distinguished from the mucoproteins are the glycoproteins. They are similar to the mucoids, except that their hexosamine content is less than 4 per cent. Mucoproteins and glycoproteins cannot be separated from each other histochemically (Pearse, 1961; Barka and Anderson, 1963).
B. The Alcian Blue Staining Reaction

Alcian blue staining was employed to demonstrate the general category of acid mucopolysaccharides. Acid mucopolysaccharides are defined by Meyer (1938) as polysaccharides containing hexosamine as one component, and occurring free or as esters of sulphuric acid. The acid mucopolysaccharides, both simple and complex, contain hexuronic acid as their second carbohydrate component.

Salt linkage with acidic groups is the mechanism responsible for Alcian blue staining. The dye is water soluble and is highly selective for acid mucopolysaccharides. In the present study, moderately heavy staining was observed in the zona pellucida of all the preimplantation stages of the hamster egg. There was less heavy staining in the vitelline membrane while no acid mucopoly-
saccharide presence was detected in either the cytoplasm or the nucleus of any of the cells.

C. **The Toluidine Blue Staining Reaction**

A histochemical reaction in which the dye selectively stains a tissue structure a color different from the color of the dilute dye solution is said to be metachromatic (Barka and Anderson, 1963). Subsequent to the original observation (Ehrlich, 1897), there have been numerous investigations of the phenomenon of metachromasia. Although the exact mechanism of the reaction is not known, Michaelis and Branick (1945) proposed the concept of polymer formation in which polymerization of the dye is induced by polymerization of the substrate, resulting in metachromasia. According to these workers, toluidine blue has an absorption spectrum with three bands. Each band represents a different isomeric form. The monomeric alpha form is blue, the dimeric beta form is purple, and the polymer gamma form is red. However, Pearse (1961) warns that the purple (beta) metachromasia is not due to a beta form, but the presence of simultaneous alpha and gamma forms.

Sylven (1954) considers metachromasia to represent a "special type of orderly dye aggregation characterized by formation of new intermolecular bonds between adjacent dye molecules." Although alcohol resistant metachromasia may be the only true representative of the staining phenomenon (Kramer and Windrum, 1955), Pearse (1953) claimed that metachromatic staining of sulphate-free
hyaluronate is not diminished by alcohol.

Bunting (1950) is of the opinion that sulphated acid mucopolysaccharides, non-sulphated acid mucopolysaccharides, and basophilic material are capable of staining metachromatically. Likewise, Wislocki, et al. (1947) claim that all metachromasia observed in mammalian tissues is due to mucopolysaccharides, since nucleoproteins and substances of unknown composition may stain metachromatically under certain conditions. They used ribonuclease to control any staining referable to yeast nucleic acids. Barka and Anderson (1963) are of the opinion that metachromatic staining, abolished by hyaluronidase treatment, can be attributed to the presence of hyaluronic acid and/or chondroitin sulphate A or C.

The Hyaluronic Acid repeating unit

The Repeating units of Chondroitin Sulphate A and C
They further state that the effect of hyaluronidase treatment cannot be detected by PAS staining since neither hyaluronic acid nor the chondroitin sulphates give significant reactions.

This investigation revealed metachromatic staining in all preimplantation hamster ova. The reaction was more intense in the early stages, with purple metachromasia visible in the zonae, in the vitelline membranes, and in the peripheral cytoplasm of the blastomeres. No purple coloration was observed in the central or inner cytoplasmic region where the staining appeared in the orthochromatic (blue) form. The nuclei were devoid of metachromasia, although one preparation did show the nucleolar metachromasia observed by Dalcq (1955) in the rat egg.

The later stages, especially the blastocyst stage, showed decreased metachromatic staining. There was a less intense coloration in the zona of the blastocyst and the individual inner cell mass and trophoblast cells exhibited staining only in the outer portion of their vitelline membrane which was exposed to the genital tract (i.e. adjacent to the zona pellucida).

As previously reported in the materials and methods sections, hyaluronidase and ribonuclease were applied to control sections. As a result of the RNAse activity, cytoplasmic basophilia was reduced in sufficient amount to clarify and enhance the metachromatic staining. The orthochromatic (blue) staining (which indicates ribonucleoprotein presence) was greatly reduced and the toluidine
blue staining revealed a metachromatic zona pellucida and a peri-vitelline ring in each stage of development. Therefore, it can be assumed that the metachromasia developed was not due to ribonucleoprotein presence.

Hyaluronidase treated sections stained orthochromatically. This would seem to indicate that the metachromasia was due to the presence of hyaluronic acid. However, in view of the apparent disagreement in opinion concerning the nature of the metachromatic substances, it would be presumptuous to make a definite statement as to their composition.

In summation, Pearse (1961) concludes that "at this very imperfect stage of our knowledge of the nature of the substances and the actions of hyaluronidase, few conclusions can be considered accurate." He concludes that metachromatic material whose metachromasia is reversible (by one to three hours treatment with a purified testes extract) is:

(1) of a mucopolysaccharide nature, and
(2) either chondroitin sulphate A or C, or hyaluronic acid itself, or a mixture of any or all of these.
SUMMARY AND CONCLUSIONS

(1) PAS-positive material is present in the zona pellucida and the cytoplasm of all the cells in the preimplantation stages of the hamster egg.

(2) Glycogen is present in the zona pellucida and the cytoplasm of all the cells in the preimplantation stages. No decrease was observed in the amount of glycogen in any of the stages.

(3) In addition, there is non-glycogen, PAS-positive material in all the preimplantation stages. This material is located in the cytoplasm of the blastomeres.

(4) Compound lipids (glycolipids and phosphatides) were not shown to be present in the hamster egg.

(5) Alcian Blue staining has revealed the presence of acid mucopolysaccharides in the zonae and in the perivitelline area of the blastomeres. Metachromatic staining with Toluidine Blue indicates that the acid mucopolysaccharides are hyaluronic acid and/or chondroitin sulphate A or C.

This investigation has shown that, although there is a large concentration of glycogen in the hamster egg and early embryos, the glycogen content does not decrease in the later stages of development. Brinster and Thomson (1966) observed a marked decrease in glycogen content in the mouse embryo (blastodyst). However,
no similar depletion of the glycogen store was observed in the hamster egg.

There could be several reasons why glycogen does not appear to be utilized as an energy source during early hamster development. The developing embryos may have an exogenous energy source (such as lactate) available for their early metabolism and growth. It is also possible that the glycogen is not metabolized until the time of implantation when the embryo's energy requirements are greater. Finally, it is possible that the glycogen store is being metabolized and replenished simultaneously. In other words, the embryo may be utilizing the endogenous glycogen while it is producing more glycogen from exogenous sources.

The acid mucopolysaccharides (especially hyaluronic acid) may function to protect the ovum from bacterial attack. It has been shown (Blumberg and Ogston, 1952) that hyaluronic acid (in connective tissues) can limit the flow of fluid, and hinder the passage of larger molecules, such as protein, through it. It is also known that, at the time of fertilization, spermatozoa release hyaluronidase to initiate their penetration of the zona pellucida.
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Cruickshank, W. 1797 Experiments in which, on the third day after impregnation, the ova of rabbits were found in the fallopian tubes; and on the fourth day after impregnation in the uterus itself; with the first appearance of the foetus. Phil. Trans. R. Soc., pt. 1, 197. (2,3). (Cited from Austin).


Deane, H. 1952 Histochemical observations on the ovary and oviduct of the albino rat during the estrous cycle. Am. J. Anat., 91; 363-413.


# TABLE 1.

## EVENTS RELATED TO PREIMPLANTATION HAMSTER DEVELOPMENT

<table>
<thead>
<tr>
<th>EVENT</th>
<th>TIME (hours)</th>
</tr>
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<tr>
<td>Coitus</td>
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<tr>
<td>Ovulation</td>
<td>6-8</td>
</tr>
<tr>
<td>Sperm penetration</td>
<td>6-8</td>
</tr>
<tr>
<td>of zona pellucida</td>
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<tr>
<td>Appearance of 2nd</td>
<td>6-8</td>
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<tr>
<td>Polar Body</td>
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<tr>
<td>Formation of the</td>
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</tr>
<tr>
<td>Male Pronucleus</td>
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</tr>
<tr>
<td>First cleavage</td>
<td>27-38</td>
</tr>
<tr>
<td>spindle</td>
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</tr>
<tr>
<td>2-cell stage</td>
<td>23-48</td>
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<tr>
<td>4-cell stage</td>
<td>48-60</td>
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<tr>
<td>8-cell stage</td>
<td>72</td>
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<tr>
<td>16-cell stage</td>
<td>66-72</td>
</tr>
<tr>
<td>32-cell stage</td>
<td>72-78</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>78-85</td>
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<tr>
<td>Implantation</td>
<td>120-150</td>
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<tr>
<td>FIXATIVE</td>
<td>SOURCE</td>
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<tr>
<td>-------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Bouin's</td>
<td>Humason</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Rossman's</td>
<td>Humason</td>
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<tr>
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</tr>
<tr>
<td>Tellye-</td>
<td>Thomson and</td>
</tr>
<tr>
<td>sniczky's</td>
<td>Brinster</td>
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</tr>
<tr>
<td>Susa's</td>
<td>Humason</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>TREATMENT</td>
<td>NUMBER OF EGGS STAINED/ STAGE</td>
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<tr>
<td>---------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td></td>
<td>Unfert.</td>
</tr>
<tr>
<td>PAS (whole mounts)</td>
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</tr>
<tr>
<td>PAS (sectioned)</td>
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<tr>
<td>Diastase-PAS (whole mounts)</td>
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<td>Diastase-PAS (sectioned)</td>
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<td>Toluidine Blue</td>
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<tr>
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<td>RNAse-Toluidine Blue</td>
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<tr>
<td>Alcian Blue</td>
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<tr>
<td>Sudan Black B</td>
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<tr>
<td>Pyridine-Sudan Black B</td>
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### TABLE 3 (continued)

**STAINING REACTIONS APPLIED TO THE HAMSTER EGGS**

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<th>TREATMENT</th>
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<td>8-cell</td>
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<tr>
<td><strong>PAS (whole mounts)</strong></td>
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</tr>
<tr>
<td><strong>PAS (sectioned)</strong></td>
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</tr>
<tr>
<td><strong>Diastase-PAS (whole mounts)</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Diastase-PAS (sectioned)</strong></td>
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</tr>
<tr>
<td><strong>Diastase-pyridine-PAS</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>Hyaluronidase-PAS</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>Toluidine Blue</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>Hyaluronidase-Toluidine Blue</strong></td>
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</tr>
<tr>
<td><strong>RNase-Toluidine Blue</strong></td>
<td>5</td>
</tr>
<tr>
<td><strong>Alcian Blue</strong></td>
<td>5</td>
</tr>
<tr>
<td><strong>Sudan Black B</strong></td>
<td>3</td>
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<tr>
<td><strong>Pyridine-Sudan Black B</strong></td>
<td>3</td>
</tr>
</tbody>
</table>
PHOTOMICROGRAPHS

(All photomicrographs are magnified x 2552)
PLATE 1. Section of an unfertilized hamster ovum, fixed with Corrosive formol fluid, sectioned and stained with Toluidine Blue. Arrow points to the metachromatic zona pellucida. The nucleus and the cytoplasm are stained orthochromatically.

PLATE 2. Unfertilized hamster ovum fixed with Susa's fixative. The zona pellucida has been removed during fixation. The metachromatic ring does not appear in this photograph.
PLATE 3. Single-cell fertilized egg, prepared as a whole mount specimen, fixed with Telyesnieszky's fixative, and stained with PAS. Note the intense staining throughout the cytoplasm.

PLATE 4. Whole mount preparation of a 2-cell hamster ovum, fixed with Telyesnieszky's fixative, and stained with PAS. The zona pellucida was removed during the fixation.
PLATE 5. Section of a 4-cell hamster ovum, fixed with Bouin's fluid and stained by the PAS reaction. Note the heavy staining zona pellucida and the lighter staining cytoplasm.

PLATE 6. Section of the same 4-cell egg which was treated with diastase for 2 hours at 37° C. Most of the stain has been removed from the zona, while some is retained by the cytoplasm.
PLATE 7. Section of an unfertilized egg fixed in Bouin's fluid and incubated in hyaluronidase for 3 hours at 37° C. There was no effect on the intensity of the PAS reaction.
PLATE 8. Section of an unfertilized hamster ovum fixed with Bouin's fluid and stained with Alcian Blue to demonstrate acid mucopolysaccharides. Note the moderately heavy staining zona pellucida and the heavy outer ring surrounding the cytoplasm.

PLATE 9. Section through one of the two blastomeres of a 2-cell hamster ovum. The section was treated with diastase. Note the decreased cytoplasmic staining.
PLATE 10. Section through a hamster blastocyst which was fixed with Bouin's fluid and stained by the PAS reaction. Heavy staining is present in the zona pellucida (ZP), while the cytoplasm of the embryoblast (E) cells and the trophoblast (T) cells is less intensely stained.
PLATE 11. Section of a hamster blastocyst which was fixed in Susa's fluid and incubated in hyaluronidase for 3 hours at 37° C. Following the enzyme treatment, the section was stained with Toluidine Blue. The dense, round areas represent the orthochromatic (blue) staining nucleoli. No peripheral metachromatic reaction is present.

PLATE 12. Section of a hamster blastocyst which was fixed in Susa's fluid and incubated in ribonuclease (RNase) for 1 hour at 37° C. Following the enzyme treatment, the section was stained with Toluidine Blue. Arrow denotes the dense peripheral metachromatic ring. The zona pellucida is not present.
PLATE 13. Section of a sperm penetrated hamster egg which was fixed in Susa's fluid and stained with Toluidine Blue. The vitelline membrane and peripheral cytoplasm are stained metachromatically. Note the male and female pronuclei which are ready to fuse and complete fertilization.

PLATE 14. Section of an 8-cell hamster embryos which was fixed in Bouin's fixative and stained with Toluidine Blue.
PLATE 15. Section of a 2-cell hamster egg which was fixed in Bouin's fluid. Following fixation, the section was treated with malt diastase for 2 hours at 37° C. After the enzyme treatment, the section was placed in a solution of cold pyridine for 48 hours at room temperature.

PLATE 16. Section of a hamster morula which was fixed in Corrosive formol solution. Following fixation, the section was treated with hyaluronidase for 3 hours at 37° C. and then stained with Toluidine Blue. No metachromatic staining is present.
PLATE 17. Section of an unfertilized hamster egg which was fixed with Bouin's fluid and stained by the PAS reaction. Note the intense reaction in the zona pellucida and the less intense staining in the cytoplasm. The nucleus is not present in this section.
PLATE 18. Section of a 2-cell hamster egg which was fixed in Bouin's solution and stained by the PAS reaction. The zona pellucida is deeply stained while the cytoplasmic reaction is less intense.
The thesis submitted by Hal D. McReynolds has been read and approved by three members of the faculty of the Graduate School.

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

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

05/29/67
Date

Signature of Advisor