Histochemical reactions in vertebrate testes

Larry Fred Cavazos

Iowa State College

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Animal Sciences Commons, Physiology Commons, and the Veterinary Physiology Commons

Recommended Citation

Cavazos, Larry Fred, "Histochemical reactions in vertebrate testes " (1954). Retrospective Theses and Dissertations. 13607.
https://lib.dr.iastate.edu/rtd/13607

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
HISTOCHEMICAL REACTIONS
IN VERTEBRATE TESTES

by

Larry Fred Cavazos

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College

1954
TABLE OF CONTENTS

INTRODUCTION .................................................. 1
REVIEW OF LITERATURE ........................................... 3

Lipids .............................................................. 3
   Definition .................................................... 3
   Histochemistry of fats and lipids ......................... 6
   Lipids in vertebrate testes ................................ 11

Carbohydrates .................................................. 26
   Periodic acid-reactive carbohydrate ....................... 26
   PAS-reactive material in the reproductive organs .... 29

METHODS OF PROCEDURE ........................................ 33

RESULTS .......................................................... 36

Class Mammalia .................................................. 36
   Bull testes .................................................. 36
   Ram testes .................................................. 42
   Rat testes .................................................. 48
   Guinea pig testes .......................................... 53

Class Aves ..................................................... 58
   Chicken testes ............................................. 58

Class Reptilia ................................................... 62
   Horned lizard testes, Phrynosoma cornutum (Harlan) .... 62

Class Amphibia .................................................. 68
   Frog testes, Rana pipiens .................................. 68

Class Pisces .................................................... 73
   Bluegill fish testes, Lepomis macrochirus Rafinesque ... 73

DISCUSSION ....................................................... 77
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
<td>77</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>86</td>
</tr>
<tr>
<td>Chemical nature of Schiff-positive substances</td>
<td>86</td>
</tr>
<tr>
<td>Schiff-reactive material in vertebrate testes</td>
<td>90</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>102</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>104</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>116</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>117</td>
</tr>
<tr>
<td>Lipids</td>
<td>117</td>
</tr>
<tr>
<td>General procedure</td>
<td>117</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>123</td>
</tr>
<tr>
<td>General procedure</td>
<td>123</td>
</tr>
</tbody>
</table>
INTRODUCTION

In recent years biologists have examined physiologic characters of vertebrates as related to their evolutionary significance. As a result of these investigations, homologous as well as analogous physiological and biochemical characteristics have been found which, in some cases, have been as striking as the morphological findings of comparative anatomists. Therefore, in order to gain some understanding of a basic process such as reproduction, it is necessary to study the fundamental biological unit, the cell. In the investigation presented here, the various tissues of the testes of different vertebrates have been examined as to the morphological distribution of their lipid and carbohydrate components. Through a histochemical approach the characteristics of an organ can be related to its structure and composition which otherwise might not be realized in studies employing only cytological, histo­logical, physiological, and biochemical procedures.

In order to demonstrate the lipids present in the vertebrate tissues examined, the following methods were used: Sudan black B, oil red O, the Ashbel-Seligman reaction, the Schultz method for cholesterol, polarization microscopy after digitonin treatment, and Baker's acid hematin test for phospholipids. For the localization of periodic acid-reactive carbo­hydrates, the staining technique was the periodic acid-Schiff method.

The nature and purpose of this investigation is threefold. First, to present an adequate conception of the histochemical morphology of
the testis and spermatogenesis in various classes of a major subphylum; secondly, to make a comparison of testicular morphology and function by histochemical investigations in various species in order to ascertain similarity as well as dissimilarity; and thirdly, to trace the course of evolution of testicular function in the classes studied.
REVIEW OF LITERATURE

Lipids

**Definition**

Lipids are important constituents of cells. For this reason numerous investigations have been carried out either through chemical analyses or histochemical observations in order to determine quantitatively the lipid content or to localize this material within tissues or cells. Therefore, it becomes necessary first to define the term "lipid."

According to Whitehead and Kay (1950), various words have been used to denote fatty substances as a group. These include: fat, lipoid, lipid, lipide, and lipin. When these terms were used in their most restricted sense, the word "fat" indicated neutral fats only, and the term "lipoids" was applied to fatty substances other than neutral fats. These authors stated further that when using this terminology in reference to tissue sections "fats" usually indicated sudanophil substances and "lipoids" denoted sudanophil material that contained cholesterol. According to Lillie (1948), the property of staining with Sudans III or IV has generally come to be designated "sudanophilia."

In this literature review the term "lipid" will be used in the same sense that "lipoid" was employed by Overton (1901), MacLean and MacLean (1927), Baker (1946), and Cain (1950). Therefore, according to Baker (1946), lipoids would refer to fats and all substances which resemble fats in solubility that occur in both plants and animals. Cain (1950)
used the same terminology in denoting all substances which could be extracted from tissue by the usual fat solvents, and which behaved as colloids. MacLean and MacLean (1927) applied the term in reference to ether-soluble substances only. Overton (1901) shared their concept of the term "lipoid."

Cain (1950) stated that all naturally occurring lipoids belonged to one of three main groups. These were composed of: (1) non-cyclic, usually straight-chain hydrocarbon derivatives; (2) the steroids; and (3) derivatives of isoprene, for example, terpenes and the carotenoids.

The substances described as fats, waxes and oils were the general constituents of group (1). The neutral fats were the esters of glycerol and fatty acids and constituted the chief stores of food of most animals. The waxes were esters of aliphatic alcohols other than glycerol with fatty acids. An important aldehyde-containing group in the first division was the substance known as "plasmal," which was freed from the lipoid mixture "plasmologen" and aided in the histochemical identification of the latter. Cain included a subgroup under group (1) and these compounds contained nitrogen and, in some cases, phosphorus. This subgroup included the phospholipoids (lecithins, cephalins, and sphingomyelins), the galactolipines, and some of the chromolipoids.

According to Cain (1950) group (2) included cholesterol, the vitamin D's and their precursors, and the hormones of the gonads, corpus luteum, and the adrenal cortex. The parent compound was perhydro-cyclopenteno-phenanthrene.

The same author included in group (3) of the lipoids those which were the derivatives of isoprene. This parent substance is not found in animals.
The terpenes, of great importance in plants, also were placed into this category. Terpenes present in animals include squalene, in some selachian livers, and the triterpene alcohol, lanosterol, which is present in wool fat. No reports of histochemical studies of animal terpenes were found by Cain (1950).

Gomori (1952a, p. 92), in his book entitled Microscopic Histochemistry, presented a general classification of lipids which included only those which were likely to be found within normal and pathological tissues of man and the common laboratory animals. This author's classification was as follows:

A. Paraffins (petrolatum).
B. Isoprene derivatives (carotenoids). This group includes carotene, vitamin A, visual purple, and some of the pigments of crustaceans.
C. Fatty acids and their derivatives.
   a) Fatty acids.
   b) Soaps, especially those of Ca. Although soaps are insoluble in fat solvents, they are included in this group for reasons of close chemical relationship.
   c) Triglycerids (neutral fats).
   d) Waxes (long-chained alcohol esters of fatty acids).
   e) Phosphatids.
      1. Lecephithins (glycerine esterified with 2 molecules of fatty acid and 1 molecule of phosphorylcholine).
      2. Cephalins (glycerine esterified with 2 molecules of fatty acid and 1 molecule of either phosphorylcolamine or phosphorylserine). Lipositol is a complicated cephalin-like substance with inositol as an additional component.
      3. Plasmalogen (glycerophosphorylcolamine (or choline) in a cyclic acetal linkage with 1 molecule of fatty aldehyde).
      4. Sphingomyelin (sphingosine esterified with 1 molecule of phosphorylcholine and in acylamide linkage with 1 molecule of fatty acid).
   f) Cerebrosides. Sphingosine galactoside or glycoside in acylamide linkage with 1 molecule of fatty acid (examples: kerasin, phrenosin) are the simplest representatives of this group; there are also more complicated ones containing several units of various sugars and unidentified amino acid components. A special group of cerebrosides contains sulfuric acid.
D. Lipid peroxides.
E. Steroids.
   a) Cholesterol.
   b) Cholesterol esters.
   c) Steroid hormones.
F. Group of chemically unidentified lipid pigments. These substances appear to be complicated polymerization products of unsaturated fatty acids. Their solubility in lipid solvents is variable; some of them will resist even embedding in paraffin.

**Histochemistry of fats and lipids**

According to Lillie (1948) one of the oldest methods for the demonstration of fatty substances in tissue sections was the reduction of osmium tetroxide (commonly miscalled osmic acid). Baker (1950) stated that osmium tetroxide was said to have been used in 1849, but that Max Schultze (1865) introduced it into cytological methods in an investigation of the luminous organs of the glowworm. Schultze and Rudneff (1865) noted its capacity to undergo reduction by animal nerves and fats. Osmium tetroxide was reduced to a black substance in the presence of fats and fatty acids, as well as by aleidin and tannin. Myelin was also blackened by osmium tetroxide (Lillie, 1948). In general this substance has not been considered specific, but it does have some value in assisting in the identification of fat-like substances.

Hoerr (1936) reported that, for a study of osmicated lipids, his method consisted of fixing tissue in formal-Zenker's or Regaud's fluid, which he found the most favorable fixative, and then mordanting in 2 percent osmium tetroxide for varying periods. He stated that lipids could reduce osmic acid to a degree, and that they in turn were oxidized and became insoluble in lipid solvents. If the osmication period was extensive,
the lipid could once more become soluble. Therefore, according to this investigator, the time of osmication was highly important. In addition, Hoerr pointed out that osmium tetroxide could undergo reduction by other reducing substances in the tissue, and that it could migrate secondarily and be adsorbed onto a suitable surface.

Lillie (1948) felt that the oil-soluble dye methods were the most satisfactory ways for the demonstration of fatty substances. Daddi (1896) introduced Sudan III for histological purposes. Michaelis (1901) brought into use Sudan IV or Scharlach R. Another excellent stain for fatty substances is Sudan black B which, according to Whitehead and Kay (1950), was introduced by Lison (1934). These authors stated that Sudan stains color fat by dissolving in it and this process involves the passage of the dye from a solvent, alcohol, into the fat in which it is more soluble. Little is known concerning the factors which might influence this transfer.

Daddi's original method (1896) employed a saturated solution of Sudan III in 70 per cent alcohol. Lillie (1948) stated that this technique required a half hour for staining and dissolved out appreciable amounts of fat. Herxheimer's technique (1903) used a saturated solution of Sudan IV in equal mixtures of acetone and 70 per cent alcohol. Nevertheless, according to Lillie and Ashburn (1943) and Lillie (1948) it also removed considerable amounts of the fat present in the tissues.

Lillie and Ashburn (1943), in order to avoid the fat loss associated with the Herxheimer method, developed the technique of using a saturated solution of Sudan IV, oil red 1B, or Sudan brown in 99 per cent isopropyl alcohol. For staining, this stock solution was diluted to a final concentration of 50 or 60 per cent with isopropanol. Frozen sections
were stained for 5 to 10 minutes and counterstained in alum hematoxylin containing 2 per cent acetic acid. French (1926) reported that oil red 0 was superior to Sudan III when used in the Herxheimer technique. Oil red 0 was found to be an excellent fat stain in the Lillie-Ashburn technique (Lillie, 1944). This author pointed out that oil red 4B and Sudan red 4B exhibited a somewhat deeper orange-red or red fat stain than Sudan IV. Sudan II gave a more intense orange-yellow fat stain and was stable longer in dilute isopropanol solutions than Sudan III.

Sudan brown, Sudan brown 5B, and oil brown D were, according to Lillie (1944), satisfactory brownish-red dyes with regard to their intensity and the stability of their dilute isopropanol solutions. Lillie (1945a) developed two new fat stains, Carycinol red and Coccinol red. Lillie felt they gave excellent deep red and deep orange-red colorations in staining periods of 10 to 20 minutes. This author used 4.0 per cent isopropanol as a solvent for these stains. Lillie (1945b), using a 4.0 per cent isopropanol solution, found that oil blue N or NA produced a deep blue coloration with fats.

Popper (1941) has demonstrated the presence of fats in tissues by means of fluorescence. A green fluorescence is produced by vitamin A-containing fats. It is possible, therefore, to differentiate between fats that carry the vitamin and those which are free of it.

Lillie (1948) recommended the use of polarized light for lipid determinations. Neutral fats, when examined in the dark field by crossing the Nicol prisms, remained dark. However, cholesterol esters, phospholipids, and cerebrosides if present appeared as luminous quadrants. This
investigator suggested that sections showing these crystals be compared with paraffin sections after treatment with fat solvents. These crystals may be regarded as lipid in nature if they are absent from sections following such a procedure. According to Prickett and Stevens (1939), polarized light may be used for the study of myelin changes. They found that these alterations could be demonstrated much earlier by this method than by either the Marchi or Sudan III technique.

In 1924 Schults developed a method which would definitely identify cholesterol and, according to Lillie (1948), was an adaptation to histology of the Liebermann-Burchardt sterol reaction. Whitehead and Kay (1950) stated that the technique of Schults (1924) constituted a specific method for steroids. In addition, Lillie (1948) wrote that cholesterol and cholesterol esters reacted with this method. The Windaus digitonin precipitation (Lillie, 1948) was classified as another method for free sterols. According to Lillie, formalin-fixed tissues were cut as frozen sections and placed in a 0.5 per cent solution of digitonin in 50 per cent alcohol. After being rinsed in 50 per cent alcohol, part of the sections were counterstained with hematoxylin, Sudan IV, or oil red 0. Then all the sections were mounted in glycerol. Polarized light with crossed Nicol prisms was used to examine the uncounterstained tissues. If needles or rosettes were observed it was concluded that cholesterol digitonides had been formed. In the counterstained slides, the cholesterol compound remained doubly refractile and did not stain, whereas the cholesterol ester was colored by the dye and had lost its birefringence.

Some fatty substances such as lipofuscin, pigments, lutein, Ciaccio's lipoids, unsaturated fats, and myolins were resistant to extraction by
alcohol, acetone, benzene, and other similar fat solvents and thus they could be demonstrated in paraffin sections with oil-soluble dyes (Lillie, 1948). Some of these substances required chromate treatment in order to render them resistant to fat solvents, whereas others needed only formaldehyde fixation. Ciaccio's method (Ciaccio, 1909) needed both formaldehyde and chromate treatment of fresh or formalin-fixed material. After paraffin embedding, sections were stained in a supersaturated solution of Sudan III or IV, counterstained, and mounted in gum syrup.

According to Baker (1946), in the Smith-Dietrich test the tissues were fixed in formaldehyde and frozen sections were prepared. This was followed by treatment with Kulitschitzky's haematoxylin and differentiation with Weigert's borax-ferricyanide. The mounting medium was levulose syrup. However, Baker felt that the Smith-Dietrich test had three drawbacks: (1) it gave a negative test with pure lecithin; (2) it was not sensitive enough to show lipin in small cell inclusions; and (3) by variation of the differentiation time, positive and negative results could be obtained with the same object. Baker (1946) presented two new histochemical methods: The acid-hematin and the pyridine-extraction procedure. The former gave a positive test for lecithin and was more sensitive. The acid-hematin test presented positive results with both lipines and also certain proteins. Therefore, a pyridine-extraction test was carried out to differentiate between the two groups of substances.

Baker (1944) presented a new method known as the formal-Sudan black technique. The tissues were fixed in an aqueous formalin and calcium chloride mixture. As a lipoid stain, Baker selected Sudan black B in preference to Sudan III or IV because of its greater solubility in lipoids.
other than triglycerides. He used a saturated solution in 70 per cent alcohol and counterstained in Meyer's carmalum. The tissues were mounted in levulose syrup or glycerine jelly.

According to Herman (1950), plasmalogens were demonstrated by the plasmal reaction of Feulgen and Voit. This method required treatment of sections with N/10 mercuric chloride and staining in leuco-fuchsin. Following this treatment the tissues were transferred to a reducing solution consisting of 1 normal hydrochloric acid, 10 per cent potassium metabisulfite, and distilled water. The sections were then washed and mounted in glycerol or glycerine jelly.

Hack (1952) developed a histochemical technique for detection of plasmal in sections prepared from frozen-dried tissues which had been infiltrated by polyethylene glycol. According to this author the cellular localization of plasmalogen and neutral fats was preserved.

Lorrain Smith (1908) introduced Nile blue into histological technique as a method for distinguishing neutral fats (triglycerides) and fatty acids from other lipoids. This method has been considered by some to be of no histochemical value until Cain (1947) reinvestigated the method and concluded that Nile blue could be used to distinguish neutral lipoids (esters and hydrocarbons) from acidic lipoids (phospholipines and fatty acids). Furthermore, he stated that cholesterol could not be detected with this technique.

Lipids in vertebrate testes

Leydig (1850) was the first to describe the presence of characteristic cells among the seminiferous tubules of the testis. These cells later
became known as the Leydig or interstitial cells. Leydig observed that there were present in the intertubular spaces masses of cells containing either colorless or yellowish fatty globules that were unchanged by acetic acid.

According to Hanes (1911), Kolliker completed a careful histological study of the Leydig cells in 1854 and observed the presence of round cells containing vacuoles, fat, and pigment granules. Furthermore, Hanes (1911) reported that fat was a variable constituent of the interstitial cells, though rarely was it entirely absent. It was present in relatively large amounts in the Leydig cells of human beings, dogs, and cats; however, in the interstitial cells of the pig, fat droplets were usually scanty.

In 1902 Ganfini published an investigation on the structure and function of the interstitial cells of the testis, noting the presence of these cells in all vertebrates with the exception of the fish and some amphibians. He stated that, as the species ascended the evolutionary zoological scale, the interstitial cells tended to become individual or to differentiate from the other elements of the testis. This author carried out his extensive work on fish, urodela, amura, reptiles, birds, and mammals, including man. Ganfini (1902) considered the interstitial cells of the testis as true gland elements of which the most important secreted products were granules which reduced osmium and were poured through the lymph into the blood.

Loisel (1903) suggested that the internal secretion of the testis was lipoidal in nature. He studied fat distribution in the testis of the dog, cat, bat, horse, rabbit, and rat. In the adult horse, Loisel found
excessive elaboration of neutral fats. There was an active production of lipids in the interstitial cells as well as in the seminiferous epithelium of this species. This was almost the same condition as those observed in the rabbit, rat, and dog with variations in the quantity of fat elaborated. According to Bouin and Ancel (1903a, 1903b), the interstitial cells in the testes of various mammals studied presented the cytological characteristics of glandular elements. These characters included the structure of the nucleus; the presence of numerous secretory products such as globules and granules, fats, pigments, as well as crystalloids; and, in addition, the existence of a secretory cycle.

Ciaccio (1909) carried out lipoidal studies on mammals and amphibians. Using the Ciaccio method he localized abundant fat in the Sertoli cells of the toad. The spermatogonia showed an endoplasm which was stained and thereby indicated a fatty substance which demonstrated only a few fine lipid granules. He reported that in various classes of vertebrates many germinal cells presented numerous lipid granules and globules, which were particularly abundant during the period of active breeding. More recently, Mancini and Burgos (1948), using Sudan III, obtained a positive lipoidal reaction in the interstitium of the toad testicle. In addition, Sluiter, van Cordt, and Wighorst (1950) reported interstitial cells with many lipoid droplets and mitochondria in the frog, Rana esculenta.

According to Whitehead (1904), no fat was demonstrable with osmic acid or Sudan III in the Leydig cells of pig embryos shorter than 14 centimeters. After this stage it was found in the shape of minute droplets situated in the cytoplasm near the nucleus. However, Whithead (1905) later suggested the possibility that the Leydig cells elaborated a specific
pabulum for the tubules during the development of the testis.

In a later publication, Whitehead (1908) studied the interstitial cells in the rat, grey squirrel, rabbit, opossum, dog, cat, bull, sheep, pig, and man. Osmic acid and oil red O were again employed as the staining agents. Frozen sections of fresh material or of tissue fixed in formalin were used. It was found that fat was not localized in the Leydig cells in sufficient amounts to justify the conclusion that they were simply adipose cells, and in some animals little or no fat was present. Whitehead (1908) pointed out that the histogenesis and arrangement of these cells, their rich blood supply, conformation, and content of specific granules strongly supported the view that they were of a glandular nature.

Whitehead (1912a), using Giaccio's method, carried out a study of the lipid in the interstitial cells of the cat testis. He indicated that the fatty globules of the Leydig cells consisted, for the most part, of phosphatid lipoid material but that cholesterol ester and neutral fat were also present.

Hanes and Rosenbloom (1911), working on normal and cryptorchid testes of the pig, found that by the use of Sudan III or osmic acid large droplets of fat were constantly present in the basal portion of the Sertoli cells. The large droplets responded to all tests for neutral fats. During the spermatogenic process, however, these droplets were broken into smaller ones and passed to the central portion of the Sertoli cells. The small droplets eventually ended in the spermatids. At this stage the spermatid fat failed to stain with osmic acid, but responded to Sudan III. The authors thus concluded that the fat droplets had undergone some type
of chemical change from a neutral fat to a lipoid. In addition, Whitehead (1912b), in an investigation of the Leydig cells, concluded that the granules in the interstitial cells of the pig, and probably of the cat, consisted of a combination, either physical or loosely chemical, of protein with fatty substance.

Loisel (1901) reported two types of epithelial-like interstitial cells in the testicle of the fowl. One of these types elaborated dark insoluble pigments in contrast to the soluble pigments seen in other interstitial cells.

Boring (1912) did not believe that the fat in the chicken testis was formed by the interstitial tissue, but thought it was brought there by circulation and deposited. She felt that "interstitial cells," in the sense that this term had previously been used, were absent from the testis of the domestic chicken. Furthermore, Boring (1912) found no evidence that an internal secretion of any kind was formed by any of the cells of the interstitial tissue.

Pearl and Boring (1912) fed Sudan III to chicks and adult fowls and noted that a portion of the metabolized fat from the food was transported to the ovary or testis and deposited in the interstitial tissue of these organs. These investigators were of the opinion that the amount of such deposited fat appeared to be sufficient to account for the greater portion, if not all, of the fat, which was observed by histological methods, in the interstitial tissue of the sex organs. However, Pearl and Boring (1912) did not feel that deposition of fat in the gonads, as stated above, had any relationship to the functional sexual activity of
these organs. According to these authors, fat deposition in the sex organs began at the time of hatching, whereas sexual activation of the testis and ovary did not begin for several weeks.

Nonidez (1921) also fed young chicks Sudan III and found remnants of the sexual cords in the juvenile testes of the cockerels. These cords appeared as clusters of cells heavily laden with fat which persisted unchanged from hatching to puberty.

Reeves (1915), using Sudan III, found abundant lipid in the interstitial cells of 3-month old cocks. Also, fat was free in the inter-tubular tissue and a small amount was observed in the tubules. However, Reeves reported that less fat was visible in older testes and Leydig cells. Nevertheless, Boring and Pearl (1917), continuing their studies of the reproductive organs of the chicken, pointed out that the characteristic, true interstitial cells were neither a necessary nor a constant element in the constitution of the testes of the male domestic fowl. According to these authors, interstitial cells could be, and usually were, totally absent from the testes of males over 6 months of age and of full sexual maturity.

Benoit (1922), investigating pubertal domestic cocks, reported much fat which was reduced by osmic acid in the interstitial cells. However, towards the end of puberty, there was a definite and profound cytological modification and the interstitial cells took on the aspect of typical glandular cells with rich chondriomes and a fuchsinophile secretion. This type of structure, according to Benoit, was present during the sexual life of the bird.
Benoit and Wenslau (1929) investigated the chemical nature of the fat substances in the interstitial cells of the rooster testis. They reported that these cells were not anisotropic under the polarizing microscope; however, the Windaus-Schultz reaction was positive in the young fowl at puberty and usually negative in the adult. Osmic acid stained the droplets clear brown and Sudan III, as well as Scharlach R, intensely colored the granules. With the Smith-Dietrich test the results obtained by these authors were not positive, but not always entirely negative.

Pfeiffer and Kirschbaum (1943) studied the relation of the hyperplasia of the interstitial cells to secretion of male hormone in the sparrow. No Leydig cells were found during the normal sexual cycle. Nevertheless, following stimulation with pregnant mare's serum (PMS) or hypophyseal extracts, Leydig cells appeared between the seminiferous tubules. In addition, these investigators injected PMS into white Leghorn chicks for 12 to 35 days after hatching. Following this period the classical Leydig cells developed. However, the production of androgen was only moderately accelerated.

Recently, Sluiter and van Oordt (1947) carried out an investigation on the interstitium of the testes of 31 cockerels whose ages varied from 2 to 200 days. Among other procedures, those authors used Sudan III and the Schultz method for their description of the main types of interstitial cells present. They observed lipoid cells which were totally packed with lipoidal globules and which they considered were not directly connected with the production of the male sex hormone, but which might have had a
related secondary function, as cholesterol derivatives were stored in these cells. The other type of interstitial cells present were secretory cells, characterized by the absence of lipid vacuoles and the presence of numerous mitochondria. According to Sluiter and van Oordt (1967), these secretory cells produced the male sex hormone.

In a study of the sexual cycle of a wild bird, *Fulmaris glacialis*, Marshall (1969) described both juvenile Leydig cells, which usually contained lipoid droplets, and lipoid Leydig cells. In the young bird the juvenile cells developed into the lipoid Leydig cells at the approach of sexual maturity. The interstitium of the adult generally consisted of lipid Leydig cells. At all seasons these cells gave a positive reaction with the Schultz test for cholesterol, which corresponded in intensity to the amount of sudanophilic material present.

Lipschultz (1924), in his book entitled *The Internal Secretions of the Sex Glands*, reviewed the general literature on the interstitial cells of the testes.

Pellegrini (1925) attributed the endocrine function of the testis to the interstitial tissue. Using Ciaccio's method for lipid and Sudan III, Pellegrini (1925) examined the testes of representatives of three classes of vertebrates including mammals, birds, and reptiles. This investigator reported that, during the period of seminal maturation, the interstitial cells contained only a small amount of lipid; however, during seminal activity the intercellular and extracellular lipids were abundant.

The lipid distribution in the bull testis was studied by Sorg (1924) using Scharlach R, Nile blue sulfate, Fischler's method, Smith-Dietrich
technique, and the polarizing microscope. He reported the presence of cerbrosides as well as phosphatides in the interstitial cells of the bovine testis. Gresson and Zlotnik (1948) observed osmiophilic granules at the posterior pole of the nucleus of the late spermatid of the bovine, and suggested that this substance probably originated from the Golgi material.

Investigations on fat deposition in the dog have been carried out by Smith (1919), Bell (1929), Collery (1944), and Gresson and Zlotnik (1945). In a study of senile changes in the testes of dogs, Smith (1919) using formalin fixation and staining with Sudan III and Nile blue found that the interstitial cells of the propubic stage contained lipoid droplets with small amounts of neutral fat. The Leydig cells at this stage formed a third of the volume of the testis. In the normal adult dog the Leydig cells were relatively few in number and contained numerous lipoid droplets and vacuoles.

Bell (1929) using Sudan III and Scharlach R contended that the fat which appeared in the spermatid stage of the dog was derived from the breakdown of the lipoidal Golgi material to neutral fat. However, Collery (1944), upon examination of the testes of 20 dogs with the aid of Sudan IV, did not agree with Bell (1929), since both sudanophilic fat and strongly argentophile Golgi remnants were co-existent. According to Collery, no evident changes in volume of the Golgi material were observed in the early stages or later during metamorphosis, as would be expected if fat were derived from this structure. In addition, Collery (1944) reported sudanophilic fat in the cytoplasm of the Leydig cells, in the intermediate neighborhood of the interstitial cells, but not from
the surrounding connective tissue. He illustrated fat droplets apparently migrating across the connective tissue membrane of the seminiferous tubules. Collery (1944) reported that small globules of lipoid were present in the Sertoli cells; none was observed in the spermatogonia or spermatocytes, and some was seen in the cytoplasm of the spermatids. Also, fat was visible in the lumen of active tubules; however, it always remained in close association with the spermatids.

Gresson and Zlotnik (1954) were in agreement with Collery (1944) in that they reported that the Golgi remnant did not give rise to fat in the dog testis. Fat globules appeared in the spermatids soon after the archoplasmic vacuole made its appearance, and a large number of fat droplets were present in the Sertoli cells, which stained black in osmic acid preparations.

A comparative investigation of male germ cells of certain mammals was carried out by Gresson and Zlotnik (1954). Following staining of testicular material with Sudan IV, they reported fat globules in the pig spermatid, Sertoli cell, and interstitial cells. Lipoidal material was visible in the sheep spermatids and Sertoli cells. Fat globules were observed in the Sertoli cells of the dog and cat, and in the rat lipid appeared at an early spermatid stage followed by an increase in size and number of the lipoidal globules. In addition, these authors examined the testes of the rabbit and golden hamster.

The phenylhydrazine technique used by Bennet (1940) was applied to testes of cats, rats, guinea pigs, mice, and rabbits by Pollock (1942). Control sections were extracted with alcohol, acetone, petroleum ether,
or were treated with semicarbazide. Other sections were treated with
digitonin and studied with crossed Nicol prisms. Pollock (1942) con-
cluded that the substance soluble in these solvents and found only in the
interstitial cells was made up of active sterones. Upon treatment of the
section with digitonin, the number of birefringent crystals increased
markedly. According to the author, this indicated the presence of
steroid compounds in the interstitial cells. Pollock (1942) suggested
that this increase in birefringence might be due to cholesterol or some
similar compound.

Albert and Leblond (1946) examined the phenylhydrazine reaction in
tissues whose activity was dependent upon the presence of ketosteroid
hormones. According to Albert and Leblond, the results of Bonnet (1940)
and Pollock (1942) presumably indicated the presence of specific keto-
steroids. Albert and Leblond (1946) used 2,4-dinitrophenylhydrazine (DNPH)
in place of phenylhydrazine. A reaction was obtained with this technique
in the seminal vesicles and the prostate glands of the rat, and a decrease
in staining intensity was observed following castration and adrenalectomy.
As the testes and the adrenal glands are the only known sources of keto-
steroids, the reaction should have disappeared completely (Albert and
Leblond, 1946). According to Albert and Leblond (1946), these results
cast doubt upon the specificity of phenylhydrazine as a test for keto-
steroids.

Albert and Leblond (1946) carried out Feulgen's reaction on the same
stages as the DNPH reaction mentioned above. These investigators found
that the results obtained with DNPH and the Feulgen reaction paralleled
each other. They stated that evidence existed that the plasmalogens produced Feulgen's plasmal reaction, and they concluded that the histo-
chemical reactions obtained with phenylhydrazine were due to agents
effective in producing the Feulgen reaction, namely plasmalogens.

Baker (1946) obtained a positive reaction with the acid hematin
test in the middle piece of the mouse spermatozoon. In addition, the
secretion droplets in the Leydig cells of the testis exhibited a very
intense positive test. Wislocki (1950) applied the Sudan black B reaction
and the Baker's acid hematin test for phospholipids to human spermatozoa.

With the aid of the Sudan black B technique, he was able to localize the
lipids in the middle piece of the sperm where it was oriented in spiral
fashion. Following Baker's method a positive coloration was also
observed in the middle piece, but the spiral filament was not apparent.
Wislocki was able to confirm the findings of Baker (1946) on the mouse
spermatozoa by the application of the acid hematin test.

Using the Sudan black B technique, Nelumpy, Cavasos, and Porter
(1952) observed a uniform positive reaction in the middle piece of bovine
spermatozoa in contrast to the spiraling noted by Wislocki (1950) in
human sperm. In the same investigation, Baker's method was employed in
order to demonstrate phospholipids in the middle piece. These results
were similar to the findings of Wislocki (1950) on the deer and Baker
(1946) and Wislocki (1950) on the mouse spermatozoon.

Asbel and Seligman (1949) in developing a new histochemical method
for the demonstration of carbonyl groups in lipids studied the testes of
one rat, one rabbit, and three dogs and noted a positive reaction in the
form of blue-stained fat droplets in the interstitial cell cytoplasm.
Evidence was presented proposing that the carbonyl-containing lipid in these tissues was ketosteroid. In a later publication Ashbel, Cohen and Soligman (1951) reported examination of the testes of frogs, roosters, mice, rats, rabbits, pigs, dogs, horses, and a goat for the presence of keto-
steroid. In addition, sections were stained with Sudan IV. A positive ketosteroid reaction was observed in the interstitial cells of all species studied with the exception of the rooster, although a sudanophilia was reported in all cases. Ketosteroid as well as a sudanophilia was visible in the frog and pig Leydig cells.

Wislocki (1949) carried out a histochcmical study on the seasonal changes in the testes, epididymides, and seminal vesicles of deer. He compared these reproductive organs in their active and inactive states and thus studied the changes in the lipids, steroid hormones, glycogen, and acid and alkaline phosphatases. Wislocki found that in the fall lipids were present in the interstitial tissues. These tissues were sudanophilic and birefringent, gave a positive plasmal reaction, presented a yellow fluorescence, and were soluble in acetone. According to Wislocki, this combination of reactions indicated that steroid hormones were formed in the Leydig cells. In June, these tests showed a much less intense reaction.

With the aid of Sudan black B, Nile blue, Schiff plasmal, the polarizing microscope, Smith-Dietrich, and Schultz method, McEnery and Nelson (1950) studied the lipids of the rat, mouse, guinea pig, cat, dog, stallion, and human testes. These authors found sudanophilic lipid in the Leydig cells of all species, although quantitative variations occurred. With the exception of the dog and cat, the basal areas of the seminiferous
tubules presented a rich sudanophilia. In the two species mentioned, the reaction was sparse. Birefringence in the interstitial cells varied from little or none in the rat to an abundant reaction in the cat. Testes of all forms contained some Schultz-positive material. The reactions varied from a slight one in a few interstitial cells of the rat to an intense coloration in the cat. In a later paper McEnery and Nelson (1951) carried out cytochemical studies on the testes of rats using Sudan black B, Nile blue, Schiff plasmal, birefringence and the Schultz test. Testicular material from normal, hypophysectomized, cryptorchid, and estrogen-treated rats were examined.

Perlman (1950) with the aid of Sudan black B, the Lieberman-Burchardt test, and birefringence carried out histochemical observations on cholesterol in rat testes. According to Perlman, cholesterol was found in the seminiferous tubules and, to a lesser degree, in the interstitial cells. Perlman did not agree with the findings of Pollock (1942) on the rat. Pollock reported that all of the steroids of rat testes were in the interstitial cells.

In addition, Lynch and Scott (1951) and Scott and Lynch (1952) with the use of oil red O reported that little or no lipid was visible in the interstitial cells of the white rat, whereas moderate amounts were noted in several wild rats investigated. Also, lipoidal droplets were observed in the Sertoli cells of the white rat, and these authors considered the possibility that this might represent a transfer of lipid material, perhaps nutritive, from the Sertoli cell to the spermatozoa.

Winiwarter (1912) carried out a cytological study of the interstitial cells of the human testis and noted the distribution of fats, pigment,
and crystalloids within these cells. He concluded that the interstitial
cells were not fixed and immovable elements, but were subject to fluctua­
tions which paralleled the evolution and involution of the seminiferous
tubules.

Montagna and Hamilton (1951) and Montagna (1952) studied the distrib­
bution of lipids in human testes. They found that lipids, reacting to
Sudan black B and the Schults test for unsaturated steroid, were localized
in the fibroblast-like cells of the interstitial tissue as well as the
Leydig cells. Montagna reported that, although in the deer (Wislocki,
1949) and in the rat (Iynoh and Scott, 1951) the intratubular lipids
appeared to be only within the Sertoli cells, in human testes they were
found in the subnuclear cytoplasm of the spermatogonia, peripheral primary
spermatocytes, and Sertoli cells. The more centrally established secondary
spermatocytes and the centripetally extending Sertoli cell cytoplasm
contained a dust-like sudanophilia.

A histochemical study was carried out by Mancini, Nolazco, and de la
Balze (1952) on testicular biopsies from normal men. These investigators
characterized glycogen, lipids, mucoproteins, ascorbic acid, and alkaline
phosphatase within this tissue. They reported lipids in the spermato­
gonia, primary spermatocytes, and Sertoli cells. Two types of Leydig
cells were observed: immature cells, without steroid but with alkaline
phosphatase, cytoplasmic basophilia, and lipids; and mature or adult cells
with lipids, steroid, ascorbic acid, but without alkaline phosphatase and
cytoplasmic basophilia. The free fibroblast located in the intertubular
connective tissue contained lipids. This is in agreement with the results
of Montagna and Hamilton (1951) and Montagna (1952).
Long and Engle (1952) studied the cytochemistry of the testes of sterility patients and observed that either a diffuse or a droplet type of lipid was present in the Sertoli cells and the interstitium following Sudan black B staining. With the Schultz method, negative results for interstitial cholesterol were obtained in three cases, whereas in six a positive coloration was demonstrated. No definite cholesterol was visible in the tubule cells.

Carbohydrates

Periodic acid-reactive carbohydrate

The principal development of the periodic acid-Schiff technique (PAS)\(^1\) has been attributed to McManus (1946), Lillie (1947a and 1947b), and Hotchkiss (1948). McManus (1946) described the histological demonstration of mucin through the employment of Schiff reagent following periodic acid treatment, and reported that this new method resembled the Molisch's test tube reaction of carbohydrates and mucoproteins. Hotchkiss (1948) critically evaluated the PAS procedure from a chemical standpoint, and found that polysaccharides were oxidized by periodic acid to give polyaldehydes which yielded colored compounds with the Schiff reagent, fuchsin-sulfite.

McManus (1948) reviewed the histological and histochemical uses of periodic acid, and presented for the first time the technique for frozen sections with the PAS technique. McManus reported in his review that periodic acid acted upon the 1,2 glycol linkage (\(-\text{CHOH-CHOH}\) of carbohydrates in tissue sections to produce aldehydes (\(\text{RCHO} + \text{RCHO}\)) which were in turn colored with the Schiff reagent.

\(^1\) Hereafter referred to as PAS.
Lison (1932) pointed out that it was necessary to control the Schiff reaction by use of other techniques which are specific for aldehydes or carbonyl groups. In addition, Jeanloz (1950) questioned the validity of the PAS technique and contended that the presence of two adjacent free hydroxyl groups within the chain of the polysaccharide did not have any relationship to a positive reaction. He considered it unsafe to use this reaction for the identification of polysaccharide structures.

Gersh (1949), using the PAS technique, observed probable glycoprotein granules in the Golgi apparatus of the intestine of both the guinea pig and the rabbit. Also, Loblond (1950), employing the same method, reported a Golgi reaction in the cells of the intestinal epithelium and the male excretory organs. However, Arzac (1950a) did not observe similar reactions with the colored fuchsin technique, and suggested that the discrepancies might have been due to different fixatives used. In a later investigation, Arzac and Flores (1952) reported that a presumptive carbohydrate component had been localized in the Golgi zones of rat, rabbit, and frog organs with three different histochemical methods, following fixation in Orth's fluid. Arzac and Flores (1952) used periodic acid with colored fuchsin, "direct" chromic acid piperazine silver, and periodic acid with leucofuchsins.

According to Pearse (1949 and 1951), following the use of ordinary aqueous fixatives, mucopolysaccharides, mucoproteins, glycogen, glycoproteins, and sugar-containing lipoids remained. Likewise, Pearse (1949) stated that the amount of leuco-dye converted in the periodic acid-Schiff procedure into the red substituted dye was dependent on the amounts of substance containing the glycol structure present in the tissue. Substances which contained less than 1 to 2 per cent of hexose or
hexosamine demonstrated only a faintly positive reaction. Pearse (1951) reviewed the methods employed in current histochemical procedures. Concerning the PAS technique, this investigator reported that 1,2 glycols or amino-glycols were oxidized to dialdehydes by periodic acid, and that carbonyl groups present were converted to the unreactive carboxylic group.

Lillie (1950) recorded a positive PAS reaction after oxidation with periodic acid in a number of tissue elements. In addition, Lillie (1951) carried out a histochemical comparison of the Casella, Bauer, and periodic acid oxidation-Schiff leucofuchsin techniques. He found that all three apparently produced aldehyde from the same general group of substances. However, the chromic acid as well as the potassium permannanate also destroyed the aldehyde which they had produced. Lillie (1951) suggested that the PAS-positive materials which did not present a distinct Bauer or Casella reaction were those with relatively few reactive 1,2 glycol; 1,2 OH,NH₂; or 1,2 OH,NHR groupings per molecule.

Wolman (1950) reported that unsaturated lipids stained with Schiff's reagent after oxidation with periodate. According to Wolman and Greco (1952), histochemical and chemical evidence indicated that formaldehyde combined with unsaturated lipids at the double bond, thus forming a free carbonyl group which probably originated from the formaldehyde. These authors reported that this reaction was visualized by the Schiff reagent as well as by the Ashbel-Seligman procedure.

In 1951, Clegg, Clermont, and Leblond presented a modified Hotchkiss spot test as an aid in the interpretation of the PAS technique, and they found that their results were in agreement with Hotchkiss' (1948) interpretation of the reaction. Following a later investigation, Clegg,
Clermont, and Leblond (1952) reported on the use of lead tetraacetate, benzidine, o-dianisidine, and a "film test" in an investigation of the PAS technique. It was concluded that the PAS procedure consisted of an oxidation of 1,2 glycols and α-amino alcohols to produce aldehyde groups which were in turn stained by the Schiff reagent. The "film test" demonstrated that these radicals were present in certain polysaccharides, mucopolysaccharides, and mucoproteins.

Hashim (1952) has included the PAS technique in a literature review of the histochemical methods employed for the identification of polysaccharides and their derivatives.

**PAS-reactive material in the reproductive organs**

Mancini and Burgos (1948) described mucopolysaccharide distribution in the toad testicle at rest, during sexual activity, and after injection of gonadotrophic hormones. The mucopolysaccharides were localized by the positive reaction to PAS method, metachromatic techniques, and a photochemical procedure.

In a histochemical investigation of the seasonal alterations in the testis of the deer, Wislocki (1949) described the acrosome and acroblast in deer spermatids following the PAS technique. In addition, the head of deer sperm stained with this method. In a later study, Wislocki (1950) applied various cytochemical reactions to human spermatozoa and seminal plasma, and observed that the seminal plasma was strongly PAS-positive but that the spermatozoa were negative. Wislocki (1950) presumed the reactive substance in the seminal plasma to be a mucopolysaccharide, in view of the fact that known mucoproteins stained relatively faintly.
Elftman (1950) carried out a study of spermatogenesis in the mouse and in man through oxidation by means of periodic acid as well as by other methods. This author reported that periodic acid oxidized constituents of the acrosome, idiosome, cytoplasmic granules in the germ cells as well as the Sertoli cells and mitotic spindles.

Leuchtenberger and Schrader (1950) and Schrader and Leuchtenberger (1951) reported that the acrosome of the sperm of Arvelius albopunctatus, after staining with the PAS technique, contained a polysaccharide with a 1,2 glycol grouping which was neither starch, glycogen, nor hyaluronic acid. Likewise, those investigators observed a Schiff-positive reactive acrosome in bull and snake sperm. The latter demonstrated PAS-positive material at the extreme tip, in contrast to the former in which the carbohydrate containing acrosome covered the anterior two-thirds of the head. The observations of Leuchtenberger and Schrader (1950) and Schrader and Leuchtenberger (1951) on bull spermatozoa were confirmed by Melampy, Cavazos, and Porter (1952) who carried out a cytochemical investigation of bovine spermatozoa.

Leblond, Clermont, and Cimon (1950), Leblond (1950), and Clermont and Leblond (1950) reported the presence of periodic acid-reactive carbohydrates in the head cap and acrosome of rat spermatozoa. These investigators suggested a relationship between the reduction of staining intensity of the acrosome and the release of the sperm from the Sertoli syncytium. According to Leblond (1950) the presence of reactive material in the acrosome and the sperm head made it possible to trace these structures back to the Golgi material of young spermatids.
Clermont (1951) applied the PAS reaction as a method for demonstration of the formation of the acrosome and head cap of sperm in mammals. This investigator described four stages for the spermiogenesis of the guinea pig, which included a Golgi, a cap, an acrosome, and a sperm maturation stage. He reported that, in other species examined, the same four stages were recognized; however, in the final two, the shape of the acrosome varied with the species.

With the PAS technique, Leblond and Clermont (1952a) followed the spermiogenesis of the rat, mouse, hamster, and guinea pig. Polysaccharide-reactive material was present in the head cap and acrosome of the spermatids. The critical staining of this method permitted a detailed description of the development of these structures, which were traced back to Golgi material. In the species examined by these investigators, it was found that spermiogenesis could be subdivided into the same four stages reported by Clermont (1951). Leblond and Clermont (1952b) published a detailed account of the spermiogenesis of the rat as demonstrated by the PAS technique. Likewise, these investigators described the cell types found in the seminiferous epithelium of rat testes.

In a preliminary report, Cavazos and Melampy (1951) noted the distribution of periodic acid-reactive carbohydrates in the testes of the bovine, rat, chicken, horned lizard, frog, and bluegill fish. The staining method was the PAS technique following Orth's fixative.

Glycogen distribution in human testicular biopsy material was studied by Arzac (1950b) with, among other methods, the aid of the PAS technique. It was found that appreciable but moderate quantities of
of glycogen were present in the cytoplasm of the Sertoli cells from normal specimens. According to Arzac, no glycogen was present in other cells of either the normal or pathologic biopsies, and, in complete atrophy, there was practically no glycogen.

In a histochemical investigation of the human testis with methods which included the PAS technique, Montagna and Hamilton (1952) and Montagna (1952) reported that glycogen was present in the spermatogonia, small and growing primary spermatocytes, and in Sertoli cells. However, it was absent in the mature primary spermatocytes, secondary spermatocytes, and spermatids. Also, glycogen was localized in the epithelium of the tubuli recti, rete, and ductuli efferentes. In the ductuli epididymides, glycogen was observed only in the basal pyramidal cells. Within the Leydig cells as well as in the epithelium of the ductuli efferentes and ductuli epididymides, Schiff-reactive, diastase-resistant granules were visible.

Elftman (1952) oxidized sections of human testes with periodic acid and stained them with leuco-fuchsine. He observed glycogen in the Sertoli cells, spermatogonia, and the primary spermatocytes. The acrosome of the developing spermatid contained material which was oxidized by periodic acid but which was not glycogen. Long and Engle (1952) confirmed the presence of glycogen in the Sertoli cells, spermatogonia, and early primary spermatocytes, which were reported as being present by Montagna and Hamilton (1952), Montagna (1952), Elftman (1952), and Mancini, Nolazco, and de la Balze (1952).
METHODS OF PROCEDURE

Testicular tissue of sexually mature males representing the classes Mammalia, Aves, Reptilia, Amphibia, and Pisces of the subphylum Vertebrata were studied as to the morphological distribution of lipids as well as periodic-reactive carbohydrates. In the class Mammalia, testes were fixed from six bulls, four rams, eight rats, and four guinea pigs. Five New Hampshire reds and two White Leghorn roosters represented the Aves. In Reptilia, 17 horned lizards, Phrynosoma cornutum (Harlan), were prepared for study. Twenty-two grass frogs, Rana pipiens, and 12 bluegill fish, Lepomis macrochirus Rafinesque, were used as examples of the classes Amphibia and Pisces, respectively.

In order to demonstrate the lipoidal components of the vertebrates investigated, testes were fixed in 10 per cent neutral formalin, washed in tap water for six hours, placed in gelatine at 37°C overnight, embedded in 10 per cent gelatine, and hardened in the refrigerator. The blocks of tissue were then cut out and left in neutral formalin for several hours in order to make the gelatine insoluble in water. Sections were cut at ten microns on a freezing microtome, and the following histochemical methods were applied: (1) Sudan black B, using the procedure according to Herman (1950). (2) Oil red O counterstained with Mayer's hemalum (Lillie, 1948). (3) The Ashbel-Seligman reaction for carbonyl groups as outlined by Seligman and Ashbel (1952). (4) For cholesterol and its esters, the Schultz reaction was employed (Gomori, 1952a).
(5) The polarizing microscope was used to study birefringence according to Gomori (1952a). Frozen sections were treated with digitonin, which forms insoluble, anisotropic esters with cholesterol and other \( \beta \)-steroids. These digitonides are birefringent.

Control sections for these lipid procedures were prepared by treatment with an equal mixture of ethyl ether and chloroform at room temperature for periods of 30 minutes to 48 hours. Sections to be used as controls for polarization microscopy were extracted in acetone overnight prior to treatment with digitonin.

Testicular material, from all species except fish, was fixed in formaldehyde calcium and other in weak Bouin's fluid as recommended by Baker (1946) for the acid hematin test for phospholipids. Sections were cut at five microns on a freezing microtome.

For the localization of periodic acid-reactive carbohydrates, testes were fixed in Orth's fluid for two days, washed overnight with tap water, dehydrated and cleared in dioxan, and embedded in paraffin. Sections were cut at seven and ten microns. The staining method was the PAS technique as developed by McManus (1946) and Hotchkiss (1948). In some instances, the reducing rinse recommended by the latter author was omitted following the suggestion of Leblond and Clermont (1952a) who stated that its only effect was to decrease the brilliance of the purple stain. Three types of controls were employed as described by Leblond (1950). These consisted of: (1) hydrolysis in salivary amylase for one hour at 37°C in order to remove glycogen, (2) extraction in
equal parts of methanol and chloroform for 16 hours at 58°C for removal of glycolipids, and (3) staining without previous treatment in periodic acid. Reactions were considered positive only if they stained after periodic acid oxidation. Counterstains employed included Delafield's hematoxylin, methyl green, and light green. As an aid in the study of general morphology, sections from each species were prepared with Delafield's hematoxylin and eosin y. On occasions, material was stained with Heidenhain's iron hematoxylin.

Details of fixation, embedding, staining reactions, and mounting methods are presented in the APPENDIX.
RESULTS

Class Mammalia

Bull testes

**Sudan black B.** Following the application of the Sudan black B technique to bovine testes, it was seen that the nuclei of the interstitial cells were unstained, whereas the cytoplasm showed rather heavy concentrations of lipid in the form of fine and large granules (Fig. 91). As indicated in Table 1, many of the larger droplets were vacuolated. The plasma within blood vessels demonstrated a few fine granules, but the erythrocytes failed to give a reaction. Only slight staining, in fine droplet form, was noted on the basement membrane of the seminiferous tubules. Fine and large granules, some vacuolated, were present in the cytoplasm of Sertoli cells as shown in Figure 92; however, the nuclei and nucleoli remained unstained.

Within the seminiferous tubules, the spermatogonia and primary spermatocytes demonstrated a rather strong reaction to Sudan black B. These cells exhibited fine droplets which at times were diffuse. The presence of an unstained area was noted adjacent to the nucleus in the spermatocyte cells. This region was generally ringed or partially enclosed by sudanophilic material. With the exception of the size differential, primary and secondary spermatocytes reacted to the lipid stain with about the same intensity.

Early stages of spermatids demonstrated the same ring of lipid about
<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B</th>
<th>Oil red O</th>
<th>Ashbel-Seligman reaction</th>
<th>Baker reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated</td>
<td>Type of</td>
<td>Estimated</td>
<td>Type of</td>
</tr>
<tr>
<td></td>
<td>amount</td>
<td>droplet</td>
<td>amount</td>
<td>droplet</td>
</tr>
<tr>
<td>Interstitial</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Fine</td>
</tr>
<tr>
<td>cell cytoplasm</td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vacuolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basement membrane</td>
<td>+</td>
<td>Fine</td>
<td>**</td>
<td>Fine</td>
</tr>
<tr>
<td>Sertoli cell cytoplasm</td>
<td>****</td>
<td>Fine</td>
<td>****</td>
<td>Fine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vacuolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Unreactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vacuolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary spermatocyte</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Unreactive</td>
</tr>
<tr>
<td>cytoplasm</td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary spermatocyte</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Unreactive</td>
</tr>
<tr>
<td>cytoplasm</td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Unreactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vacuolated</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Technical Localisation of Lipids in Bovine Testes

<table>
<thead>
<tr>
<th>Ashbel-Seligman reaction</th>
<th>Baker’s acid hematin</th>
<th>Schultz reaction</th>
<th>Polarization microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Type of amount droplet</td>
<td>Estimated Type of amount droplet</td>
<td>Estimated Type of amount droplet</td>
<td>Birefringent crystals</td>
</tr>
<tr>
<td>++ Fine</td>
<td>++ Fine</td>
<td>+ Diffuse</td>
<td>Absent</td>
</tr>
<tr>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td>Unreactive</td>
<td>++ Fine, and occasionally large</td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+ Fine</td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+ Fine</td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+ Fine</td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+ Fine</td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
</tbody>
</table>
the unstained region, and occasional vacuolated or fine lipoid droplets were dispersed in the cytoplasm. During later developmental stages the cytoplasm commenced to pass from the nucleus and carried Sudanophilic material with it.

**Oil red O.** The distribution of lipid material reacting to oil red O was observed scattered in the cytoplasm of the interstitial cells of bull testes as fine or large granules (Table 1). As was the case in other species studied, the amount of lipid within individual cells of this type was quite variable, but the over-all reaction was strong. A few fine granules of lipoidal material were often noted in the serum of the blood elements remaining in some of the vessels, and the basement membrane surrounding the seminiferous tubules. Adjacent to this area were observed the Sertoli cells, which demonstrated numerous fine as well as large lipoidal droplets in their cytoplasm. The cytoplasmic strands of these cells extended well into the center of the tubule and contained clustered spermatozoa. It was especially in this region of sperm lodging that large amounts of oil red O-positive material could be seen. A few fine lipoidal granules were noted in the cytoplasm of the spermatogonial cells and primary spermatocytes. However, in the case of the latter cell type, a diffuse cytoplasmic coloration was also seen. Much the same was true for secondary spermatocytes. Fine and diffuse granulation, with an occasional large droplet, was seen in the cytoplasm of these cells. An unstained area was visible in the juxtanuclear position. Generally, as in primary spermatocytes, this zone was surrounded by lipoidal material.

The cytoplasm of the spermatids demonstrated fine as well as large lipid droplets. However, the quantity of lipid was variable in these
cellular types. Many large and some fine granules were noted in the cytoplasm at this time. These cells were oriented towards the basement membrane of the tubule and appeared to cluster in the Sertoli cells during subsequent developmental stages. In addition, large or fine lipid droplets were seen during the caudal movement of the cytoplasm at the time of spermiogenesis.

Ashbel-Seligman reaction. Following the application of this method to bull testes, it was observed that a few fine granules of reactive substance were present in the cytoplasm of some of the interstitial cells. These droplets were bluish-black in color. No other cellular components of bovine testis demonstrated a positive reaction.

Schultz reaction. Very few cells of bull testes gave the blue-green coloration indicative of a positive reaction. These instances were limited to some of the interstitial cells which demonstrated a diffuse coloration.

Polarization microscopy. As indicated in Table 1, no birefringent crystals were present in bull testes.

Baker's acid hematin test. A positive reaction indicating the presence of phospholipids was noted in the cytoplasm of the interstitial cells of bovine testes. However, the quantity of reactive substance in these cells was quite variable. The nucleus and nucleolus were unstained. An intense reaction was visible in the stroma of the erythrocytes, but occasionally unstained cells were seen. No phospholipid was found in the basal areas of the seminiferous tubules, but fine cytoplasmic granules were observed around the unstained nucleus of the Sertoli cells. However,
in their cytoplasmic extensions, an intense reaction was localized in the region of maturing spermatozoa. Spermatogonia contained a few droplets of bluish coloration in the cytoplasm as did the spermatocytes and spermatids.

**PAS technique.** In a study of this reaction in bull testes, it was observed that the cytoplasm of the interstitial cells, walls of blood vessels, lamellated connective tissue, and basement membrane of the seminiferous tubules gave a positive PAS reaction. Scattered throughout the interstitial cell cytoplasm were numerous brilliant granules whose staining intensity was not decreased by saliva or methanol-chloroform treatment. Within the seminiferous tubules, the long cytoplasmic strands of the Sertoli cells demonstrated a positive reaction for carbohydrate, whereas the nuclei were unstained. Schiff-positive granules were scattered throughout the faintly staining cytoplasm, and spermatozoa were often lodged in these cells. Spermatogonia did not demonstrate a Schiff-positive reaction.

The development of the acrosome was traced as far as its earliest indication in the Golgi region of the primary spermatocytes. Although the nuclei and cytoplasm of these cells were unstained, it was noted that a faintly reactive area was present in the Golgi zone. This material was comparable to the idiosome of the Golgi zone, and was also observed in the spermatids as a faintly stained area in the juxtanuclear position (Fig. 1). The cytoplasm of the spermatids was rather weakly reactive, whereas the nucleus remained unstained. In later developmental stages, the idiosome gave rise to the proacrosomic granules, which were
considered the precursors of the acrosome (Fig. 2). The number of granules within the idiosome varied, but did not exceed two or three. As illustrated in Figure 3, there were other granules of a carbohydrate nature present in the cytoplasm outside of the idiosome. Aggregation of proacrosomic granules took place within the idiosome, and the newly formed acrosomic granule, larger in size than those previously described, moved adjacent to the nucleus, as shown in Figures 4 and 5. As development proceeded, the newly formed acrosomic granule gave rise to PAS-positive material which spread over a small portion of the still rounded nucleus. Nevertheless, the acrosomic granule remained within the idiosome (Figs. 6 and 93). As illustrated in Figures 7, 8, 9h, and 95, at a later stage the acrosomic granule was freed of the idiosome, and the spreading of carbohydrate material over the nucleus continued. During these stages (Figs. 7 and 8), an aggregation of reactive material was seen in the cytoplasm which was in the process of passing toward the caudal portion of the cell. This might have represented excess Golgi material which eventually contributed, in part, to the formation of the protoplasmic droplet often seen in ejaculated immature spermatozoa. As shown in Figures 9, 10, 96, and 97, spreading of carbohydrate material and nuclear elongation took place. In the latter process, the excess PAS-positive substance was present near the apical end of the cell. As nuclear elongation proceeded, the acrosome became evident, but the bead remained in its original position. Also, as shown in Figures 10 and 97, the Schiff-positive material spread over the anterior two-thirds of the nucleus. The cytoplasm commenced to pass from the nucleus in a caudal direction, and
the material contributing to the formation of the protoplasmic droplet remained massed at the basal portion of the nucleus. Figure 11 illustrates the partial flattening of the nucleus during this stage of spermatid metamorphosis. Figure 12 is differentiated from Figure 10 by further flattening of the nucleus and by the reduction of the acrosomic bead in both size and staining intensity. Many of the spermatozoa lodged within the Sertoli cell cytoplasm were noted as represented in Figure 13. The cytoplasm continued caudally over the tail, and remnants of PAS-reactive material massed to form the Schiff-positive protoplasmic droplet. This structure, in addition to the acrosome, was the only Schiff-reactive carbohydrate material present in bovine spermatozoa (Figs. 14, 15, and 98).

Ram testes

Sudan black B. The presence of lipids was observed with this method in the cytoplasm of the interstitial cells of ram testes as shown in Figure 99. Although the amount was variable, most of these cells contained numerous droplets which ranged in size from fine to large. Whereas the cytoplasm of the interstitial cells demonstrated sudanophilia, the nucleus and nucleolus remained unreactive. A fine lipoidal granulation was noted in the walls of arterioles and venules. Plasma within these vessels also contained fine lipid droplets, whereas the erythrocytes demonstrated none. Extraction for 16 hours in ether-chloroform mixture removed most, but not all, of the reacting lipids in the interstitial cells and blood vessels. A few fine granules of lipid were present in the basement membrane of the seminiferous tubules, but a
heavier concentration of droplets was dispersed about the tubular periphery. Many of these lipoidal droplets were vacuolated.

Close to the basement membrane were noted the Sertoli cells extending into the lumen of the tubule. As indicated in Table 2, these cells demonstrated a strong cytoplasmic reaction in the form of fine as well as large droplets which were principally in the region containing sperm. As in the other species studied, the nucleolus and nucleus were unreactive. Treatment with the fat solvent removed most of these lipids.

Spermatogonia were seen in the vicinity of the tubule periphery and were noted to contain a few large lipid globules as well as a diffuse cytoplasmic coloration. Primary spermatocytes presented numerous large as well as diffuse lipoidal granules. In some instances, a clear unstained zone was distinguished in the cytoplasm containing one or two fat-staining droplets. The lipid distribution of secondary spermatocytes was quite similar to that of the primaries in that a moderately strong reaction was noted in the cytoplasm. In some secondary spermatocytes, a small round sphere in the juxtanuclear position reacted more intensely to the Sudan black stain than did the cytoplasm. Also, the unstained clear zone mentioned above was often visible in these cells.

In early stages of spermiogenesis it was noted that the strong cytoplasmic lipoidal reaction was rather evenly dispersed about the unstained nucleus and that occasionally an unstained region was visible adjacent to this structure. The fat globules appeared as fine, diffuse and moderately large cytoplasmic inclusions. In many of these cells large aggregations of lipoidal material were localized in the Golgi region. In later stages, but prior to elongations, most of the spermatid cytoplasm passed in a
Table 2. Histochemical Localization of Lipids

<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B</th>
<th>Oil red 0</th>
<th>Ashbel-Seligman reaction</th>
<th>Baker's hematoxylin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated amount</td>
<td>Type of droplet</td>
<td>Estimated amount</td>
<td>Type of droplet</td>
</tr>
<tr>
<td>Interstitial cell cytoplasm</td>
<td>*** Large</td>
<td>Fine</td>
<td>*** Large</td>
<td>Diffuse</td>
</tr>
<tr>
<td>Basement membrane</td>
<td>±± Fine</td>
<td></td>
<td>±± Fine</td>
<td></td>
</tr>
<tr>
<td>Sertoli cell cytoplasm</td>
<td>*** Large</td>
<td>Fine</td>
<td>*** Large</td>
<td>Fine</td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>±± Large</td>
<td>Diffuse</td>
<td>±± Diffuse</td>
<td>Large</td>
</tr>
<tr>
<td>Primary spermatocyte cytoplasm</td>
<td>±± Large</td>
<td>Diffuse</td>
<td>±± Diffuse</td>
<td>Fine</td>
</tr>
<tr>
<td>Secondary spermatocyte cytoplasm</td>
<td>±± Fine</td>
<td>Diffuse</td>
<td>±± Fine</td>
<td>Diffuse</td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>*** Diffuse</td>
<td>Fine</td>
<td>*** Large</td>
<td>Fine</td>
</tr>
</tbody>
</table>
### Chemical Localization of Lipids in Ram Testes

<table>
<thead>
<tr>
<th>Ashbel-Seligman reaction</th>
<th>Baker's acid hematin</th>
<th>Schults reaction</th>
<th>Polarization microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Type of droplet</td>
<td>Estimated Type of droplet</td>
<td>Estimated Type of droplet</td>
<td>Birefringent crystals</td>
</tr>
<tr>
<td>Fine</td>
<td>Fine</td>
<td>Fine</td>
<td>Diffuse</td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td>Very fine birefringent crystals throughout the tubules and interstitium. However, slides treated with acetone first showed the same birefringence</td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
</tbody>
</table>
caudal direction carrying with it much of the Sudan black-reactive material.

Oil red O. Numerous granules of oil red O-stained material were dispersed in the cytoplasm of the interstitial cells (Table 2). Diffuse, fine, and large droplets were seen with variable amounts in these cells. Generally a discrete coloration was noted in the walls of arterioles and venules. Along the basal areas of the seminiferous tubules were observed varied deposits of lipoidal material. The Sertoli cells of ram testes contained a cytoplasmic sudanophilia especially in the region of maturing spermatocytes. Spermatogonia demonstrated a diffuse cytoplasmic staining with occasional larger droplets. Primary and secondary spermatocytes were observed to contain comparably reactive diffuse and fine cytoplasmic granules. Spermatids, possibly because of their small size, contained a greater quantity of oil red O-reacting material.

Ashbel-Seligman reaction. A fine, weak, bluish granulation was observed in the cytoplasm of some of the interstitial cells of ram testes following this method. However, the amount of reactants was quite variable and differed from cell to cell. In many cases the interstitial cells did not demonstrate a positive reaction.

Schulte reaction. As shown in Table 2, a weak diffuse cytoplasmic coloration was observed in some of the interstitial cells.

Polarization microscopy. Very fine birefringent crystals were noted throughout the tubules and interstitium. However, slides treated with acetone prior to digitonin demonstrated equivalent birefringence.

Baker's acid hematin test. The interstitial cell cytoplasm demonstrated a weak, fine granulation, whereas the nucleolus and nucleus were unreactive. Many of the erythrocytes seen within the blood vessels were
deeply stained; however, in other cases they did not show a positive coloration. The basement membrane of the seminiferous tubules showed a very fine granulation. A fairly strong reaction in the form of some fine as well as a few large droplets was visible in the Sertoli cell cytoplasm, but a somewhat stronger reaction was noted in the region of the cell which contained lodged spermatozoa. Spermatogonia, primary as well as secondary spermatocytes, and spermatids contained a few droplets of phospholipid in their cytoplasm (Table 2).

**PAS technique.** A faint cytoplasmic reaction was noted in the interstitial cells of ram testes after the PAS method. In some cases fine granules of Schiff-positive material were dispersed in the cytoplasm of these cells; however, treatment with saliva or methanol-chloroform did not remove them. The walls of arterioles and venules within the interstitium demonstrated an intense carbohydrate reaction as did the basement membrane of the seminiferous tubules. A strong coloration was visible in the Sertoli cell cytoplasm, and in many instances the cytoplasm of these cells contained prominent, brightly-staining granules which were not hydrolyzed by saliva nor were they of a glycolipid nature. The cytoplasm of spermatogonia and spermatocytes was weakly stained to a comparable degree. However, in the latter cells the precursor of the acrosome, the idiosome, was often seen in these preparations as a faintly reactive area adjacent to the nucleus and in the region of the Golgi apparatus.

With the aid of the periodic acid-Schiff technique, the alterations taking place during spermiogenesis in the ram were followed. As shown in Figure 16 the idiosome of the ram was not as homogeneous as that of other species studied, for a diffuse granulation was often seen. In Figures 17
and 18 these granules became more pronounced becoming the proacrosomic granules and in some cases 6 to 8 were present in the idiosome. In a later developmental stage they seemed to coalesce (Fig. 19) and eventually formed the single, rather large, acrosomic granule as illustrated in Figure 20. At this time the acrosomic granule was seen adjacent to the nucleus but still within the idiosome. As shown in Figure 21 the idiosome commenced to pass from the acrosomic granule, but, before this movement was completed, PAS-positive material had begun the formation of a cap-like structure over the rounded spermatid nucleus (Fig. 22). This activity continued until a major portion of the anterior part of the nucleus was covered (Figs. 23, 24, 100, and 101). In some instances the idiosome and other granules of carbohydrate-reactive material were dispersed in the cytoplasm. Also, it was observed that the cytoplasm had commenced to pass in a caudal direction (Fig. 24). After this stage a gradual nuclear elongation took place; however, the acrosomic granule remained quite prominent as illustrated in Figures 25, 26, 27, and 28. During this time of elongation and caudal cytoplasmic passage, the nucleus became somewhat flattened, as shown in Figure 28, which is a side view of Figure 27. Schiff-positive substances, probably formed by remnants of the Golgi material, were often massed in the caudal regions of these cells. Following the dissolution of the acrosomic granule, the only PAS-substances in ram spermatozoa were the faintly staining acrosome and the protoplasmic remnant located on the tail (Figs. 29 and 30). Figure 30 was prepared from a side view of spermatozoa within the seminiferous tubules.
Rat testes

Sudan black B. The presence of Sudan black B-reactive lipid was observed in the cytoplasm of the interstitial cells of the rat. As shown in Figure 102, the degree of sudanophilia in these cells varied considerably. In some cases the interstitial cytoplasm was filled with fine or large lipoidal globules which were sometimes vacuolated (Table 3), whereas, in others, none or only small amounts were present. Nuclear and nucleolar material were unstained. Within large blood vessels, the presence of lipid material was observed. In this case the chief distribution was in the leucocytes and plasma of the blood, whereas the erythrocytes were unreactive.

The basal areas of the seminiferous tubules showed a sudanophilia in the form of large as well as fine globules (Table 3). In the long cytoplasmic strands of the Sertoli cells were fine granules of Sudan-positive material. The nuclei and nucleoli of these cells were unreactive. Fine and diffuse cytoplasmic granules of sudanophilic material were present in the spermatogonia and spermatocytes. However, those regions of reactivity were quite sparse. There was a positive coloration often observed in the Golgi region of the spermatids. This stained mass appeared to be formed by the aggregation of lipoid droplets. Nevertheless, other fine cytoplasmic globules were present.

Oil red O. Following the use of this method, varying amounts of sudanophilic material were present in the interstitial cells of the rat. As indicated in Table 3, this substance was dispersed in the cytoplasm and appeared principally as diffuse staining, but numerous fine as well
Table 3. Histochemical Localization of Lipids in Rat

<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B</th>
<th>Oil red O</th>
<th>Ashbel-Seligman reaction</th>
<th>Baker's acid hematin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated amount</td>
<td>Type of droplet</td>
<td>Estimated amount</td>
<td>Type of droplet</td>
</tr>
<tr>
<td>Interstitial cell cytoplasm</td>
<td>+++</td>
<td>Fine</td>
<td>+++</td>
<td>Large</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Often</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vacuolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basement membrane</td>
<td>+++</td>
<td>Fine</td>
<td>+++</td>
<td>Large</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Often</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vacuolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sertoli cell cytoplasm</td>
<td>+++</td>
<td>Fine</td>
<td>+++</td>
<td>Fine</td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
</tr>
<tr>
<td>Primary spermatocyte</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
</tr>
<tr>
<td>cytoplasm</td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary spermatocyte</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
</tr>
<tr>
<td>cytoplasm</td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
</tr>
</tbody>
</table>
Istochemical Localisation of Lipids in Rat Testes

<table>
<thead>
<tr>
<th>Ashbel-Seligman reaction</th>
<th>Baker's acid hematin</th>
<th>Schults reaction</th>
<th>Polarization microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>of droplet</td>
<td>Estimated amount</td>
<td>Type of droplet</td>
<td>Estimated amount</td>
</tr>
<tr>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
</tr>
<tr>
<td>Unreactive</td>
<td></td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td>Occasional-</td>
<td>Fine</td>
<td>Fine</td>
<td>Absent</td>
</tr>
<tr>
<td>Unreactive</td>
<td>++</td>
<td>Fine</td>
<td>Unreactive</td>
</tr>
<tr>
<td>Unreactive</td>
<td>++</td>
<td>Fine</td>
<td>Unreactive</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+</td>
<td>Fine</td>
<td>Unreactive</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+</td>
<td>Fine</td>
<td>Unreactive</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+</td>
<td>Fine</td>
<td>Unreactive</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+</td>
<td>Fine</td>
<td>Unreactive</td>
</tr>
<tr>
<td>Unreactive</td>
<td>+</td>
<td>Fine</td>
<td>Unreactive</td>
</tr>
</tbody>
</table>
as large granules were also observed. However, the amount reacting to the fat stain proved to be quite variable from cell to cell.

Oil red 0-positive globules were noted in the plasma of blood remaining in the arterioles and venules. In the basal areas of the seminiferous tubules, including the lamellated connective tissue, large and fine fat droplets were present. The cytoplasm of the Sertoli cells demonstrated a positive reaction in the form of a fine granulation. Spermatogonia, and primary as well as secondary spermatocytes, were almost equally stained by the oil red 0. Most of these cells contained either fine globules, a diffuse sudanophilic reaction, or a combination of the two. Within the cytoplasm of the spermatids many finely granulated lipid droplets were seen, whereas the nucleus and nucleolus were unreactive. On occasions, in the region of the Golgi apparatus, some of these cells contained a bead-like cluster of small lipid granules.

Ashbel-Seligman reaction. Reactive material was observed in the interstitial cells of rat testes (Fig. 103). It appeared as fine dark granules scattered within the cytoplasm. The nucleus and nucleolus were unstained. As in the case of the Sudan black B and oil red 0 reactions, the amount of reactive material within these cells was variable.

Schults reaction. With this method the presence of a few positive globules was demonstrated in the cytoplasm of the interstitial cells of the rat. However, the amount present was quite limited, and most of the cells observed were unreactive to this procedure.

Polarization microscopy. As shown in Table 3, sections treated with digitonin prior to observation with the polarizing microscope demonstrated no birefringent material.
**Baker's acid hematin test.** The presence of fine granules of phospholipid material was noted in the cytoplasm of rat interstitial cells (Table 3). However, no coloration was observed in the nucleus or nucleolus. The walls of arterioles and venules established in the interstitium often contained discrete globules of reactive material. In most cases the erythrocytes were deeply stained, indicating the presence of phospholipid in the stroma. A few fine and occasional large globules of reactive substance were dispersed about the basal area of the seminiferous tubules. Cytoplasmic granulation was localized about the unreactive nuclei of the Sertoli cells and extended toward the region in which sperm were embedded. The presence of a few fine globules was noted in the cytoplasm of spermatogonia, primary and secondary spermatocytes, as well as of the spermatids.

**PAS technique.** A weak positive reaction was observed in the cytoplasm of the interstitial cells of rat testes following the periodic acid-Schiff technique. Numerous small, brightly staining granules, unaffected by saliva or chloroform-methanol treatment, were present in some of these cells. Reactive carbohydrate material was located in the walls of blood vessels, especially in the elastic interna of the arterioles, as well as in the lamellated connective tissue and basement membrane of the seminiferous tubules. Schiff-positive granules were noted in the cytoplasm of the Sertoli cells. However, this granulation was not limited to the zone about the unstained nucleus, but was seen to extend along the cytoplasmic strands with a tendency to aggregate in the cellular region where spermatozoa had embedded.

The cytoplasm of spermatogonia and the spermatocytes was only faintly stained. In spermatocytes, about the region of the Golgi zone adjacent to
the nucleus, an oval, weak positive reaction was discerned. This reaction was also visible in the early-phase spermatids and represented the idiosome or the central body of the Golgi apparatus (Fig. 31). Often brightly staining Schiff-positive granules were scattered throughout the cytoplasm of these cells. In a later stage (Fig. 32) the idiosome gave rise to two carbohydrate-reactive bodies, termed proacrosomic granules by Loblond and Clermont (1952a), which condensed to form the single acrosomic granule as shown in Figure 33. In a later developmental stage (Fig. 34) the acrosomic granule moved adjacent to the nucleus, and, as maturation proceeded, commenced spreading over its surface (Figs. 35 and 10b). Nevertheless, the acrosomic granule was still within the pale-staining idiosome as illustrated in Figure 35. Throughout these developmental stages bright carbohydrate-reactive granules were often scattered in the cytoplasm of the spermatids. As the acrosomic granule spread over the nucleus as seen in Figures 36 and 105, the idiosome was seen to have started moving from the Golgi region of the cell (Fig. 36) and eventually it passed along one side of the nucleus. This distribution of PAS-positive carbohydrate continued until well over one-half of the cell was covered (Figs. 37, 38, and 106) and the cytoplasm began to pass caudally from the cell (Fig. 38). In further developmental stages, the cell became somewhat asymmetrical as the acrosomic material extended further towards one side than the other (Fig. 39). This was the first indication of the mode of formation of the hooked acrosomic cap over the sperm head. Flexing of the nucleus and the acrosome continued until the reactive material appeared to have passed quite extensively over one side, whereas the other was only partially covered. At the same time that the nucleus was
undergoing flexure, elongation commenced, and the cytoplasmic material continued to flow towards the distal portion of the cell (Fig. 40). At this time the acrosomal granule was still visible, and the nucleus was not completely covered. The basal portion was somewhat rounded as shown in Figure 40, but in the following stage (Fig. 41) the nucleus became even more elongated and the apex quite rounded. In addition, the caudal portion of the nucleus was more angular than that of the previous stage.

As illustrated in Figure 42, the nucleus, which now governed the dispersion of the acrosomal material by its changing morphology, continued to flatten, elongate, and become more angular. Following this stage, the nucleus contracted to a smaller size and continued to curve slightly. The head cap was slightly rounded over the apex of the nucleus (Fig. 43), but extended caudally only to the dorsal angle, leaving the other portion of the nucleus uncovered by PAS-positive material. During the period illustrated in Figure 44 the sperm was essentially mature in its morphology; however, during these last stages there was contraction of the head cap material which resulted in loss of contact of the dorsal angle of the sperm nucleus and the acrosome (Fig. 45). Occasionally the birefringent perforatorium was visible within the apex of the sperm. It was observed that spermatozoa in the last stages of maturation underwent a reduction in staining of the acrosome by the Schiff reagent.

**Guinea pig testes**

Sudan black B. Following the application of this method to guinea pig testes numerous lipoidal droplets were visible in the cytoplasm of the interstitial cells as seen in Figure 107 and Table 4. Staining
<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B Estimated amount</th>
<th>Type of droplet</th>
<th>Oil red O Estimated amount</th>
<th>Type of droplet</th>
<th>Ashbel-Seligman reaction Estimated amount</th>
<th>Type of droplet</th>
<th>Baker's hematoxylin Estimated amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial cell cytoplasm</td>
<td>+++</td>
<td>Fine</td>
<td>+++</td>
<td>Fine</td>
<td>+++</td>
<td>Fine</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Diffuse</td>
<td>Large</td>
<td>Many vacuolated</td>
<td></td>
</tr>
<tr>
<td>Basement membrane</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occasionally</td>
<td></td>
<td>Occasionally</td>
<td>large</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sertoli cell cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>*</td>
<td>Diffuse</td>
<td>Unreactive</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary spermatocyte cytoplasm</td>
<td>+++</td>
<td>Fine</td>
<td>++</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary spermatocyte cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>*</td>
<td>Diffuse</td>
<td>Unreactive</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>*</td>
<td>Diffuse</td>
<td>Unreactive</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Leal Localisation of Lipids in Guinea Pig Testes

<table>
<thead>
<tr>
<th>Ashbel-Seligman reaction Estimated Type of droplet</th>
<th>Baker's acid hematin Estimated Type of droplet</th>
<th>Schults reaction Estimated Type of droplet</th>
<th>Polarisation microscopy Birefringent crystals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine Large</td>
<td>Fine</td>
<td>Diffuse</td>
<td>Fine birefringent crystals throughout interstitium and tubules. Acetone treatment prior to digitonin does not remove the reaction</td>
</tr>
<tr>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
</tbody>
</table>
appeared as fine, diffuse, or large granules with varying amounts of lipid being present in each cell. When this lipid was massed in a few cells, which was often the case, a very intense sudanophilia was observed. Along the basement membrane of the seminiferous tubules, a few droplets of lipoidal material were present. However, as in the Leydig cells, not every tubule demonstrated equal amounts of fatty material. The cytoplasm of the Sertoli cells was moderately stained, with the sudanophilia appearing diffuse or as fine beads. This reactivity included not only the cytoplasmic portion about the unstained nucleus, but also the long ramification which often extended to the lumen of the tubule. Spermatogonia contained a small amount of dust-like, fine particles of lipoidal material. Primary and secondary spermatocytes as well as spermatids had many fine granules of Sudan-positive substance dispersed about the cytoplasm. It was possible to discern the Golgi material in many of these cells as a faintly reactive, oblong bead in the juxtanuclear position.

**Oil red O.** A strong oil red O reaction was localized in the interstitial cells of guinea pig testes. This sudanophilia was visualized as diffuse, fine or large granules scattered in the cytoplasm of these cells. Some of the lipoidal droplets were vacuolated. Oil red-positive substances were observed in the plasma portion of blood remaining in arterioles and venules, whereas the erythrocytes were unstained. A fine granular reaction was noted in the basal areas of the seminiferous tubules. Many fine sudanophilic droplets were present in the Sertoli cell cytoplasm both about the unstained nucleus and in the ramifying portions of the cell. As seen in Table 1, spermatogonia, as well as primary and secondary spermatocytes and spermatids, were weakly reactive. Generally this appeared as a diffuse
or finely granulated coloration.

Ashbel-Seligman reaction. Through the employment of this method the carbonyl-containing compounds were visualized as moderately strongly staining granules localized in the cytoplasm of the interstitial cells (Fig. 108). The reactive material consisted of fine or large droplets with the quantity of reactive substances in individual cells being quite variable. Treatment in an ethyl ether–chloroform mixture for 4.5 hours did not remove all reactants, but in many cases the granules became vacuolated or husk-like in appearance.

Schulte reaction. With this technique a weak positive reaction as a diffuse blue-green coloration was observed in the cytoplasm of some of the interstitial cells of guinea pig testes.

Polarization microscopy. Very fine, dust-like birefringent crystals were scattered throughout the interstitium and tubules. Acetone treatment prior to digitonin did not remove the reaction.

Baker's acid hematin test. Fine bluish granules which indicated one of the localizations of phospholipids were seen dispersed in the cytoplasmic portion of most of the interstitial cells following the use of this technique. Most of the erythrocytes were deeply stained; however, at times others were observed to be unreactive. As indicated in Table 4, the basal areas of the seminiferous tubules did not contain phospholipid, but a fine granulation was visible in the cytoplasm of the Sertoli cells. Spermatogonia, primary and secondary spermatocytes, as well as spermatids contained small amounts of fine cytoplasmic droplets stained by the acid-hematin test. On occasions a rather large aggregation of this material was seen in the cytoplasm of some of the cells of the seminiferous tubules.
PAS technique. Following the application of the PAS reaction, it was noted that staining of a positive nature was localized in the cytoplasm of the interstitial cells, walls of blood vessels, lamellated connective tissue and the basement membrane of seminiferous tubules. Bright, granulated material was present in some of the blood vessel walls. However, these granules were not affected by methanol-chloroform, whereas most of them were removed by treatment with saliva. The cytoplasm of the spermatogonia as well as of the spermatocytes gave a slight reaction, and numerous brightly staining granules were observed in the cytoplasm of the latter group of cells.

Throughout spermiogenesis a weak cytoplasmic staining was evident. It was observed that the idiosome was present as a slightly reactive region in the Golgi zone of the cytoplasm. At this stage, as shown in Figure 16, the granulation was quite fine and rather diffuse. As development proceeded, aggregation of PAS-positive material took place until several large granules were present in the idiosome and established adjacent to the nucleus (Figs. 17, 18, and 19). These early proacrosomal granules fused to form one large bead, the acrosomal granule (Figs. 50 and 109). Nevertheless, as illustrated in Figure 50, the idiosome was still present at this time. Spreading of reactive material over the nucleus followed (Figs. 51, 52, 53, 110, 111, and 112), and continued until the anterior two-thirds of the nucleus was covered as seen in Figure 54. Excess acrosomic material and the lightly stained idiosome were often seen in the cytoplasm during these stages. During later development these materials, and the cytoplasm, passed caudally from the nucleus. This was followed by formation of a rather pointed acrosomic cap (Figs. 55, 56,
and 113), and eventually by flattening and elongation of the nucleus and acrosome as shown in Figures 57, 58, 114, and 115. As metamorphosis proceeded, it was observed that the acrosome had spread further and the nucleus had continued to flatten until it was quite thin when viewed laterally (Figs. 59, 60, 61, and 109 to 111).

Class Aves

Chicken testes

Sudan black B. With the aid of this technique numerous fine sudanophilic granules were seen in the tunica albuginea enclosing the seminiferous tubules of rooster testes. Of the few interstitial cells seen in these specimens, many contained rather large cytoplasmic droplets of Sudan-positive material; however, the nucleoli and nuclei were unreactive. On occasions a few fine granules were dispersed in the cytoplasm of erythrocytes. As shown in Table 5, the basement membrane of the seminiferous tubules contained numerous fine lipoidal granules and the Sertoli cells demonstrated a rich cytoplasmic sudanophilia. Spermatozoa were lodged in these cells and numerous globules of lipoidal material were scattered about them. Spermatogonia were observed to contain fine as well as large Sudan black-positive globules in their cytoplasm. Primary and secondary spermatocytes, as well as spermatids, also gave a very strong reaction following this method. In these cells a heavily-stained droplet established in the juxtanuclear position was often visible in the Golgi zone. At times it contained a clear, somewhat vacuolar space within the central portion.
<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B</th>
<th>Oil red O</th>
<th>Ashbel-Seligman reaction</th>
<th>Baker's ac hematii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated</td>
<td>Type of</td>
<td>Estimated</td>
<td>Type of</td>
</tr>
<tr>
<td></td>
<td>amount</td>
<td>droplet</td>
<td>amount</td>
<td>droplet</td>
</tr>
<tr>
<td>Interstitial cell cytoplasm</td>
<td>****</td>
<td>Large</td>
<td>****</td>
<td>Large</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td></td>
<td>Fine</td>
</tr>
<tr>
<td>Basement membrane</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Fine</td>
</tr>
<tr>
<td>Sertoli cell cytoplasm</td>
<td>****</td>
<td>Fine</td>
<td>****</td>
<td>Fine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Fine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
</tr>
<tr>
<td>Primary spermatocyte cytoplasm</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Fine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
</tr>
<tr>
<td>Secondary spermatocyte cytoplasm</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Fine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Fine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
</tr>
</tbody>
</table>
**Biochemical Localization of Lipids in Chicken Testes**

<table>
<thead>
<tr>
<th>Ashbel-Seligman reaction</th>
<th>Baker's acid hematin</th>
<th>Schults reaction</th>
<th>Polarisation microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Type of droplet</td>
<td>Estimated Type of droplet</td>
<td>Estimated Type of droplet</td>
<td>Birefringent crystals</td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td>Very fine dust-like particles in interstitial and tubules. Reaction unchanged by acetone.</td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
<tr>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
</tr>
</tbody>
</table>
Oil red O. Although there were only a few interstitial cells in chicken testes, they contained numerous cytoplasmic granulations of lipid following the use of oil red O. The basement membrane of the seminiferous tubules also demonstrated fat globules. Within the Sertoli cell cytoplasm a very heavy sudanophilia was observed, which consisted of fine as well as large lipoidal droplets. In most cases these cells retained clusters of spermatozoa, and in this region there was also an abundance of material stained with oil red O. Spermatogonia contained fine and large granules in their cytoplasm, but the amount present in these cells was quite variable. Primary and secondary spermatocytes were stained in a comparable degree to the spermatogonia (Table 5). However, in the Golgi zone of the spermatocytes and adjacent to the nucleus, there was often observed reactive lipid in the form of a rather large globule. Generally this structure was oriented toward the basal portion of the tubule. The spermatids contained fine and large granules in their cytoplasm and, as in the case of spermatocytes, the reactive material of the Golgi region was also seen in spermatids. It appeared bright orange in color and was globular, but somewhat smaller in size than that seen in spermatocytes. In later stages, lipid was observed in the cytoplasm of the cell as the nucleus underwent changes. Within the lumen of the seminiferous tubules were numerous droplets of lipoidal substances as well as spermatozoa.

Ashbel-Seligman reaction. As indicated in Table 5, this method was found to be negative when applied to the testes of mature roosters.

Schults reaction. No coloration that could be considered as a positive one was observed in the testes of the fowl after the use of this test.
Polarization microscopy. With this method very fine dust-like particles were observed in the interstitium and tubules. However, the reaction was unchanged when treated with acetone prior to placement in digitonin (Table 5).

Baker's acid hematin test. Through the application of this method, a weak positive reaction in the form of fine bluish-black granules was observed in the cytoplasm of the interstitial cells. However, not all of these cells demonstrated this type of staining, and in some cases the reactive material in the interstitium contained what appeared to be clear halos surrounding them. There was a strong cytoplasmic coloration in some of the erythrocytes. A few fine droplets lined some of the region of the basement membrane of the seminiferous tubules; nevertheless, the overall staining was quite sparse. Some phospholipid was evident in the Sertoli cell cytoplasm in the region containing spermatozoa. Spermatogonia, primary and secondary spermatoocytes, as well as spermatids showed weak cytoplasmic staining as a dispersed granulation.

PAS technique. A faint cytoplasmic reaction, involving a few brightly-stained granules, was observed in the interstitial cells of chicken testes following the periodic acid-Schiff technique. These granules were not obliterated by treatment with saliva or methanol-chloroform prior to staining. The walls of arterioles and venules within the interstitium showed a strong Schiff-positive staining. Carbohydrate-reactive material was present in the lamellated connective tissue and the basement membrane of the seminiferous tubules. The cytoplasm of Sertoli cells demonstrated numerous reactive granules, which were not removed by saliva hydrolysis or treatment with a glycolipid solvent. Weak PAS-positive staining was noted.
in the cytoplasm of spermatogonia, and in that of primary as well as secondary spermatocytes. The faintly-stained idiosome was visible in the region of the Golgi apparatus of the spermatocytes.

In the preliminary stages of spermiogenesis the idiosome was often seen in the juxtanuclear position (Fig. 62), but as development proceeded a homogeneous mass was sometimes present as shown in Figure 63. This stage was followed by formation of the proacrosomic granules within the idiosome (Fig. 64) and eventual condensation of this material to produce the single acrosomic granule as illustrated in Figure 65. As development continued, the idiosome passed caudally from the region of the acrosomic granule (Fig. 66 and 67), but the acrosomic granule did not spread as was the case in some species studied. Figure 68 illustrates the apparent condensation of the nucleus in a later period of metamorphosis; this was followed by nuclear elongation as well as passage caudal-ward of the cytoplasm (Figs. 69 and 70). These changes in nuclear morphology continued (Figs. 71, 72, 73, and 74) with elongation being the principal alteration. Eventually the long, thin, fine spermatozoon of the fowl was formed, which contained only a small amount of PAS-reactive acrosome at the extreme tip as shown in Figures 75 and 76.

Class Reptilia

Horned lizard testes, Phrynosoma cornutum (Harlan)

Sudan black B. An abundance of lipid material was seen in the interstitial cell cytoplasm (Table 6). It appeared as dark blue-black droplets of varying sizes; however, the amount of sudanophilia was variable
Table 6. Histochemical Localization of Lipids in Sertoli Cell

<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B</th>
<th>Oil red O</th>
<th>Ashbel-Seligman reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated amount</td>
<td>Type of droplet</td>
<td>Estimated amount</td>
</tr>
<tr>
<td>Interstitial cell</td>
<td>+++ Fine</td>
<td>or Large</td>
<td>or Large</td>
</tr>
<tr>
<td>cytoplasm</td>
<td>or Large</td>
<td></td>
<td>or Large</td>
</tr>
<tr>
<td></td>
<td>+++ Some</td>
<td>vacuolated</td>
<td>Some vacuolated</td>
</tr>
<tr>
<td>Basement membrane</td>
<td>++</td>
<td>Fine</td>
<td>++ Fine</td>
</tr>
<tr>
<td>Sertoli cell cytoplasm</td>
<td>+++ Fine</td>
<td>or Large</td>
<td>or Large</td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>+++ Fine</td>
<td>Large</td>
<td>Large</td>
</tr>
<tr>
<td>Primary spermatocyte cytoplasm</td>
<td>++ diffuse</td>
<td>or Fine</td>
<td>or Fine</td>
</tr>
<tr>
<td>Secondary spermatocyte cytoplasm</td>
<td>+++ Large</td>
<td>or Fine</td>
<td>or Fine</td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>+++ Fine</td>
<td>or Large</td>
<td>or Large</td>
</tr>
</tbody>
</table>
**Table: Localisation of Lipids in Horned Lizard Testes**

<table>
<thead>
<tr>
<th></th>
<th>Ashbel-Seligman reaction</th>
<th>Baker's acid hematin</th>
<th>Schults reaction</th>
<th>Polarisation microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>droplet</td>
<td>Estimated amount</td>
<td>Type of droplet</td>
<td>Estimated amount</td>
<td>Type of droplet</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unreactive</td>
<td>Fine</td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>Fine</td>
<td></td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>Fine</td>
<td></td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>Fine</td>
<td></td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>Fine</td>
<td></td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>Fine</td>
<td></td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>Fine</td>
<td></td>
<td>Unreactive</td>
<td>Absent</td>
</tr>
</tbody>
</table>
in these cells. Fine droplets of reactive lipid material were also noted in the basement membrane of the seminiferous tubules. Within these tubules, fine granules were present in the long cytoplasmic strands of the Sertoli cells, whereas the nuclei and nucleoli were unreactive to the fat stain. Numerous granules were noted in the cytoplasm of the spermatogonia as shown in Figure 116 and Table 6. These lipid granules were usually arranged about the unreactive nucleus and were diffusely scattered. In contrast, the lipid in the primary spermatocytes was often grouped in the Golgi region of the cell with granules scattered rather generally throughout the cytoplasm. Still further cytoplasmic aggregation of lipid in the Golgi region was noted in the secondary spermatocytes, thus presenting the appearance of a small cap adjacent to the unreactive nucleus. It was observed in the spermatids that the lipid was condensed in the form of a small black bead. Nevertheless, other granules were seen scattered in the cytoplasm. Following metamorphosis and prior to their loss of cytoplasm the late spermatids demonstrated numerous cytoplasmic lipid inclusions.

Oil red O. Large amounts of orange-staining fat material were found in the testes of the horned lizard with the principal site of distribution being in the cytoplasm of the interstitial cells. As observed in Figure 117, the lipid material in these cells was visualized as orange globules, whereas the nuclei and nucleoli were unstained. Globules of lipid material were noted in the basement membrane of the seminiferous tubules. Adjacent or near to this membrane were seen the Sertoli cells, which also demonstrated reactive lipoidal granules in the cytoplasm. These long cytoplasmic strands, in which the sperm heads were embedded, were very rich
in substances of a lipid nature (Table 6).

In the spermatogonia cytoplasm, large orange-staining lipid bodies were scattered throughout. In the primary and secondary spermatocytes the presence of lipid components within the cytoplasm was readily evident. However, in the former, the cytoplasmic granules were more diffuse, whereas in the latter stage there appeared to be an aggregation of lipid in the Golgi region of the cells. This lipid material underwent further condensation until there was formed in the cytoplasm of the spermatids a bead in the Golgi region of the cell. In addition to this lipid material, other substances of a sudanophilic nature were noted in the cytoplasm. At this time the nucleus of the spermatid was quite rounded and, as development proceeded, underwent elongation. Lengthening left the sperm bullet-shaped. However, during later metamorphosis, it became more rod-like with a tip as contrasted to the blunt posterior portion of the sperm head. When the spermatozoon was still within the cytoplasm, lipid material was quite evident. Elongation of the nucleus continued until the sperm head became quite long and slender with a sharp pointed acrosomic tip. Lipid granules were present in the cytoplasm which enclosed the sperm nucleus. Eventually the cytoplasm was cast, but even at this period lipid granules were observed distributed along the length of the spermatozoon.

Control slides demonstrated that treatment for one hour at room temperature in equal parts of a mixture of ethyl ether and chloroform extracted almost all of the lipid material, and only a small fraction remained in a few cases.

Ashbel-Seligman reaction. Positive staining was noted in the cytoplasm of the interstitial cells, whereas the nuclei and nucleoli were
unstained. Within these cells, the reaction was evidenced as fine granules which varied in color from bluish to purple or pink. Variable amounts of reactants were present within individual cells.

**Schultz reaction.** Application of the Schultz reaction showed a few fine green granules in the cytoplasm of the interstitial cells.

**Polarization microscopy.** No birefringent crystals were noted in the testes of the lizard with this method (Table 6).

**Baker's acid hematin test.** Numerous fine droplets of phospholipid were demonstrable in the interstitial cell cytoplasm of horned lizard testes (Fig. 118). However, the quantity of visible reactants in these cells was quite variable and fluctuated from only a small amount in some cells to a very rich sudanophilia in others. It was observed that the cytoplasm of the erythrocytes was deeply stained, whereas the nucleus was unreactive. In many cases a fine granulation was localized along the basement membrane of the seminiferous tubules. The Sertoli cells contained very fine droplets both in the cytoplasm about the nucleus and in the projections extending towards the lumen of the tubule. In these cells there appeared to be a heavier concentration of phospholipid in the zone of maturing spermatozoa than the region about the nucleus. The germ cells contained several fine cytoplasmic droplets as well as occasional larger granules.

**PAS technique.** Following the use of this method a weak positive staining was observed in the tunica albuginea surrounding the testes. In some cases Schiff-positive granules, which could be removed through saliva hydrolysis, were dispersed in this connective tissue sheath. Carbohydrate material was also present in the interstitial cell cytoplasm;
however, saliva treatment removed only a portion of these reactants. PAS-reactive granules were seen in the cytoplasm of these cells one hour after saliva or 16 hours after methanol-chloroform treatment. The walls of arterioles and venules were strongly reactive and often contained numerous brightly stained granules which were removed by saliva but not by methanol-chloroform.

The basement membrane of the seminiferous tubules contained PAS-positive material. Within the cytoplasm of the germ cells was abundant carbohydrate, of which most was removed following saliva treatment. The Sertoli cells, and the clusters of spermatosoa within their cytoplasm, demonstrated a heavy deposition of a glycogen material. In the spermatocytes a lightly stained area, the idiosome, was sometimes present about the Golgi region of the cells.

With the aid of this method the various morphological changes taking place during spermiogenesis were followed. In the first stage, as illustrated in Figure 77, a lightly stained idiosome was seen in the region of the Golgi apparatus. This structure formed the small Schiff-positive acrosomic granule which enlarged and moved adjacent to the nucleus but still within the idiosome (Figs. 78, 79, 80, and 81). The idiosome passed from the Golgi region, as shown in Figures 82 and 83, and in a later stage it was noted that condensation of nuclear material had taken place, forming a somewhat smaller nucleus (Fig. 84). At times, remnants of Golgi material were present in the caudal region of the cell. In addition, passage of cytoplasm towards the distal part of the cell was observed. As seen in Figures 85, 86, and 87, this migration continued during the elongation of the nucleus and in many cases granules
of carbohydrate material were clumped in the cytoplasm. At a later developmental stage, spreading of the acrosome over the sharp pointed tip of the sperm nucleus occurred (Fig. 88 and 89). Following the loss of cytoplasm, only the acrosome of the horned lizard demonstrated PAS-positive substance (Fig. 90).

**Class Amphibia**

**Frog testes, Rana pipiens**

Sudan black B. As seen in Figure 119 and Table 7, this method revealed a large amount of lipid material within frog testes. In the cytoplasm of the interstitial cells were observed sudanophilic substances in the form of fine, as well as large, droplets. Fine granules were noted in the basement membrane of the seminiferous tubules. The cytoplasm of the Sertoli cells demonstrated numerous granules. This granulation extended down the cytoplasmic sheath that enveloped the lipid filled spermatozoa, and was much greater in these cells than previously noted in other species (Fig. 119).

Fine, diffuse, and large granules were seen in the cytoplasm of spermatogonia (Table 7). Primary spermatocytes exhibited a more diffuse cytoplasmic lipid coloration and finer granulation than did either the spermatogonia or Sertoli cells. However, occasional large droplets were noted. In these preparations it was difficult to distinguish secondary spermatocytes with any degree of certainty. Spermatids were present, generally demonstrating a diffuse lipid reaction in the cytoplasm with occasional fine granules. In some instances, there was noted a darkly
<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B</th>
<th>Oil red O</th>
<th>Ashbel-Seligman reaction</th>
<th>Basophilic</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated</td>
<td>Type of droplet</td>
<td>Estimated</td>
<td>Type of droplet</td>
<td>Estimated</td>
</tr>
<tr>
<td></td>
<td>amount</td>
<td></td>
<td>amount</td>
<td></td>
<td>amount</td>
</tr>
<tr>
<td>Interstitial cell</td>
<td>****</td>
<td>Fine</td>
<td>***</td>
<td>Large</td>
<td>***</td>
</tr>
<tr>
<td>cytoplasm</td>
<td></td>
<td>Large</td>
<td></td>
<td>Fine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
</tr>
<tr>
<td>Basement membranes</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Fine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td>Large</td>
<td></td>
</tr>
<tr>
<td>Sertoli cell cytoplasm</td>
<td>***</td>
<td>Fine</td>
<td>***</td>
<td>Large</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td>Fine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>**</td>
<td>Fine</td>
<td>**</td>
<td>Diffuse</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse</td>
<td></td>
<td>Few</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>large</td>
<td></td>
</tr>
<tr>
<td>Primary spermatocyte</td>
<td>**</td>
<td>Diffuse</td>
<td>**</td>
<td>Diffuse</td>
<td>**</td>
</tr>
<tr>
<td>cytoplasm</td>
<td></td>
<td>Fine</td>
<td></td>
<td>Fine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occasional</td>
<td></td>
<td>Occasional</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>large</td>
<td></td>
<td>large</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>globule</td>
<td></td>
<td>globule</td>
<td></td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>**</td>
<td>Diffuse</td>
<td>**</td>
<td>Diffuse</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some fine</td>
<td></td>
<td>Some fine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>granules</td>
<td></td>
<td>granules</td>
<td></td>
</tr>
</tbody>
</table>
### Staining Results

<table>
<thead>
<tr>
<th>Type of Droplet</th>
<th>Ashbel-Seligman Reaction</th>
<th>Baker's Acid Hematin</th>
<th>Schults Reaction</th>
<th>Polarisation Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Type of Droplet</td>
<td>Large</td>
<td>Fine</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Estimated Type of Droplet</td>
<td>Large</td>
<td>Fine</td>
<td>**</td>
<td>Absent</td>
</tr>
<tr>
<td>Estimated Type of Droplet</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>Estimated Type of Droplet</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
<td>Absent</td>
</tr>
<tr>
<td>Estimated Type of Droplet</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
<td>Absent</td>
</tr>
<tr>
<td>Estimated Type of Droplet</td>
<td>Fine</td>
<td>Unreactive</td>
<td></td>
<td>Absent</td>
</tr>
</tbody>
</table>
stained bead in the cytoplasm, usually in the Golgi region of the cell and somewhat adjacent to the unreactive nucleus. Spermatozoa lodged within the Sertoli cell cytoplasm contained numerous lipid granules which extended the length of the head. Often the acrosome could be distinguished as a dark staining, lipid-reactive cap.

**Oil red O.** The cytoplasm of the interstitial cells of frog testes demonstrated large quantities of lipid following staining. The major granules observed were of the large globular type; however, small and fine droplets were also present. Nearly all of the interstitial cells demonstrated lipoidal staining, but the amount visible was quite variable. There was no reaction in the nucleus and nucleolus of these cells or of any other cells studied in frog testes; however, an abundance of large and small lipid droplets were located in the basement membrane of the seminiferous tubules. Within the cytoplasm of the Sertoli cells, large and fine lipid droplets were numerous. It was observed that spermatogonia contained a moderate number of fine granules of lipid material; however, staining was generally diffuse. Occasionally a few droplets of fat were observed in these cells (Table 7). The primary spermatocytes also presented a moderate sudanophilia, principally as diffuse and fine granules. As in the case of spermatogonia, some large droplets were visible in these cells. The spermatids demonstrated a diffuse cytoplasmic granulation with an occasional fine droplet of lipid within the cytoplasm. It was observed that spermatozoa which were embedded within the Sertoli cells demonstrated an oil red O-positive acrosome.

**Ashbel-Seligman reaction.** As shown in Table 7, a strong positive reaction was visible within the cytoplasm of many of the interstitial
cells of frog testes. This site demonstrated fine as well as large droplets varying in coloration from blue to light purple. The nuclei and nucleoli were unstained, and with the exception of the Leydig cells there were no other cells showing a positive reaction for this method.

**Schulte reaction.** Following the application of this technique, fine blue-green granules were visible in only a few cases in the cytoplasm of the interstitial cells (Table 7).

**Polarization microscopy.** Birefringent crystals were present in the cytoplasm of the interstitial cells and Sertoli cells, as well as in the basement membrane of the seminiferous tubules.

**Baker's acid hematin test.** The cytoplasm of the interstitial cells contained large quantities of phospholipid material after the use of this method (Fig. 120). This technique visualized the lipoidal cytoplasmic inclusions as fine granules which varied according to quantity of material reactive within individual cells. The nuclei were unstained, but the nucleoli appeared as homogenous blue-black masses, thus indicating one of the sites of phospholipid material. The cytoplasm of frog erythrocytes was observed as deeply stained cells. There was no visible reaction in the basement membrane of the seminiferous tubules (Table 7). The Sertoli cells contained numerous fine granules of lipid substance in their cytoplasm as well as deeply stained nucleoli. Spermatogonia also contained fine droplets of reactive substances dispersed in their cytoplasm and a heavily-stained nucleolus. Primary and secondary spermatocytes as well as spermatids were observed to contain phospholipid in the cytoplasm and nucleolus to a degree comparable to that in spermatogonia. The majority of the Amphibian sperm was unstained, with the exception of a very small
amount of lipid at the anterior tip of the head and at the middle-piece. Large masses of intensely reactive phospholipid were dispersed in the lumen of the seminiferous tubules.

**PAS technique.** A slight positive reaction was observed in the cytoplasm of the interstitial cells of the frog following the PAS technique. In addition, brightly-stained granules were dispersed in the cytoplasm of these cells, and, though they were not affected by treatment with methanol-chloroform, saliva treatment removed many of them. A deep coloration, combined with a heavy Schiff-positive granulation, characterized the walls of arterioles and venules; however, salivary amylase prior to staining also obliterated these inclusions. It was found that the basement membrane of the seminiferous tubules contained PAS-reactive material. As shown in Figure 121, heavy concentrations of glycogen were localized within the tubules. This substance was dispersed throughout the cytoplasm of all cellular types within the tubules, and appeared as droplets which were quite variable in size. Fine granular inclusions of glycogen outlined spermatozoa embedded within the Sertoli cells, and very large amounts were present in the distal projection of these cells into the tubular lumen. Furthermore, deposits of glycogen were visible within the lumen proper. Figure 122 shows the removal of glycogen from the tubules of the frog testis following treatment with salivary amylase.

With this method it was not possible to trace the development of the acrosome of frog spermatozoa, for no idiosome was visible in these preparations. However, on occasions spermatids containing one to four small PAS-positive beads were observed (Fig. 123). These beads were seen only from the acrosomic or apical pole of the cell and never from
lateral view. Spermatozoa did not exhibit an acrosome with this method, but it was found that in many cases one to three Schiff-positive granules appeared in line along the side of the sperm head.

Class Pisces

Bluegill fish testes, Lepomis macrochirus Rafinesque

Sudan black B. A weak positive reaction was present as a few fine granules in the testicular sheath of connective tissue which invests the testis of the fish, and in the interlobular connective tissue (Table 8). Sudanophilia was also observed in the cytoplasm of the spermatagonia as fine granulations; however, the amount present was quite small. The nuclei and nucleoli of these cells were unstained. It was noted that a few fine granules of lipid-reactive material were situated in the Golgi region of the primary spermatocytes. These inclusions were in a juxta-nuclear position. Nevertheless, not all of the lipoidal substances were located in this region, for it was observed that a few granules were scattered within the cytoplasm and remained somewhat removed from the above mentioned region. In the secondary spermatocytes the reacting lipids in the Golgi material were not as diffuse as those observed in the primary cells. In addition fine granules were dispersed in the cytoplasm in a position other than the Golgi zone. The spermatids showed the lipoidal portion of the acrosomic granule clearly and in some cases it appeared to have spread slightly over the nuclear surface. Following the metamorphosis of these cells into spermatozoa, it was observed that the acrosome was present as a small and rather diffuse lipid-reactive area
<table>
<thead>
<tr>
<th>Site</th>
<th>Sudan black B</th>
<th>Oil red O</th>
<th>Ashtel-Seligman reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated</td>
<td>Type of</td>
<td>Estimated</td>
</tr>
<tr>
<td></td>
<td>amount</td>
<td>droplet</td>
<td>amount</td>
</tr>
<tr>
<td>Testicular sheath connective tissue</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
</tr>
<tr>
<td>Interlobular connective tissue</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
</tr>
<tr>
<td>Spermatogonium cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
</tr>
<tr>
<td>Primary spermatocyte cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
</tr>
<tr>
<td>Secondary spermatocyte cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
</tr>
<tr>
<td>Spermatid cytoplasm</td>
<td>++</td>
<td>Fine</td>
<td>++</td>
</tr>
</tbody>
</table>
**Tochemical Localisation of Lipids in Fish Testes**

<table>
<thead>
<tr>
<th>Type of droplet</th>
<th>Red O reaction Estimated amount</th>
<th>Type of droplet</th>
<th>Schults reaction Estimated amount</th>
<th>Type of droplet</th>
<th>Polarisation microscopy Birefringent crystals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine</td>
<td>Unreactive</td>
<td>Unreactive</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
on the nucleus. The small size of this acrosome and the possible masking by the hematoxylin counterstain probably account for the fact that it was not seen in oil red O preparations. It was noted that the adipose digitative processes within the intertubular connective tissue reacted to the Sudan stain rather diffusely. However, treatment with an equal mixture of ethyl ether-chloroform did not always remove all of the lipoidal material in these areas. The same was true for some of the lipid material in the germ cells.

**Oil red O.** Fine granules of oil red O-positive material were visible scattered in the connective tissue sheath surrounding the testes of the blue gill fish and dividing it into lobules. The spermatogonial cells were found in cysts which were arranged near or adjacent to the periphery of the lobulo. Within these cells was observed a variable number of fine granular lipid scattered about the cytoplasm. The primary spermatocytes were noted to be somewhat smaller than the spermatogonia but were still located within cysts. Both these cells and the secondary spermatocytes demonstrated lipoidal staining. In the primary cells an accumulation of larger reactive granules generally established in the juxtanuclear position was noted. The spermatids showed a lipoidal portion adjacent to the nucleus in the region of the Golgi apparatus. In these preparations it was seen as a small orange bead; nevertheless, other fine lipoidal granules were situated in the cytoplasm. In later developmental stages, it was noted that the spermatid nucleus elongated slightly and then began to bow. This continued until the nucleus was U-shaped. It was not possible to follow the metamorphosis of the acrosome in this species, for, soon after changes commenced in the spermatid nucleus, it disappeared.
Lipid was observed in the digitative processes of adipose tissue in the septa between the lobules. Treatment with chloroform-ether did not remove all of the material stained by oil red 0.

Ashbel-Seligman reaction. As indicated in Table 8, this reaction was negative when applied to fish testes.

Schultz reaction. This test was negative.

Polarisation microscopy. No birefringent crystals were noted in the testes of the bluegill fish (Table 8).

PAS technique. A strong Schiff-positive reaction was visible within the connective tissue sheath covering and separating the testis of the bluegill into lobules. As shown in Figure 124, numerous brightly-stained granular masses were dispersed throughout this tissue; however, treatment with salivary amylase decreased the intensity of reaction as well as obliterating the granular inclusions. Methanol-chloroform had no effect upon this substance. Staining of the walls of arterioles and venules indicated the presence of carbohydrate-reactive substances. Spermatogonia, spermatocytes, and spermatids were unstained. Large masses of carbohydrate material were established at random throughout the connective tissue that separated the lobules, and in some cases this Schiff-positive substance was seen within the region of the germ cells. This material was not affected by treatment with glycolipid solvents or by saliva hydrolysis. It was not possible to trace the development of the acrosome with this method as there was no indication of an idiosome or acrosomal granule in these preparations. Spermatozoa observed within the lobules did not demonstrate an acrosome.
DISCUSSION

Lipids

Sudan black B, which was introduced as a histological stain by Lison (1931), is considered to be an excellent stain for the demonstration of fatty substances (Whitehead and Kay, 1950). Cain (1950) reviewed the available methods for the histochemical analysis of lipids, and stated that the Sudan black B technique was valid for all liquid or grease-like lipids. Therefore, this classification would exclude cholesterol. In the present investigation, Sudan black has been found to be superior to oil red O for the demonstration of lipids in tissue sections. Although the reactions of these stains generally paralleled each other, as shown in Tables 1 through 8, it was easier to interpret results obtained with Sudan black than with oil red O. The latter dye has a tendency to precipitate within a short time after staining, and, if a counterstain is applied, there is the possibility that sudanophilic material may be masked.

Scott and Lynch (1952) reported that they were able to find little if any interstitial cell lipid in the white rat, but that considerable quantities were present within these cells in the mouse, guinea pig, and wild rat. In the present investigation, the results of Scott and Lynch (1952) were confirmed as to Leydig cell lipids in the white rat and guinea pig, as seen in Figures 102 and 107. Moreover, these authors observed sperm heads embedded in the apex of the Sertoli cells, and numerous fine
lipids located adjacent to these spermatozoa. Scott and Lynch (1952) considered the possibility as to whether or not this distribution might represent transfer of lipid material of a nutritive nature from the Sertoli cells to the spermatozoa. Following the use of the oil red 0 technique, these authors were unable to demonstrate lipid in the sperm. The results obtained in the present investigation concerning the localization of lipoidal material in the male gametes of the rat and guinea pig are in agreement with those reported by Scott and Lynch (1952).

It is of interest that, through the use of these Sudan dyes, lipids were discerned in the interstitial cell cytoplasm, the basement membrane, and the cytoplasm of the Sertoli cells, spermatagonia, spermatocytes, and spermatids of all species studied, with the exception of the fish. Figures 91 and 92 illustrate lipid distribution in the interstitial cells and seminiferous tubules of the bull following the Sudan black technique. Figure 99 demonstrates sudanophilia in the Leydig cells of the ram. In the bluegill, there are no structures which are comparable to the basement membrane or to the Sertoli cells. However, it must be emphasized that the amount of lipid observed in those cells was quite variable within the same cell types (Figs. 102, 107, 116, 117, and 119), between the different species, and even among individuals of the same species. As shown in Tables 1 through 8, these sudanophilic substances appeared as diffuse, fine, or large droplets.

Intratubular lipids have been described in different animals including the dog, cat, bat, horse, rabbit, and rat (Loisel, 1903); the chicken (Reeves, 1915); the ram (Gresson and Zlotnik, 1945); and in man, guinea pig, and mouse by McEnerney and Nelson (1950).
Montagna and Hamilton (1951) have carried out a comprehensive investi-
gation of lipids in the human testis, and reported that all of the germinal
cells, with the exception of the transforming spermatids, contain fine
lipid granules. According to these investigators, most authors believe
that the only intratubular lipids present are in the Sertoli cells. It
is of interest that the results obtained in the present investigation are
comparable to those of Montagna and Hamilton (1951) for the human testes.
Moreover, in the various species examined, intratubular lipids were present
in all of the cell types, including the spermatids.

Deane and Seligman (1953) have reviewed and evaluated the methods
employed for the histochemical demonstration of ketosteroids. In 1949,
Ashbel and Seligman introduced their method for the cytochemical locali-
sation of active carbonyl groups. Their technique consisted of the
reduction of aldehydic and ketonic groups with 2-hydroxy 3-naphthoic
acid hydrazide, followed by coupling of tetrazotised diorthoanisidine
into the naphtholic ring. This resulted in the formation of a blue,
insoluble azo compound. Ashbel and Seligman (1949) presented evidence
that the carbonyl-containing lipid was ketosteroid in nature. According
to these investigators, the carbonyl-reacting lipid was present in the
interstitial cells of the testis. Ashbel, Cohen, and Seligman (1951)
studied the testes of frogs, roosters, mice, rats, rabbits, pigs, dogs,
horses, and of a goat with the aid of the Ashbel-Seligman test. The
results obtained by these investigators on the carbonyl reaction and
sudanophilia in general, were confirmed in the present study on the rat
(Fig. 103), the rooster, and the frog. Figures 108 and 118 illustrate
the cytoplasmic reaction obtained with the Ashbel-Seligman test in the
Leydig cells of the guinea pig and horned lizard, respectively.

Boscott, Mandl, Danielli, and Shoppee (1948) reported that it has been claimed by other investigators that $\alpha$-hydroxyketones gave a purple color when reduced with fuchsin in Foulgon's plasmal reaction. Boscott, Mandl, Danielli, and Shoppee (1948) studied this reaction with pure desoxycorticosterone and were unable to find evidence of oxidation of this substance to an aldehyde by aqueous mercuric chloride. These authors came to the conclusion that the plasmal technique could not be relied upon to demonstrate the cytological localization of $\alpha$-hydroxyketones.

Moreover, Deane and Seligman (1953) emphasized that the hydrazide reaction was not specific for ketosteroids or for ketones. These investigators pointed out that with each tissue studied it becomes necessary to demonstrate the following: (1) by other techniques that the substance reacting was lipid, (2) that it was ketonic, and (3) that from chemical and biological analyses ketosteroids had previously been found in the tissue examined. Deane and Seligman (1953) stated that, if these requirements were met, then it could be presumed that ketosteroids or their ketonic precursors were being demonstrated. The battery of tests employed in the present investigation indicated the localization of lipids; however, as far as the demonstration of ketosteroid substance in the interstitial cells of the various vertebrates studied is concerned, it might be best to await further refinement of the techniques in order that a more positive identification of steroids be established.

Gomori (1952b) is not in agreement with the current histochemical methods for ketosteroids. According to this investigator, the "battery
of reactions" which were thought to be specific for ketosteroids were probably due to unsaturated fatty acid esters of cholesterol. Gomori (1952b) stated that the carbonyl reactions as they were observed in tissue sections were due to compounds which constituted a substantial percentage of lipid material. In addition, he pointed out that, at the present time, the ketosteroids could not be demonstrated with the methods available.

Baker (1946) developed a histochemical technique for the demonstration of phospholipids in tissue sections. This investigator reported the presence of reactive secretion droplets in the Leydig cells of the mouse testis and in the middle-piece of the mouse spermatozoon. The results obtained in mouse Leydig cells by Baker (1946) are comparable to those observed in the same cells in the rat (Table 3). In addition, in the present study, a positive phospholipid reaction was noted in the Leydig, Sertoli, and germ cells of all species studied with the exception of the fish. This technique was not applied to the bluegill testis. As indicated in Tables 6 and 7, the heaviest concentrations of phospholipid were observed in the Leydig cells of the horned lizard and of the frog. Figure 120 illustrates the localization of phospholipid material in the cytoplasm of the interstitial cells and the sperm middle-piece of the frog.

In a later study on the histochemical recognition of phospholipid, Baker (1947) reported that purified galactolipine had scarcely any tendency to give a positive reaction with this method. According to this investigator, the acid hematin test was a method for the localization not of lipid, but of phospholipid.
Cain (1947) critically examined Baker's acid hematin test for phospholipids, and reported that this method was specific, provided that only a definite positive result was considered. In a discussion of the mechanism for this test, this investigator stated that the phospholipid was not fixed by the formaldehyde calcium, and that it was restrained from passing into solution by the calcium ions which entered into the reaction in no other manner. Then, according to Cain (1947), the phospholipid combined with chromium from the mordant, thus rendering it insoluble. In a later investigation, Cain (1950) listed Baker's test as valid for phospholipids and possibly acetalphosphatides as well.

Casselman (1952) reported that studies with synthetic enantiomeric α-phosphatidic acids, α-phospholipines, and related compounds, and with purified lipid fractions confirmed Baker's acid hematin test when pyridine extraction was employed as the control method. He considered the acid hematin technique as specific for phospholipids. In addition, Casselman (1952) stated that a true positive reaction was also given by certain phosphatidic acids. Elftman (1953) reported that lipids, including phospholipids, were an essential part of the Golgi material.

It is of interest that phospholipids were localized in all testicular cell types of all species studied in the present investigation. As in the case of the over-all lipid reactions, the phospholipids were variable in amount among the various species as well as between individuals of the same species. This substance is considered to play an important role in the over-all metabolic function of the cells of the testis.

Schultz (1924) introduced the histochemical method for the localization of cholesterol in tissue sections. In the present investigation,
a positive Schultz reaction was observed in the interstitial cells of all species studied with the exception of the chicken and fish testes. In view of the question which exists concerning the presence of functional Leydig cells in the fowl and the fish testes, it is of interest that a positive reaction was not noted in the testicular interstitium of these species, as indicated in Tables 5 and 8. However, it is possible that, if the cyclic breeding season of the bluegill is considered, the specimens employed in this study may have been at a stage during which a reactive quantity of testis cholesterol was absent.

According to Everett (1947), the Schultz test is more specific than the Liebermann-Burchard reaction, which is the biochemical method for cholesterol. Everett (1947) reported that the substances which gave the color response of a positive Schultz test were limited to the diols, such as those formed from cholesterol by mild oxidation. Moreover, Everett (1947) stated that, with regard to the significance of a negative Schultz test, this method was not as sensitive as the Liebermann-Burchardt reaction. When this investigator applied the Schultz test to gelatin sections containing cholesterol emulsified with lecithin and other fats, the lower range of sensitivity was reached when the amount of cholesterol to other fats was approximately 1 to 10.

Perlman (1950) reported that chemical analyses of rat testes demonstrated that, in cases in which spermatogenesis was impaired, as after hypophysectomy or cryptorchidism, a characteristic effect was an over-all loss of testis cholesterol, an increase in the ester/free cholesterol ratio, and an increase in the concentration of cholesterol present. According to this investigator, these events took place without regard
to the functional status of the interstitial cells.

Reiner (1952, 1953) carried out studies on the Schultz histochemical test for cholesterol and concluded that, although the method was very insensitive, it was nevertheless a useful one. This investigator reported that the substance, or one of the substances, which was responsible for the positive Schultz reaction was 7-hydroxycholesterol, and that sterols convertible to a compound of this or similar structure under the conditions of the reaction would, in all probability, give a positive reaction. Kent (1952a, 1952b) studied the specificity of the Schultz test and reported that it was not absolutely specific for cholesterol, but that it was, however, more specific than the Liebermann-Burchard histochemical test from which it was derived.

According to Greep and Deane (1949), Bennett (1940) employed the method of pretreatment of frozen sections with digitonin, which resulted in the formation of insoluble, anisotropic esters with cholesterol and other β-steroids, thereby producing more birefringence.

In 1942, Pollock carried out a histochemical study on the interstitial cells of the testes of the cat, rat, guinea pig, mouse, and rabbit. According to this investigator, substances with solubility properties similar to those of the testes steroids were demonstrated in the interstitial cells. Pollock (1942) used the digitonin reaction followed by polarization microscopy, and reported that in the cat testis, as well as those of other animals, compounds with the chemical properties of testosterone were localised exclusively in the interstitial cells and not present elsewhere in the gland. However, according to Claesson and Hillarp (1947), the digitonin-precipitable sterol occurring in the
interstitial cells of the testis was probably cholesterol or some related sterol. These investigators concluded that it was not possible, at that time, to demonstrate histochemically any carbonyl groups belonging to a ketosteroid.

Gomori (1952a) pointed out that the method for polarization microscopy used to be considered as a means for the distinction of doubly refractile cholesterol from other fat substances. However, this investigator reported that Lison (1933) demonstrated that the results obtained by this method could not be interpreted chemically. Furthermore, Gomori (1952a) stated that, in the case of lipid, birefringence depended principally on factors other than chemical constitution. These included, among others, the state of aggregation, supercooling, and the nature of the mounting medium. According to Yoffey and Baxter (1947) a positive reaction was not necessarily evidence of the localization of cholesterol. Also, these investigators reported that sudanophilic and crystalline birefringent material could occur in the same cell, but that the crystals themselves were never sudanophilic.

In this investigation a few birefringent crystals were noted in the Leydig cells and within the seminiferous tubules of the frog testis (Table 7). In other cases, the dust-like birefringent material observed in the case of the ram (Table 2), the guinea pig (Table 4), and the chicken (Table 5) was comparable to that shown by control slides which had been previously treated with acetone. According to Deane and Seligman (1953), treatment with acetone at room temperature will dissolve pure steroids. However, Gomori (1952a) stated that the sensitivity of the polarization microscopy method is poor, and lipid mixtures which contain less than 5
per cent cholesterol showed no birefringence.

It is of interest that, in a comparative examination of vertebrate testes with the aid of various lipid techniques, the results obtained within certain structures were more or less comparable to lipid localization observed in other species within the same class or belonging to other classes. These findings suggest the possibility of the universality of lipoidal distribution within the Leydig, Sertoli, and germ cells of vertebrate testes. Roberts and Szego (1953) have reviewed the importance of steroids in the metabolic processes of reproductive target organs.

Carbohydrates

Chemical nature of Schiff-positive substances

Hotchkiss (1948) has made a critical evaluation of the PAS technique and has found that a positive reaction was given by any substance which complied with certain requirements. Those included: (1) the presence of a 1,2 glycol grouping (-CHOH-CHOH-) in unsubstituted form, or the equivalent structure in which hydroxyl groups were replaced by amino or alkyl-amino groups, or its oxidation product -CHOH-CO-; (2) a compound which did not diffuse during fixation or give an oxidation product which was diffusible; and (3) the presence of the substance in sufficient concentration originally to give a detectable final color. In addition, Hotchkiss (1948) pointed out that the carbohydrate component of desoxyribonucleic acid and ribonucleic acid, as well as the hydroxyamino acid residues of most proteins, was chemically substituted so that the free glycol group was absent, and thus did not give a positive reaction.
Hotchkiss (1948) stated that nearly all of the known naturally occurring compounds which oxidize the 1,2 glycol linkage were classed as carbohydrates, but only the high-molecular substances such as polysaccharides, hyaluronic acid, mucoproteins, and mucins were likely to be present in sufficient amounts to give a visible coloration.

Leblond (1950) reported that the PAS method involved two successive steps: the liberation of the carbonyl groups by periodic acid, and the identification of these groups through their characteristic formation of purple addition compounds with the Schiff reagent. According to Leblond (1950), the oxidation of 1,2 glycols by periodic acid is highly specific at a pH lower than 6, and no further oxidation occurs after the initial aldehyde formation.

Furthermore, it has been reported (Leblond, 1950) that, under the conditions that the PAS technique is used, the substances detected are glycogen, which can be hydrolyzed from sections through the action of salivary amylase; mucoproteins; and such mucopolysaccharides as mucopolysulphuric acid, heparin, and hyaluronic acid.

Cain (1949) found that, if ordinary unsaturated lipids were exposed to air, they attained a capability of producing a color with the Schiff reagent. Herman and Dempsey (1951) have shown that the Ashbel-Seligman reaction for carbonyl compounds stains the same structure revealed by the PAS technique after analogous procedures. According to these authors, this reaction suggests that carbonyl groups are responsible configurations in the group of methods employing the Schiff's reagents for localization of various lipids, carbohydrates, and mucoproteins. Likewise, Deane and
Andrews (1953) suggested that in some cases the aldehydic substance which reacted with the Schiff reagent was derived from unsaturated lipids.

According to Mogg and Wenger (1952), all tissues which contained enough phosphatase to give a clear-cut Gomori reaction also contained mucopolysaccharide as demonstrated by the PAS technique. These investigators suggested that the phosphatase-associated mucopolysaccharide constituted part of a dynamic cytoskeletal mechanism which oriented the enzyme and furnished the favorable chemical conditions for its activity.

In a comparative study of the staining affinities of aldehyde-fuchs in and the Schiff reagent, Scott and Clayton (1953) reported that, after periodic acid oxidation, acrosomes stained as intensely with the Schiff reagent as with aldehyde-fuchs in. However, without oxidation, the acrosomes exhibited only a slight reaction with the Schiff reagent. These investigators concluded that there was a similarity in affinity for aldehyde groups on the part of aldehyde-fuchs in and the Schiff reagent, but that these reagents differed in that the former appeared to possess an affinity which the latter did not demonstrate for strong sulfur acids.

Ihotka (1953) cautioned that, when carbohydrates were localized by the PAS technique, the specificity with 1,2 glycols in the formation of aldehyde radicles could be destroyed by excessive oxidation with involvement of amino acids, especially those with hydroxylamino linkages.

Using solubility as the basis, Leblond (1950, p. 15) divided the naturally occurring compounds which react with periodic acid into three groups. These include:
(a) Water-soluble substances, such as monosaccharides, amino acids, and some proteins.
(b) Fat-soluble substances, such as cerebrosides and steroids.
(c) Water-insoluble and fat-insoluble substances.

Through the fixation and embedding procedure employed, reactants which might be classified in the first two groups would be removed. Moreover, the prolonged treatment of tissue sections in hot methanol-chloroform would serve to remove any persistent fat-soluble substance which might have remained. However, throughout the course of this investigation, no differences were observed between methanol-chloroform treated sections and those which had gone through the routine PAS technique.

According to Leblond (1950), the third group listed above is composed primarily of carbohydrates, proteins, or carbohydrate-protein complexes. Although these substances are known to give some PAS-positive coloration in vitro, the intensity of staining reaction in tissue sections would be dependent upon a sufficient concentration of reactive glycol groups (Leblond, 1950).

Glycogen is identified by the PAS technique. Following salivary amylase hydrolysis, glycogen is removed from the site of reaction. Thus, in the present investigation, abundant glycogen deposits were localized in the tunica albuginea of the horned lizard testes; in the Leydig, Sertoli, and germ cells of lizard and frog testes (Fig. 121 and 122); and in the connective tissue sheath which covers and separates the testis of the bluegill fish into lobules (Fig. 124).
Schiff-reactive material in vertebrate testes

Leblond and Clermont (1952a) have demonstrated that the PAS technique is an excellent staining method for tracing the various stages of rodent spermiogenesis. These investigators carried out detailed studies on spermatid metamorphosis in the rat, mouse, hamster, and guinea pig, and were able to subdivide spermiogenesis into four phases: a Golgi, a cap, an acrosome, and a maturation phase. Likewise, Leblond and Clermont (1952a) found that the acrosomic system existed as a fundamental structure among all species which had been examined by them. In addition, a PAS-positive acrosome has been reported in the deer (Wislocki, 1949), mouse (Elftman, 1950), bovine, reptile, and Arvelius albopunctatus (Leuchtenberger and Schrader, 1950, and Schrader and Leuchtenberger, 1951).

Melampy, Cavazos, and Porter (1952) confirmed the findings of a Schiff-positive acrosome in the bull. Elftman (1952) observed PAS-reactive material in the human spermatid.

It is evident that PAS-positive substances are present in widely diversified species. Therefore, it becomes of interest to examine a number of species within a major sub-phylum in order to note the presence or absence of an acrosomic system containing the 1,2 glycol linkage, and to follow acrosome formation. In the present investigation, within the class Mammalia, the results obtained by Leblond and Clermont (1952a) on the rat and guinea pig were confirmed, and a detailed description of spermiogenesis in the bull and ram was obtained with the PAS technique. Moreover, as in the case of the mammals studied, the mode of acrosomic formation in the representatives of the classes Aves and Reptilia was
traced to its precursors in the Golgi region of the primary spermatocytes. Following the PAS technique, Gersh (1949) classified a portion of the Golgi apparatus as a carbohydrate-protein complex, and discussed the possibility that such a glycoprotein played a role in cell metabolism. However, it is of interest that, in the lower forms of Vertebrata examined, the frog and the fish, no acrosome was discerned with the staining methods employed in the present investigation.

The similarity of spermiogenesis in all species examined, with exception of the frog and fish, is quite striking. From a comparative viewpoint, the acrosome arises in the central portion of the Golgi body, or idiosome, of the primary spermatocyte in most of the species studied. The proacrosomic granules generally are formed during the initial phases of spermiogenesis. This development is followed by formation of the acrosomic granule, and eventually spreading of PAS-reactive material over the nucleus takes place. The similarity which exists in spermiogenesis in the bull and ram and that found in the fowl and horned lizard is readily seen by comparison of Figures 1 through 15 and 16 through 30, as well as Figures 62 through 76 and 77 through 90.

The presence of testicular glycogen in some of the vertebrates studied is of interest. It was observed that glycogen was not localized in the testes of mammals and birds examined, whereas abundant deposits of this polysaccharide were readily noted in the tunica albuginea of the horned lizard testes; in the Leydig, Sertoli, and germ cells of the lizard and frog (Figs. 121 and 122); and in the connective tissue sheath of the bluegill fish testis (Fig. 124). Thus a marked distinction exists in
glycogen distribution among the various species examined in this investigation.

In the human, glycogen has been localized with the PAS technique by various investigators including Arzac (1950b), Montagna and Hamilton (1952), Montagna (1952), Elftman (1952), Long and Engle (1952), and Mancini, Kolazco, and de la Balze (1952). Wislocki (1949) noted the presence of a small amount of glycogen in the Sertoli cells of the deer. However, using the PAS technique, the glycogen-iodine method, and the Bauer method, Mancini and Burgos (1940) found glycogen in the interstitium of the toad testicle. This finding is in agreement with the results obtained in the present study on the frog testis. However, in this investigation, glycogen was also observed in the Sertoli cells and germ cells of the frog.

In Amphibia and Reptilia it is possible that the glycogen found in the testes of the species examined may be of physiologic significance to the Sertoli cells and the spermatozoa during the period of sexual inactivity which precedes the release of the latter the following spring. Montagna and Hamilton (1952) have suggested that, in view of the fact that human Sertoli cells are rich in glycogen, they may act as "nurse cells" for the transforming spermatids and spermatozoa. The results obtained in the present study with regard to glycogen in the lizard and frog tend to support this concept of Montagna and Hamilton (1952). However, in view of the fact that estrogens as well as androgens are produced by the testis, Huggins and Moulder (1945) have suggested that the Sertoli cells may be the source of estrogens. These investigators found, by histochemical and bioassay procedures, a high estradiol
content in Sertoli-cell-tumor tissue of dogs. Likewise, Ashbel, Cohen, and Seligman (1951) reported histochemical localization of estrogen in Sertoli cell tumors and in normal cells. Nevertheless, Maddock and Nelson (1952) have presented evidence that the Leydig cells produce estrogen. They found that injection of chorionic gonadotrophin brought about an increased urinary estrogen output, interstitial cell hypertrophy, and tubular atrophy.

With the exception of the bluegill fish, a PAS reaction was observed in the Leydig or interstitial cell cytoplasm of all species examined in this investigation. Dempsey (1948), in a review of the histochemistry of the endocrine glands, reported that the Leydig cells were Schiff-positive. However, some question exists as to whether true interstitial cells of a secretory nature are present in the adult fowl (Boring, 1912, Pearl and Boring, 1912, and Boring and Pearl, 1917). Nevertheless, according to Sturkie (1954) the consensus of opinion is that the Leydig cells of the rooster produce androgen. This author stated that it has been found that the increase in the interstitial tissue of the testis coincides with the growth and development of the comb. Sturkie (1954) considers this an indication of androgenic activity.

Following examination of 17-day-old cockerels, Taber (1951) reported interstitial cells in the testis which were grouped in clusters and dispersed between the tubules. These cells demonstrated relatively large, osmic-stained droplets. According to this author his experiments provided additional evidence that the interstitial cells of the testis were responsible for the production of androgen in the Leghorn. However, Munro (1938) isolated sperm in the ductus deferens of the fowl and found
that these gametes retained the capacity for movement in physiological saline for an average period of 26 to 28 days, irrespective of the presence or absence of functional testicular tissue. According to Munro (1938) this finding demonstrated that the testis hormone was not directly concerned with the maintenance of sperm life in the ecurrent reproductive ducts of the rooster.

This controversy relative to the presence or absence of Leydig cells in a species also occurs regarding the class Pisces. Canfini (1902) reported the absence of these cells in the fish. Turner (1919), in a comprehensive work on the testis of the perch, made no mention of Leydig cells. Craig-Bennet (1931) found that the germ cells and interstitial and connective tissue of the testes of the three-spined stickleback, Gasterosteus aculeatus Linn., underwent an annual cycle of development and regression. James (1946) observed distinctive cells in the interlobular tissue of the bluegill fish testis, but did not investigate them further. In a study of the gametogenesis of Polyden spathula, Larimore (1950) stated that, although the occurrence of interstitial tissue in fishes was a point of considerable controversy, this tissue was clearly evident in material which he had examined.

With the PAS technique and lipid stains employed in the present study, interstitial cells were not observed in the bluegill fish testis. It is possible that the bluegills used were at a period of sexual activity which caused regression of the interstitial cells; therefore, these cells might not have been noted. Nevertheless, in view of the contradictory results regarding the interstitium of the fish testis, the question of the elaboration of the male sex hormone in Pisces is
an interesting one which remains open to further investigation.

Within the PAS-reactive cytoplasm of the interstitial cells of the bull, rat, ram, chicken, horned lizard, and frog were noted Schiff-positive granules which were not removed by treatment with saliva or methanol-chloroform mixture. Comparable granules were not visible in the Leydig cell cytoplasm of the guinea pig testis. In addition to the above mentioned granulation, glycogen was discerned in the interstitial cells of the lizard and frog testes. It is of interest that a specific histochemical reaction demonstrates a distinctive pattern of localization within definite cell types in representatives of classes which compose a group as inclusive as a subphylum.

In many cases only a weak coloration was observed in the Leydig cells, walls of blood vessels, basement membrane of the seminiferous tubules, and the germ cell cytoplasm. Hale (1953) demonstrated that certain mucinous substances which were weakly positive after the PAS technique stained more strongly following treatment with sodium hydroxide. According to Hale (1953), this deeper staining was produced by making available for aldehyde production by periodic acid more -CHOH-CHOH-, -CHOH-CHH2-, or -CHOH-CHNR groups.

In the various species studied in the present investigation, the results obtained, with regard to the Schiff-reactive basement membrane, are comparable to those of Montagna and Hamilton (1952), who reported the demonstration of this structure in the human testis following the PAS method.

Schoenfeldt (1902) carried out some of the early studies on the spermatogenesis of the bull. Wodsedalek (1920) also did extensive work
on bovine spermatogenesis; however, his study dealt principally with the chromosome numbers and the method of sex determination in the bull. In a later investigation, Gresson and Zlotnik (1948) examined the cytoplasmic components which were present during the gametogenesis of the bovine. These investigators briefly described the formation of the acrosome in the bull with the aid of Flemming's fluid without acetic acid, Kolatchev's fluid, and Aoyama's fixative. Sections prepared in Kolatchev's fluid were stained with acid fuchsin or with hematoxylin. Gresson and Zlotnik (1948) reported that at the spermatid stage an archoplasmic vacuole, containing an archoplasmic granule, was present. Essentially, this report has been confirmed in the present investigation. The proacrosomic granules were seen to originate from within the idiosome of the Golgi apparatus, and eventually these structures gave rise to the acrosome. The PAS technique is of extreme value in the determination of the details of the acrosomic system.

According to Leblond and Clermont (1952a), these authors following the suggestion of Növes (1899) generally adopted the subdivision of spermiogenesis into four phases. The four phases established by Leblond and Clermont (1952a) are equivalent to the four distinct periods during spermiogenesis. These investigators reported that the Golgi phase takes place during the formation of the acrosomic granule within the idiosome; the cap phase occurs at the time of head cap formation; the acrosome phase lasts throughout the orientation of the cell and the establishment of the acrosome, and the changes needed to complete spermiogenesis constitute the maturation phase.
It is of interest that the results obtained in the present investigation on the bull, as seen in Figures 1 through 15 and 93 through 98, closely fit into the four phases which have been established for rodents. In addition, Leblond and Clermont (1952a) state that this breakdown into four phases is a natural classification, and that these phases had been recognizable in the four species of rodents which they had described as well as in the ram and the bull. However, as yet, no report has appeared on the details of spermiogenesis in the latter two species.

Bretschneider (1950) investigated by electron microscopy the head caps in various animals and was able to differentiate in those structures two parts, an inner portion which had its origin from the acrosome and an outer part which arose from the acroblast. Hancock (1952) studied the microscopical features of living and dead bull sperm and reported that the cap of the living cell possessed a characteristic structure which was identical with the acrosome. The altered cap structure of dead sperm was identical to a second cap-like structure, the galea capitis. Hancock (1952) concluded that the terms galea capitis and acrosome had been used to describe the same structures, and suggested that the cytoplasmic cap of the bull sperm be termed "acrosome."

Wodeckalek (1922) and Ahmed (1940) studied the chromosomal behavior in the ram during spermatogenesis. Gresson and Zlotnik (1945) carried out an investigation of the male germ cells of certain mammals, including the sheep. According to Gresson and Zlotnik (1945), the acrosome of the ram had its origin from the proacrosome and archoplasmic vacuole. These investigators reported that the nucleus of the early spermatid was depressed by the growth of the archoplasmic vacuole, and
that later it grew out in an anterior direction. The results obtained with the PAS technique in the present study are in agreement with Gresson and Zlotnik (1945) on the origin of the acrosome. However, in the present investigation, and with the method employed, no depression of the spermatid nucleus was observed.

Randall and Friedlaender (1950) used the electron microscope in an investigation of the microstructure of ram spermatozoa. Their study included an examination of the acrosome and the presentation of evidence for a skeletal framework within the sperm head. Gresson (1951) found with the electron microscope that a thin sheath-like structure, the galea capitis, was present external to the acrosome of the ram sperm. In addition, this investigator reported that the acrosome of mammalian sperm arose from the proacrosome and probably from a substance within the archoplasmic vacuole. Gresson (1951) stated that the Golgi material was related with the formation of the proacrosomic granules, the proacrosome, and the archoplasmic vacuole.

It is of interest that the formation of the acrosome of the bull and the ram, as observed in the present investigation with the PAS technique, was comparable to the same structure in man, as described by Gatenby and Beams (1935).

Brown (1885), Lenhossek (1898), Rekau (1901), Duesberg (1908), Allen (1918), and Minouchi (1927) are among the earlier investigators who contributed to the knowledge of the spermatogenesis and spermogenesis of the rat. As pointed out by Leblond and Clermont (1952a), the work of Duesberg (1908) most closely parallels the results on spermogenesis as obtained with the PAS method. More recently, Gresson and Zlotnik (1945)
included the white rat in a study of the cytoplasmic components of the male germ cells of mammals. These investigators reported that the acrosome in the rat arose from the proacrosome and substances within the archoplasmic vacuole. Later, Grosson (1950) observed the archoplasmic vacuole, proacrosome, developing acrosome, and the acrosome of the rat with the phase-contrast microscope. Studies with the PAS technique on the spermiogenesis of the white rat have been carried out by Leblond (1950), Clermont and Leblond (1950), Leblond, Clermont, and Cimon (1950), Clermont (1951), and Leblond and Clermont (1952a, 1952b). As shown in Figures 31 through 45, the results of these investigators have been confirmed in the present study.

Investigations on the gametogenesis in the male guinea pig have been carried out by Lenhossek (1898), Heves (1899), Papanicolaou and Stockard (1918), and Gatenby and Woodfer (1921). Bookhout (1937) studied the postnatal development of the testis in relation to the germ cell cycle in the guinea pig and found that mature sperm were produced at 58 days of age. The results of Clermont (1951) and Leblond and Clermont (1952a), who used the PAS technique as a method of study of the spermiogenesis of the guinea pig, are in agreement with those obtained in the present investigation (Figs. 46 through 61).

Ouyer (1916) and Miller (1938) worked on the spermatogenesis of the chicken. The latter author reported that the acrosome was formed from idiosome material which migrated around the nucleus to the anterior end before elongation commenced. According to Miller (1938), sperm in the tubules demonstrated an acrosome. Zlotnik (1947) stated that the acrosome of the chicken was formed from the proacrosome which originated
within the archoplasmic vacuole inside of the Golgi material of the spermatid. Zlotnik (1947) reported that the acrosome covered the anterior tip of the sperm head and was conical in shape. The results obtained in the present investigation with the PAS technique are, generally, in agreement with Miller (1938) and Zlotnik (1947), who employed different staining and fixing methods.

The spermatogenesis of the horned lizard, Phrynosoma cornutum, has been investigated by Cavazos (1951). Bloant (1929) studied the seasonal variation in the number and morphology of the interstitial cells of the testis and found a definite interstitial cell cycle. In the present investigation, the application of the PAS technique to the testes of the horned lizard readily demonstrated the mode of acrosome formation in this vertebrate. Of particular interest is the similarity which exists in spermiogenesis of the fowl and the lizard. Even the spermatozoa are comparable in that in both species only the extreme tip of the long, slender nucleus contains the acrosome, as shown in Figures 75, 76, and 90.

Following staining with the PAS technique, no acrosome, or structures giving rise to an acrosome, were visible in the germ cells of the frog and the fish. It is possible that the inability to demonstrate a Schiff-reactive acrosome in these two species may be either due to complete absence, or to the presence of only a small amount of substances containing the 1,2 glycol linkage in the acrosome system. Bowen (1922) observed an idiosome in the primary and secondary spermatocytes as well as in the spermatid stage in the amphibian Plethodon cinereus Green. According to Bowen (1922) the acrosome arose in relation to the Golgi
Bowen (1924) suggested that the acrosome of animal spermatozoa was essentially a secretory product which had as its chief function the initiation of the physiochemical reactions of fertilization. However, Leblond and Clermont (1952a) stated that little was known of the role of this structure in fertilization. These authors pointed out that, if the reactive substances in the acrosomic system were composed of polysaccharides, they probably had the high viscosity characteristic of this type of carbohydrate. Therefore, this viscosity might be involved in retention of the spermatids in contact with the Sertoli cells. In addition, according to these investigators, the loss of staining intensity of the acrosome during the later part of the maturation phase could account for the release of the cells from the Sertoli elements.

It is of interest that the acrosomic system has been clearly demonstrated in a comparative study of vertebrate testes. However, the exceptions to this, the frog and the fish, remain unexplained. Nevertheless, the value of the PAS technique in this type of investigation is without question. The four phases of spermiogenesis established by Leblond and Clermont (1952a) were discerned in all animals studied, except in the two species mentioned above. It is possible that, through the use of the PAS technique, those four phases will be found in the spermiogenesis of other species which represent even more diversified classes than those examined in the present investigation.
A comparative histochemical study of vertebrate testes was carried out on sexually mature males representing the classes Mammalia, Aves, Reptilia, Amphibia, and Pisces. Lipid and periodic-reactive carbohydrate distribution were examined as to their morphological localization in the bull, ram, rat, guinea pig, rooster, the horned lizard, Phrynosoma cornutum (Harlan), the frog, Rana pipiens, and the bluegill fish, Lepomis macrochirus Rafinesque.

In order to demonstrate the lipid components of the vertebrates studied, the following methods were employed: Sudan black B, oil red O, the Ashbel-Seligman reaction, the Schultz test for cholesterol, polarization microscopy after digitonin treatment, and Baker's acid hematin test for phospholipids. For the localization of periodic acid-reactive carbohydrates, the staining technique was the periodic acid-Schiff method (PAS).

Following use of the Sudan dyes, lipids were noted in the interstitial cell cytoplasm, the basement membrane, and the cytoplasm of the Sertoli cells, spermatogonia, spermatocytes, and spermatids of all species studied with the exception of the bluegill fish. In the bluegill, lipids were visible in the germ cells and in the interlobular connective tissue sheath. A positive Ashbel-Seligman reaction was obtained in the Leydig cells of all species examined, except in the case of the rooster and the fish. With the aid of Baker's acid hematin test, phospholipid was found in the testicular cell types of the species.
studied. This method was not applied to the bluegill fish testis. Schultz-positive material was noted in the Leydig cells of the bovine, ram, rat, guinea pig, horned lizard, and frog. Birefringent crystals were present in the Leydig cells and seminiferous tubules of the frog.

In this investigation, the comparable results obtained as to lipid localization in the various species studied suggest the possible widespread distribution of these substances in the Leydig, Sertoli, and germ cells of closely related species as well as those which are even more distant in taxonomic classification.

The chemical nature of substances giving a positive PAS reaction has been discussed. With the aid of the PAS technique, glycogen was identified in the tunica albuginea of *Phrynosoma cornutum*; in the Leydig, Sertoli, and germ cells of the lizard and frog, as well as in the connective tissue sheath of the bluegill fish testis. Schiff-positive material was localized in the interstitial cells, walls of blood vessels, basement membrane, and the cytoplasm of the Sertoli and germ cells of the various species examined with exception of the fish. In the case of the latter species, PAS-positive staining was visible in the interlobular connective tissue. The acrosomic system, arising in the idiosome and forming the acrosome of spermatozoea, was described in the bovine, ram, rat, guinea pig, chicken, and horned lizard.

The possibility is suggested that the four phases of spermiogenesis, as observed with the PAS technique (Golgi, cap, acrosome, and maturation phase), might be found in even more diversified species than those studied in this investigation.


———. 1950 Cytological technique. 3rd ed. N. Y., John Wiley and Sons, Inc.


. and . 1952 The use of lead tetraacetate, benzidine, o-dianisidine and a "film test" in investigating the periodic acid-Schiff technique. Stain Techn. 27:277-305.


1951 The structure and formation of the mammalian spermatozoon. La Cellule. 54:80-102.


Herman, E. 1950 Histochemical and cytological techniques. Department of Anatomy, Harvard Medical School, Boston. (Mimeographed).

and Dempsey, E. W. 1951 The demonstration of compounds containing carbonyl groups in tissue sections. Stain Techn. 26: 185-191.


Hotchkiss, R. D. 1948 A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem. 16:131-141.


--- 1945a Carycineal red and coccineal red, oil soluble anthraquinone dyes, as fat stains in animal histology. Stain Techn. 20:73-75.

--- 1945b Oil blue N or NA as a fat stain for animal histology. Stain Techn. 20:7-9.


and Ashburn, L. L. 1943 Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degenerations not shown by Herxheimer technique. Arch. Path. 36:32-135.

Lipschütz, A. 1924 The internal secretions of the sex glands. Baltimore, Williams and Wilkins Co.


MacLean, H. and MacLean, I. 1927 Leicithin and allied substances. 2nd ed. London, Longmans, Green and Co. Ltd.


_________ and Greco, J. 1952 The effects of formaldehyde on tissue lipids and on histochemical reactions for carbonyl groups. Stain Techn. 27:317-321.


ACKNOWLEDGMENTS

The writer wishes to express his sincere appreciation to Doctor Robert M. Kelampy who suggested the problem, directed the course of its investigation, and gave his constant aid and encouragement.

Acknowledgement is made of support of this study through a contract with the Office of Naval Research, Department of the Navy, NR 16h-141.
Testicular material for lipid studies was fixed in 10 per cent neutral formalin, washed in tap water for six hours, placed in gelatine at 37° C. overnight, embedded in 10 per cent gelatine, and hardened for several hours in the refrigerator. Tissue blocks were cut out and stored in neutral formalin until ready for use. The neutral formalin was prepared as follows:

Formaldehyde (36 to 38 per cent) ................. 10 cc.
Distilled water ..................................... 90 cc.
This mixture was neutralized with calcium carbonate.

Sudan black B. The stain was prepared as a saturated solution in 70 per cent alcohol. Procedure:

1. Sections mounted on glass slides were dipped into 70 per cent alcohol for a moment.
2. Staining was carried out in freshly filtered stain for seven minutes.
3. Slides were dipped momentarily into 70 per cent alcohol, in order to remove excess stain, and then washed in distilled water.
4. No counterstaining methods were employed.
5. Sections were mounted in glycerine jelly.
Oil red 0. A stock saturated solution of oil red 0 and 99 per cent isopropyl alcohol was prepared, using 250 to 500 mg. of dye per 100 cc. Procedure:
1. Six cc. of the stock solution were diluted with 4 cc. of water.
2. The diluted stain was permitted to stand for ten minutes and then filtered.
3. Frozen sections were stained for ten minutes, followed by washing in several changes of distilled water.
4. Material was counterstained for five minutes in undiluted Mayer's hemalum. The counterstain was prepared as follows:
   Hematoxylin............................................. 1 gram
   Ammonium aluminum sulfate......................... 50 grams
   Distilled water........................................ 1,000 ml.
   Ripening agent......................................... 0.2 gram NaI03
   Acetic acid.............................................. 20 ml.
   Preservative............................................ 50 grams
5. Sections were blued in tap water, rinsed in distilled water, and mounted in gum syrup. The gum syrup mounting medium was prepared according to the method of Lillie and Ashburn (1914): Fifty grams of gum arabic and 50 grams of cane sugar were dissolved in 100 cc. distilled water by frequent stirring at 55° to 60° C. Thymol crystals were added as a preservative. The mixture was placed in a vacuum chamber for a few minutes while warm in order to remove air bubbles.

Ashbel-Seligman reaction. Procedure:
1. Sections mounted on microscope slides were washed in
several changes of distilled water for several hours. This step removed the formalin from the frozen sections.

2. The sections were incubated in the hydrazide solution at room temperature for two hours. The hydrazide solution was prepared as follows:

One gram of 2-Hydroxy-3-naphthoic acid hydrazide was dissolved in 50 cc. of hot glacial acetic acid in a volumetric flask. To this was added 950 cc. of 50 per cent alcohol prepared just before use from absolute ethyl alcohol.

3. After the hydrazide treatment, the slides were washed in several changes of 50 per cent alcohol for two hours.

4. Incubation followed in 0.5 n hydrochloric acid at room temperature with subsequent washing in several changes of distilled water.

5. Sections were rinsed in 1 per cent sodium bicarbonate and washed in water.

6. The slides were placed in alcohol-buffer solution prepared from a phosphate buffer at pH 7.2, 1/15 N, mixed with an equal volume of absolute ethyl alcohol just before use. To this was added tetrazotized diorthoanisidine powder followed by stirring. The staining reaction was carried out for ten minutes.

7. The material was then washed in several changes of distilled water, and mounted in glycerine jelly.

**Schultz reaction.** Frozen sections were placed in a 2 per cent solution of ferric ammonium sulfate for 24 hours. The sections were rinsed in distilled water and blotted dry. A cover slip was added, and a few drops of a mixture of equal parts of glacial acetic acid and
concentrated sulfuric acid were allowed to flow underneath the cover slip. Generally, a change of colors took place, but only a blue-green one was considered diagnostic for cholesterol.

**Polarization microscopy.** Frozen sections were placed for six hours in a 0.5 per cent solution of digitonin in 50 per cent ethyl alcohol. This step was followed by washing in 50 per cent alcohol, and then in water. Sections were mounted in glycerine jelly and observed under the polarizing microscope for birefringence.

**Baker's acid hematin test.** For the localization of phospholipid within testicular tissue, fixation was in formaldehyde-calcium for at least 6 hours and not longer than a week. The formaldehyde-calcium was prepared as follows:

- Formaldehyde (36 to 38 per cent) ....................... 10 cc.
- 10 per cent aqueous calcium chloride .............. 10 cc.
- Distilled water .......................................... 80 cc.
- Calcium carbonate was kept in the solution.

**Procedure:**

1. After fixation, and without washing, tissues were transferred to dichromate-calcium at room temperature for 18 hours. Preparation of the dichromate-calcium solution was as follows:
   - Potassium dichromate ......................... 5 grams
   - Calcium chloride ................................. 1 gram
   - Distilled water ................................. 100 cc.

2. Tissues were then placed in dichromate-calcium at 60° C. for 24 hours.
3. Material was washed under tap water for six hours, placed in 10 per cent melted gelatine at 37° C., and left overnight.

4. The gelatine was solidified in the refrigerator, cut out in rectangular blocks, and left in formalin-calcium to make it insoluble, or until ready to cut.

5. Blocks were washed for 30 minutes and cut at 5 microns on a frozen section microtome. Sections were mounted on slides with a 1 per cent gelatine solution.

6. If it was not convenient to stain the sections immediately, they were left in formalin-calcium. If left in this solution, the sections were washed in distilled water, and placed in hot dichromate calcium for one hour at 60° C.

7. Sections were washed in several changes of distilled water for five minutes, and placed in acid hematin at 37° C. for five hours. Acid hematin was prepared as follows:

   Hematoxylin........................................... 0.05 gram
   Distilled water........................................... 48 cc.
   Potassium iodate (1 per cent solution)........... 1 cc.

   The mixture was heated until the water just began to boil. After cooling, 1 cc. of glacial acetic acid was added. The stain was used on the day of preparation.

8. Following staining, the sections were rinsed in distilled water and placed in borax-ferricyanide at 37° C. for 18 hours. Borax-ferricyanide:

   Potassium ferricyanide............................... 0.25 gram
Borax (sodium tetraborate crystallized with ten molecules of water and powdered) .......... 0.25 gram
Distilled water ........................................ 100 cc.

9. Sections were washed in several changes of distilled water for ten minutes, and mounted in glycerine jelly.

Controls for this method were extracted in pyridine in order to distinguish the lipids from certain proteins. Fixation of material for the control procedure was in "weak Bouin's fluid":

Picric acid, saturated aqueous solution ............... 50 cc.
Formaldehyde (36 to 38 per cent) .................. 10 cc.
Acetic acid (glacial) ........................................ 5 cc.
Distilled water ........................................ 35 cc.

Tissues were fixed overnight in this fluid.

1. Following fixation, the tissues were placed in 70 per cent alcohol for one hour, transferred to 50 per cent alcohol for 30 minutes, and washed under tap water for 30 minutes.

2. Tissues were then placed in pyridine for one hour, and subsequently transferred to fresh pyridine for one hour.

3. This was followed by pre-warmed pyridine at 60° C. for 24 hours, washing for two hours in running water, and transferring to dichromate-calcium at room temperature for 18 hours. From this step onward, the tissue was treated as in the previously described acid hematin test.
Carbohydrates

General procedure

For the study of glycogen and other polysaccharide complexes testicular material was fixed in Orth's fluid and stained by the periodic acid-Schiff technique. Orth's fluid was prepared as follows:

Potassium bichromate................................. 2.5 grams
Sodium sulfate............................................... 1 gram
Distilled water............................................. 100 cc.

To 100 cc. of the above mixture 10 cc. 36 to 38 per cent formaldehyde were added at the time of use.

Following fixation the tissue was washed overnight in tap water, dehydrated and cleared in dioxan, and embedded in paraffin with a melting point of 54° to 56° C. Sections were cut on a rotary microtome at seven and 10 microns, and adhered to the slide with Meyer's egg albumin. The Schiff reagent was prepared according to the following method:

One-half gram of basic fuchsir was dissolved by pouring over it 100 cc. of boiling distilled water. This solution was cooled to 50° C., filtered, and 10 cc. of 1 Normal hydrochloric acid and 0.5 gram of anhydrous potassium metabisulfite were added to the filtrate. This mixture was left in the dark overnight. Subsequently, charcoal was added followed by thorough shaking and immediate filtration. Care was taken that the Schiff's reagent was completely decolorized. The solution was then tightly stoppered and stored in the refrigerator.
Sections were stained according to the following procedure:

1. Slides were deparaffinized and passed down to the second absolute alcohol.

2. In some cases sections were covered with a solution of 0.5 per cent celloidin in equal parts of absolute alcohol and ether, transferred to 70 per cent alcohol for ten minutes, and washed in distilled water. At times the celloidin covering was not applied; especially in sections which were to be treated with saliva for removal of glycogen.

3. Sections were placed in a 1 per cent solution of periodic acid for five minutes, washed under tap water for ten minutes, followed by staining in the Schiff reagent for 15 minutes.

4. From the stain the slides were transferred directly to two changes of:
   
   10 per cent potassium metabisulfite .......... 5 cc.
   1 Normal hydrochloric acid ..................... 5 cc.
   Distilled water ............................... 100 cc.
   
   for five minutes each. In some instances step 4 was eliminated from the procedure.

5. Sections were washed under tap water for ten minutes, dehydrated, cleared, and mounted.
Figures 1 through 15. Spermiogenesis in the bovine as revealed by the PAS technique.
Figures 16 through 30. Spermogenesis in the ram as demonstrated by the PAS technique.
Figures 31 through 45. Acrosome formation and spermatid metamorphosis in rat testes. PAS technique.
Figures 46 through 61. Spermiogenesis in the guinea pig as revealed by the PAS technique.
Figures 62 through 76. Acrosome formation in the rooster.
PAS technique.
Figures 77 through 90. Spermiogenesis in the horned lizard, *Phrynosoma cornutum*, as demonstrated by the PAS staining technique.
Figure 91. Localization of Sudan black B-reactive droplets in the interstitial cell cytoplasm of the bovine testis. Note the presence of unstained nuclei. (x1125).

Figure 92. Distribution of lipids in the seminiferous tubules and Leydig cells of the bull testis. Concentrations of Sudan black B granules are visible in the Sertoli cells. (x125).

Figure 93. The PAS technique applied to bull testis. A positive reaction is discerned in the basement membrane of the seminiferous tubule as well as in the germ and Sertoli cell cytoplasm. A Schiff-positive reaction is visible during this early stage of acrosome formation. The reactive material has commenced spreading over the spermatid nucleus. (x90 objective and x7.5 ocular).

Figure 94. PAS-reactive material in bull testis. Again note the strongly reactive basement membrane. The spermatids are in a later developmental stage than in Figure 93. Faintly outlined spermatozoa are visible embedded in the Sertoli cytoplasm. (x90 objective and x7.5 ocular).
Figure 95. The PAS technique applied to bull testis. Schiff-positive spermatozoa are lodged in the Sertoli cell cytoplasm and the reactive headcaps are in an early stage of acrosome formation. (x90 objective and x7.5 ocular).

Figure 96. A later stage in acrosome formation in the bull testis as discerned with the PAS technique. The acrosomic granule and the spreading Schiff-reactive material are visible in the spermatids. (x90 objective and x7.5 ocular).

Figure 97. An advanced stage in bovine spermiogenesis as demonstrated by the PAS technique. (x90 objective and x7.5 ocular).

Figure 98. PAS-reactive protoplasmic droplets in bovine spermatozoa. The remaining portion of the sperm tail is unstained. (x1100).
Figure 99. Sudan black B-reactive granules in the cytoplasm of the ram interstitial cells. Note the presence of the arteriole containing unstained erythrocytes. (x1000).

Figure 100. PAS-reactive acrosomic caps during spermatid metamorphosis in the ram. (x1125).

Figure 101. Various stages of spermiogenesis in ram testis as demonstrated by the PAS technique. (x1125).
Figure 102. Lipid granules in the cytoplasm of the Leydig cells of the rat testis as demonstrated by the Sudan black B technique. The nuclei and nucleoli are unstained. Of interest is the variability in reaction within the same cell types. (x1125).

Figure 103. Ashbel–Seligman technique applied to the rat testis. Note the reactive granules in the Leydig cell cytoplasm, and the unstained nuclei and nucleoli. (x90 objective and x5.0 ocular).
Figure 104. PAS-reactive material commencing movement over the spermatid nucleus. Rat testis. (x90 objective and x7.5 ocular).

Figure 105. Late developmental stage in rat spermiogenesis as demonstrated with the PAS technique. Note the formation of acrosomic granules and the passage of the head cap over the unstained surface of the nucleus. Spermatozoa are located within the Sertoli cell cytoplasm. Reactive cytoplasmic granules are visible in the primary spermatocytes. (x90 objective and x7.5 ocular).

Figure 106. Head cap formation in rat spermatids following the application of the PAS technique. (x90 objective and x7.5 ocular).
Figure 107. Sudan black B-reactive substance in the cytoplasm of the interstitial cells of the guinea pig testis. Note the differential response of these cells and the presence of diffuse, fine, and large granules of sudanophilic material. (x1000).

Figure 108. The Ashbel-Seligman technique in the interstitial cell cytoplasm of the guinea pig. The nuclei and the cells within the seminiferous tubules remain unstained. (x1000).
Figure 109. Schiff-reactive material in guinea pig spermatids and spermatozoa. Observe the early indication of the acrosomic granule. (×1100).

Figure 110. A later developmental stage than the previous figure. PAS technique applied to guinea pig testes. (×1100).

Figure 111. Acrosomic formation in the guinea pig following the PAS technique. (×1000).

Figure 112. Spreading of Schiff-reactive substance during spermiogenesis in the guinea pig. (×1100).
Figure 113. Head cap formation in guinea pig spermatids. PAS technique. (x1100).

Figure 114. Elongation and spreading of Schiff-reactive material in guinea pig spermatids. (x1100).

Figure 115. Later developmental stage than previous figure. PAS technique applied to guinea pig testes. (x1000).
Figure 116. Sudan black B staining in the germ cells of *Phrynosoma cornutum*. Note the reactive granules in the cytoplasm of these cells, whereas the nuclei and nucleoli are unstained. (x90 objective and x7.5 ocular).

Figure 117. Reactive lipoidal granules in the cytoplasm of the Leydig cells of the horned lizard as demonstrated by the oil red O technique. The nuclei are unstained. (x90 objective and x7.5 ocular).

Figure 118. Ashbel-Seligman-reactive material in the cytoplasm of the horned lizard Leydig cells. The nuclei and nucleoli are unstained. (x90 objective and x7.5 ocular).
Figure 119. Sudan black B method applied to the frog testis. Observe the cytoplasmic distribution of reactive granules of varying size. Of interest is the staining of the frog spermatozoon middle-piece. (x90 objective and x7.5 ocular).

Figure 120. Localization of phospholipid material in frog testis following Baker's acid hematin test. Reactive lipid is present in the Leydig cell cytoplasm and in the germ cells. The nuclei and nucleoli are unstained. (x1000).

Figure 121. Glycogen as demonstrated in the frog seminiferous tubules following the PAS technique. Compare to Figure 122. (x150).

Figure 122. PAS reaction following treatment of frog testis with salivary amylase prior to staining. (x150).

Figure 123. Schiff-reactive droplets on frog spermatozids. (x90 objective and x7.5 ocular).
Figure 12h. Glycogen as demonstrated in the connective tissue
sheath which separates the testis of the bluegill fish into
lobules. PAS technique. (x90 objective and x7.5 ocular).