

DEVELOPMENT AND HISTOGENESIS OF
THE AVIAN DIGESTIVE TRACT WITH SPECIAL
REFERENCE TO THE HISTOCHEMISTRY
OF THE CONNECTIVE TISSUE

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DEVELOPMENT AND HISTOGENESIS OF THE AVIAN
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By
Pierson J. Van Alten

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan
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THESIS ABSTRACT

Specimens used in this study were obtained from single-comb white leghorn hatching eggs which were incubated from 11 to 20 days. The digestive tract was removed from the fetuses and the histogenesis of the alimentary canal and histochemistry of the connective tissues within it were studied. Histogenesis was studied in tissues which were fixed in Bouin's solution, sectioned at seven microns and stained in one of the following: hematoxylin and eosin, Mallory's triple stain, Masson's trichrome stain, Weigert's elastic tissue stain, Dempsey's orcein procedure, and Van Gieson's collagenous tissue stain. The histochemistry of connective tissues was studied with the Schiff procedure of Hotchkiss (1948) and with selective staining by toluidine blue at various pH levels. Basic proteins were also used to determine what effect they had on selective staining of mucopolysaccharides by basic dye.

Comparison of the geometric growth in weight of the fetus and alimentary canal indicated that the alimentary canal was growing at a faster geometric rate than was the fetus between the 11th and 20th day. This was based on weights of approximately 270 fetuses and digestive tracts.

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It was demonstrated that the epithelial layer was the first distinct layer to differentiate. The circular muscle fibers of the external muscular layer were the next tissue to differentiate. The lamina propria and submucosa differentiated after the muscle layer and the muscularis mucosae was the last layer to differentiate. The epithelium of the esophagus differentiated into stratified squamous epithelium and the epithelium of the remainder of the tract differentiated into simple columnar. The muscularis mucosae and the lateral muscle fibers of the muscularis externa were absent in the gizzard.

The Schiff procedure for soluble polysaccharides was positive in all tissues of all ages of fetuses studied. The staining reaction was diminished but not completely abolished if tissues were treated with saliva before oxidation with periodic acid. This positive reaction soluble in saliva was attributed to glycogen. The connective tissues were found to stain with the Schiff reagent for insoluble polysaccharides. The staining of these tissues was not diminished by treatment with saliva and it was concluded that they were "neutral" polysaccharides.

The connective tissues of the gut stained pink (metachromatic) with toluidine blue at pH 5.5 and 8.5 and blue (basophilic) at pH 2.5 and 3.5. Basic proteins, albumin and globulin, inhibited staining at pH 3.5. Protamine sulfate

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inhibited staining with basic dye at pH 5.5. Metachromasia persisted in connective tissues after treatment with ribonuclease.

It was concluded that connective tissues of the digestive tract of the fetus contained "neutral" polysaccharides and acid mucopolysaccharides.

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INTRODUCTION

Calhoun (1954) extensively reviewed the literature and described in detail various cells and tissues in the avian digestive tract (Galus domesticus) in specimens from 36 hours subsequent to hatching to 2 years of age. Other workers have made noteworthy contributions to the histology and gross anatomy of the avian digestive tract. McLeod (1939), Bradley and Grahame (1951) and Sturkie (1954) limited their description to the microscopic anatomy of adult birds, while on the other hand, Kaupp (1918) limited his description to the gross anatomy of the tract.

Other species have been used to limited extent for investigations of this type. Kendall (1947) described the histological characteristics of the digestive tract in the adult pigeon. Rosenberg (1941) identified chief cells, mucous cells, and basal granular cells in the duodenal epithelium of the adult turkey. An abundant number of basal granular cells were also found in the tunica propria mucosa.

Embryology and organogenesis of the chicken digestive tract has been studied in great detail. Patten (1952) summarized and described the development of the tract in embryos from fertilization of the ovum through 96 hours incubation. Hamilton (1952) described both early development and organogenesis of the tract in various ages of fetuses.

Schumacher (1926) limited his study to the origin and development of the epithelium and esophageal glands in fetuses ranging in age from 5 to 21 days. Esophageal epithelium was classified as columnar at 5 days, cuboidal at 9 days, and stratified at about 14 days. Glands arose as solid outgrowths of epithelium which acquired a lumen by coalescence and confluence of intercellular vacuoles. More recently, Ivey and Edgar (1952) confirmed Schumacher's observations and extended the study with a description of the development of the lamina propria mucosa and the lamina muscularis mucosae. Mayberry (1935) found that the distal end of the esophagus evaginated to form a recognizable crop in 7-day fetuses and that in 9-day fetuses its diameter was increased about 8 times. In 10-day fetuses the wall of the crop was thinner; growth rate was retarded but it continued through 17 days of incubation. Dilation of the crop on the right side was pronounced on the 18th day of incubation. Epithelium lining the crop consisted of three layers of cuboidal cells (7th day of incubation); fully formed glands appeared on the 17th day of incubation and stratified squamous epithelium was recognizable on the 18th day of incubation.

Formation of the mucous and epithelial layers of the proventriculus and gizzard was described by Hibbard (1942). Glands appeared in the proventriculus on the 7th day of incubation, and the distal ends of the glands forked to form

simple branched tubular glands on the 10th day of incubation. Compound and tubular glands with mucus secreting cells were recognized on the 18th day of incubation and the glandular layer was doubled by the time of hatching. On the 9th day of incubation epithelial cells lining the gizzard were arranged to form a stratified-like, columnar layer of tissue. On the 11th day of incubation a coagulum-like secretion appeared on the distal surface of the epithelial cells. Secretory material was first identified in the lumen of the tubular glands on the 14th day of incubation. Keratinoid materials which were produced by the glands rapidly increased to form as a distinct layer on the epithelial surface in 18-day fetuses.

Innervation of the avian digestive tract was described by Van Camperhout (1933). In the 6-day fetus the esophagus, proventriculus, and gizzard were innervated by the pneumogastric nerve and the sympathetic chain; the duodenum and remainder of the small intestine was innervated by the preaortic plexus, and the large intestine by the nerve of Remak.

Minot (1900) found that in 4-day fetuses the mesoderm of the large intestine was not differentiated into layers. In 6-day fetuses the epithelium lining the large intestine proliferated rapidly to occlude the lumen of the intestine. On the 9th day of incubation the distal end of the rectum was in contact with the epithelium of the proctodaeum and

differentiation of the muscular layers in the large intestine was beginning.

Boyden (1922) found that the cloaca developed from aggregates of disintegrating cells in the tail-bud region of 41-50 somite fetuses. Separation of the cloaca was initiated during degeneration of these cells and completed on the 4th day of incubation. The bursa appeared as a swollen region on the caudal wall of the cloaca. It developed from coalesced vacuoles within the urodaeal membrane between the 5th and 6th day of incubation. Boyden (1924) found that the cloaca consisted of an anterior coprodaeum, a middle urodaeum and a posterior proctodaeum. Development of the urodaeum was induced when the Wolffian duct contacted the lateral wall of the cloaca.

The cytology of the various portions of the digestive tract of the chick has been studied by some investigators. Chodnick (1947) found that the golgi apparatus in epithelial cells of the proventriculus was located between the nucleus and lumen of the gland cells. However, in zymogenic cells it was identified in the vicinity of the intercellular clefts. Mitochondria appeared as delicate filaments in surface epithelium and in the neck cells, and as thick rods and granules in the zymogenic cells. Secretory granules arose in close association with the golgi material. In the gizzard, the golgi apparatus did not change subsequent to feeding.

Golgi bodies in intestinal epithelial cells, subsequent to feeding, moved from the proximal to the distal surface of the cells, and secretory granules increased in number and were arranged in the meshes and on the surface of the golgi apparatus. The mitochondria appeared as filaments, rods, and granules and were in constant polar arrangement. In the goblet cells the golgi material was distal to the nucleus and the mitochondria collected on the border of the mucous mass and in the cytoplasm of the narrow part of the cell. Previously, Hibbard (1942) had described the golgi apparatus in the epithelial layer of the proventriculus and gizzard of the embryonic chick as an aqueous vacuole which is an artefact. Argeseanu and May (1938) showed that chondriosomes were filamentous and uniformly distributed in the cytoplasm of embryonic syncytial cells. In adult chickens chondriosomes were concentrated and formed aggregates at the distal end of the epithelial cells. The supra-nuclear chondriosomes remained in the vicinity of the nucleus throughout the developmental period. Simard and Van Campenhout (1932) observed that argentaffin cells appeared in the intestine on the 11th day of incubation and they were first identified in the epithelium near the umbilical pedicle and ultimately migrated into the submucosa. It was also demonstrated by chorio-allantoic grafts that differentiation of argentaffin cells occurs even in the absence of nervous elements, but

there was a hypertrophic development of connective tissue and muscular tissue was reduced and this was attributed to the absence of nervous elements. Dawson and Moyer (1948) identified argentophile cells in the epithelium of the chick proventriculus on the 8th day of incubation. The number of argentophile cells reached a maximum on the 13th day of incubation. They first appeared in the compound glands on the 12th day and in the gizzard on the 18th day.

Huxley (1932) and Weiss (1939) used Schmalhausen's data and gave mathematical formulation of the relative growth between some digestive organs and the total chick. They made the interesting observation that some of the digestive organs were growing at a faster rate than the total fetus.

Postnatal growth changes in single-comb white leghorn chickens were studied by Latimer (1924, 1925). He observed that in 1-day chicks the digestive tract constituted 4 percent of the net body weight; in 2-day chicks it was 12.1 percent and in 3-day chicks it was 15.8 percent. In the adult it comprised only 2 percent of the total body weight.

The chicken fetus has been used to only a limited extent for histochemical investigation. Moog (1950) found that in the duodenum of 18-day fetuses there was an accumulation of alkaline phosphomonoesterase in the striated borders and in the crypts of Lieberkühn. More recently Moog and Wenger (1952) have found a "neutral" mucopolysaccharide which was

also found at sites of high alkaline phosphatase activity. On the 17th day of incubation she noticed that a thin periodic acid-Schiff-positive line appeared on the surface of the developing villi in essentially the same position as alkaline phosphatase was found. The Schiff material and the alkaline phosphatase developed parallel until maximum levels were reached. The Schiff-positive material was located at sites of high phosphatase activity. It was difficult to hydrolyze and it was resistant to diastase and hyaluronidase. Metachromasia was absent and on the basis of this and other observations, she concluded that the Schiff-positive material was a "neutral" mucopolysaccharide. It functioned as a mechanism for orientation of alkaline phosphatase and provided a suitable environment for enzyme activity.

From the above review of the literature it is evident that investigations concerned with the histogenesis and histochemistry of the digestive tract in the embryonic chick have been fragmentary.

It is the purpose of this study to: (1) ascertain relative growth of the embryonic digestive tract, i.e. the esophagus, crop, proventriculus, gizzard, duodenum, jejunum-ileum, and rectum in comparison to the total embryo; (2) to ascertain when tissue elements of the tract differentiate; and (3) make a study of the histochemical nature of the

connective tissue of the tract for such things as basophilia with establishment of "pH signatures", and the reaction of these tissues to the periodic acid-Schiff reagents.

MATERIALS AND METHODS

Fetuses used in this study were obtained from single-comb white leghorn hatching eggs. Incubation time ranged from 11 through 20 days. In order to show the relation between body weight and weight of the digestive tract, the fetus was removed from the egg along with the yolk sac. The latter was severed near its junction with the small intestine in all fetuses with the exception of 20-day chicks. Wet weights of all fetuses were ascertained and recorded. The alimentary canal in each fetus was severed just posterior to the pharynx and the entire tract including the anus was removed. The liver was excised but the main portion of the pancreas was left in the loop of the duodenum. The gut was rinsed in cold physiological Ringer's solution and the excess moisture was removed with a towel and then the entire tract was weighed and the weight was recorded.

Tissues from the esophagus, crop, proventriculus, gizzard, duodenum, jejunum-ileum, and large intestine were obtained from fetuses in which the incubation age varied between 11 and 20 days. In all routine studies, tissues were fixed from 8 to 12 hours in Smith's modification of Bouin's picro-formol solution (Guyer, 1953). The tissues were then dehydrated, cleared, and embedded in paraffin (melting point 50-52° C.). Sections were cut at approximately

seven microns. They were affixed to clean glass slides and were stained with hematoxylin and eosin. The exact location of connective tissue, muscle, and epithelium in the digestive tract was demonstrated with various connective tissue stains, namely: Mallory's connective tissue stain (Cason, 1950), Masson's trichrome stain (Lillie, 1940), and with the Foot (1933) modification of Masson's stain. In the latter, tissues were left in Regaud's iron-hematoxylin for five minutes prior to the trichrome staining procedure.

For more exact study of connective tissue components, Weigert's resorcin fuchsin was used (McClung, 1939). The orcein staining procedure of Dempsey et al (1952) was also used for the demonstration of elastic fibers. Collagenous tissue was stained with Van Gieson's picro-acid fuchsin (Guyer, 1953).

The periodic acid-Schiff method (Hotchkiss, 1948) was used for study of polysaccharides. Some of the tissues used for polysaccharide tests were fixed in Bouin's fixative and others were fixed in acetic-alcohol-formalin for 24 hours at 0° (Lillie 1954). Tissues which were fixed in acetic acid-alcohol-formalin were oxidized for 5 minutes in Hotchkiss' periodic acid A and those fixed in Bouin's solution were oxidized for 5 minutes in Hotchkiss' periodic acid B.

Basophilic components of the cytoplasm was studied with 0.1 percent toluidine blue. French and Benditt (1953) have

stated that mucopolysaccharides will react with basic dyes. Tissues were left in various buffered solutions for 3 hours. Acid phosphate buffer was used in solution maintained at pH 2.5; acetate buffer in solutions maintained at pH 3.5 and 5.5, and phosphate buffer at pH 8.5. The slides were left in toluidine blue from 10 minutes to 24 hours. The proteins used for blocking staining reactions were protamine sulfate, globulin (from serum), and albumin (bovine plasma). (These were supplied by Bios Laboratories, Inc., New York 23, N. Y.) The proteins were dissolved in a concentration of 1 mg. per ml. of buffered solution. In order to determine if the basophilia was due to ribonucleic acid, the slides were treated with 1:100,000 crystalline ribonuclease (supplied by General Biochemicals, Inc. of Chagrin Falls, Ohio) which was added to the buffered pre-treatment solutions.

RESULTS

1. GEOMETRIC GROWTH OF THE FETUS AND ALIMENTARY CANAL

Latimer (1928) compared postnatal growth of the chick with growth rate of the gizzard from date of hatching to 19 days of age. Further, this author completed his study by the addition of Schmalhausen's data on growth of the fetus (4th day of incubation to hatching) so that a comparison could be made between prenatal and postnatal growth.

Table 2 shows the mean weights, standard deviation, and the maximum and minimum weights of approximately 270 alimentary canals from chick fetuses ranging in age from 11 to 20 days of incubation. It is evident in Table 1 that the mean weight of the fetus increased from 3.35 grams on the 11th day to reach a maximum of 41.96 grams on the 20th day.

Table 2 shows that the mean weight of the alimentary canal increased from 0.12 grams on the 11th day of incubation to a maximum of 3.44 grams on the 20th day. The geometric rate of growth was calculated by dividing the mean weight of the fetus at the end of any 24-hour period by the mean weight of the fetus at the preceding 24-hour period.

Geometric growth rates of both the alimentary canal and fetus are given in Table 3. It is evident in text-figure 1 and Table 3 that the geometric growth rate of the alimentary

canal was greater than that of the fetus. For the period from 11 to 12 days of incubation, geometric growth rate was 55 percent in the fetus and 58 percent in the alimentary canal. In the succeeding 24-hour period (12th to 13th day of incubation), the geometric growth rate of the alimentary canal reached 68 percent while the geometric growth rate of the fetus decreased to 32 percent. From 13 to 14 days of incubation geometric growth rate in the fetus and alimentary canal was 28 percent and 59 percent respectively. In the subsequent 24-hour period (14th to 15th day of incubation) geometric growth rate in both the fetus and alimentary canal was 27 percent. This was succeeded by decreased geometric growth rates in both the fetus and alimentary canal from the 15th to 19th day of incubation. In the former it decreased from 36 percent to 2 percent while in the latter the decrease was from 69 percent to 10 percent. On the 20th day of incubation the geometric growth rate of the fetus was 67 percent and in the alimentary canal it was 30 percent. The pronounced increase in geometric growth rate of the fetus on the 20th day was attributed to the withdrawal of the yolk sac into the peritoneal cavity. For a further comparison in size of the fetus and the alimentary canal see figures 1, 2, and 3.

TABLE 1

THE MEAN WEIGHT OF CHICK FETUSES RANGING
IN AGE FROM 11 TO 20 DAYS INCUBATION

Days of Incubation	Mean Weight of Fetus	Standard Deviation	Maximum	Minimum
11	3.35	± 1.90	4.25	2.69
12	5.19	± 0.40	5.61	4.52
13	6.85	± 0.66	7.65	4.72
14	8.78	± 0.18	10.40	8.14
15	11.18	± 1.35	13.00	10.05
16	15.60	± 1.56	17.86	12.23
17	17.64	± 1.87	20.93	12.08
18	24.57	± 2.76	31.00	18.70
19	25.12	± 6.91	28.80	21.15
20	41.96	± 4.56	53.69	32.29

TABLE 2

THE MEAN WEIGHT OF THE ALIMENTARY CANAL OF FETUSES
RANGING IN AGE FROM 11 TO 20 DAYS INCUBATION

Days of Incubation	Mean Weight of Alimentary Canal	Standard Deviation	Maximum	Minimum
11	.12	± 0.5	.07	0.15
12	.19	± 0.04	.25	0.13
13	.32	± 0.05	.42	0.18
14	.51	± 0.08	.66	0.39
15	.65	± 0.0002	.75	0.58
16	1.10	± 0.02	1.75	0.76
17	1.55	± 0.02	1.82	1.25
18	2.39	± 0.19	3.48	1.37
19	2.63	± 0.35	3.35	2.00
20	3.44	± 0.36	4.22	2.73

TABLE 3

GEOMETRIC RATE OF GROWTH

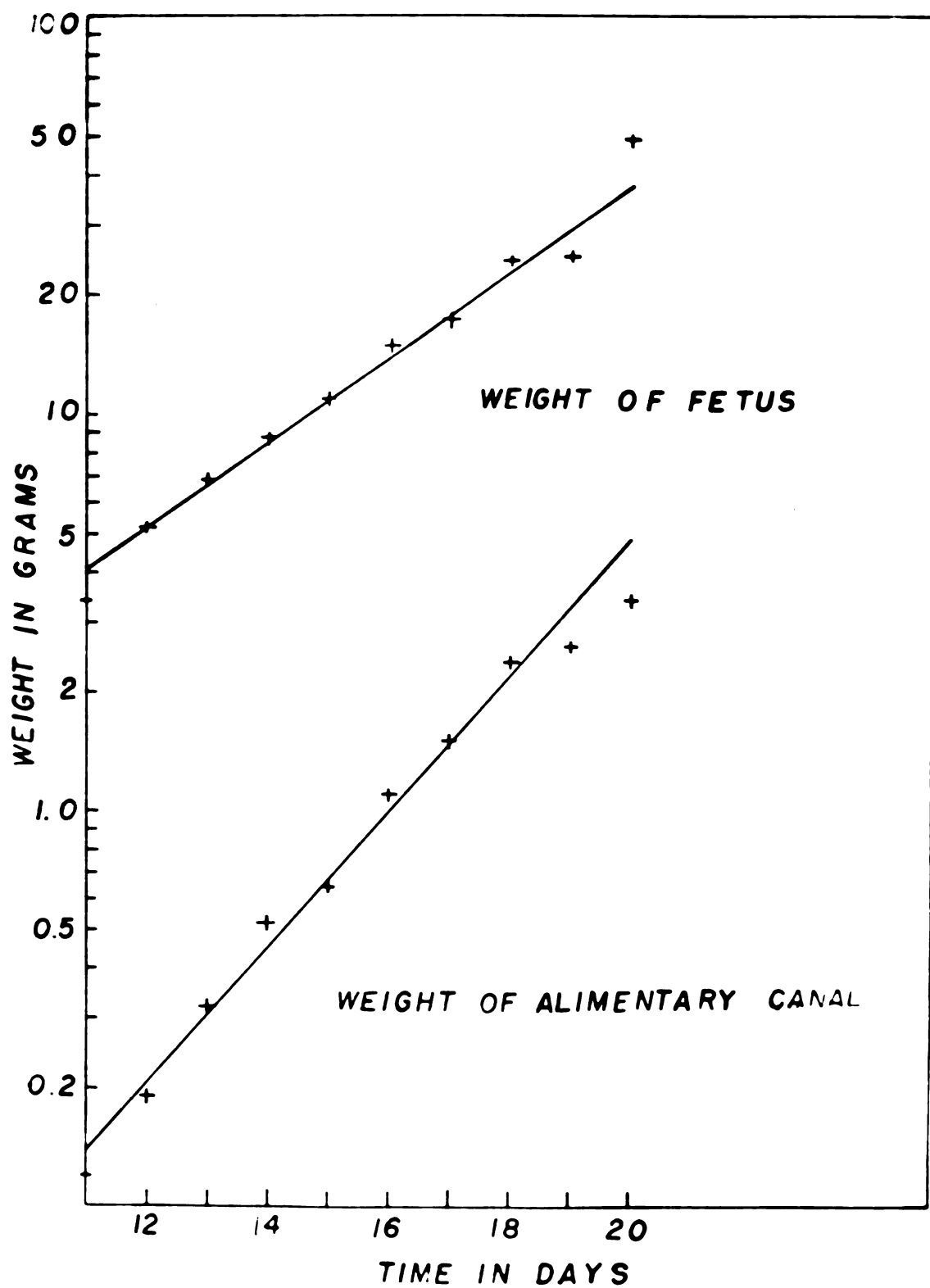
Period	Fetus	Alimentary Canal
11 - 12	55 %	58 %
12 - 13	32 %	68 %
13 - 14	28 %	59 %
14 - 15	27 %	27 %
15 - 16	36 %	69 %
16 - 17	16 %	41 %
17 - 18	39 %	54 %
18 - 19	2 %	10 %
19 - 20	67 %	30 %

TEXT-FIGURE 1

Comparison of the rate of growth between the fetus and the alimentary canal.

In the upper portion of the figure the logarithmic weight in grams of the fetus is plotted against the age of the fetus in days.

In the lower portion of the figure the logarithmic weight in grams of the alimentary canal is plotted against the age in days.



2. GROSS MORPHOLOGY OF THE ALIMENTARY CANAL IN THE ELEVEN-DAY FETUS

The various regions in the adult digestive tract are established in the fetus on the 11th day of incubation. The esophagus which is invested by loose connective tissue was located on the right lateral surface of the neck. The crop which develops on the right side in the distal third of the esophagus was a thin walled sac-like structure located just anterior to the thoracic cavity. That portion of the esophagus distal to the crop, joined the proventriculus and the latter was continuous with the gizzard. The gizzard was a solid muscular organ about 0.5 cm. long and 0.5 cm. wide which along with the liver, almost completely filled the abdominal cavity of the fetus. The duodenal loop was found inside the cavity but the jejunum-ileum was coiled and extended into the umbilical hernia. The distal end of the small intestine joined the large intestine at the junction of the caecum. The length of the small intestine was greater but the width was essentially the same in both. The large intestine was continuous with the cloaca. The latter consisted of a single chamber. The bursa was seen on the dorsal surface of the cloaca and was imbedded in the mesoderm of the body wall.

3. GROSS MORPHOLOGY OF THE ALIMENTARY CANAL OF THE TWENTY-DAY FETUS

The esophagus (fig. 4A) was cylindrical, approximately 5 cm. long, and extended from the pharynx to the proventriculus. It was located in the right lateral region of the thorax and neck. The crop which was about 1.5 cm. long and 1 cm. wide developed as a lateral outpocketing of the esophagus in the right side of the thoracic cavity. The distal end of the esophagus joined the proventriculus (fig. 4C) which was 0.9 cm. long and 0.7 cm. wide and this in turn led into the gizzard. The gizzard (fig. 4D) which was 2.5 cm. long and 1.5 cm. wide was a solid muscular mass which with the liver occupied the anterior portion of the abdominal cavity. The duodenum (fig. 4E) springs from the anterior lateral portion of the gizzard and formed a loop about 4 cm. long. Within the loop was the major portion of the pancreas. The jejunum-ileum (fig. 4F) which occupies the distal portion of the abdominal cavity, exhibited numerous coils and was attached to the body cavity by the dorsal mesentery. When uncoiled it measured about 20 cm. in length. The large intestine (fig. 4G) was somewhat wider than the small intestine but was only 1.8 cm. in length. It was located distal to the junction of the small intestine and the caecum. The distal end of the large intestine was continuous with the cloaca (fig. 4H). The length of the cloaca was approximately

1.2 cm. and the width increased from 0.5 cm. at its anterior end to approximately 0.7 cm. at the posterior end. It was divided into three regions: an anterior coprodaeum, which joins the large intestine; a middle urodaeum which receives the ureters and genital ducts; and a distal proctodaeum. The bursa of Fabricius, a sac-like structure, is located on the dorsal wall of the proctodaeum.

4. THE HISTOGENESIS OF THE DIGESTIVE TRACT OF THE VARIOUS AGES OF THE FETUSES.

Stains. Ehrlich's hematoxylin and eosin, Mallory's triple stain, and Masson's trichrome stain were used for routine staining of tissue sections. Weigert's elastic tissue stain and Dempsey's orcein procedure were utilized for staining elastic tissue. Van Giesen's stain was used for collagenous tissue.

Esophagus. The epithelium on the 11th day of incubation (fig. 6) consisted of pseudostratified columnar epithelium which was arranged to form numerous longitudinal folds of tissue. On the 15th day of incubation it was essentially the same as it was on the 11th day although there was an inverse ratio between the number and size of the folds, i.e., the number decreased and the size increased. Between the 15th and 16th day (fig. 7) the pseudostratified columnar epithelium differentiated into stratified epithelium. The distal layer appeared to consist of a single layer of

cuboidal cells, while the proximal layer was columnar. On the 17th day of incubation, the proximal layer of cells was columnar and the intervening layers between this layer and the most distal layer of cells appeared to be of the squamous type. Between the 17th and 19th day inclusive, the differentiation of the distal cells continued toward the squamous type of epithelium. On the 19th day the epithelium was established as stratified squamous and on the surface of the most distal layer of cells, a horny layer of material was recognizable.

An abundant number of cilia were evident on distal surfaces of the pseudostratified columnar epithelial cells of 11-day fetuses. The number of cilia was greatly reduced in the 16-day fetuses and they disappeared entirely in 17-day fetuses.

The thickness of the various layers of tissue in the esophagus of fetuses varying in age from 12 to 20 days of incubation inclusive, was measured with an ocular micrometer. All the measurements were made on sections of tissue seven microns in thickness. Measurements of the thickness of the various layers were made in five to six different sections of tissue.

It is evident in Table 4 that the thickness of the stratified squamous epithelium of the esophagus increased

from about twelve microns on the 12th day to reach a maximum thickness (60-100 microns) on the 20th day of incubation.

Mucous glands were first identified in 13-day fetuses at which time the epithelium inpocketed to form small buds of cells which differentiated to form simple tubular glands with a distinct lumen in 16-day fetuses. By the 18th day of incubation (fig. 8) the distal ends of the glands were forked to form compound structures and the lumen in each fork exhibited mucus. In 20-day fetuses (fig. 9) the glands were differentiated into compound tubular glands which extended through the lamina propria mucosae to reach the surface of the muscularis mucosae.

The lamina propria mucosae and muscularis mucosae consisted of closely packed mesenchymal cells in 11 and 12-day fetuses. There was a tendency for the mesenchymal cells to loosen-up in 13-day fetuses, and in 16-day fetuses (fig. 7) the mesenchymal tissue adjacent to the epithelium was loosely arranged while the mesenchyme next to the external muscular layer was more compact and exhibited small bundles of muscle cells. Tissues in the former region (loosely arranged mesenchyme) differentiated into the lamina propria mucosae while mesenchymal cells in the latter, differentiated to form the muscularis mucosae and submucosa in 19-day fetuses. The lamina propria mucosae became compact and vascularized between the 16th and 19th day of incubation. In 20-day

fetuses (fig. 9) the mucosa was made up of stratified squamous epithelium, compact lamina propria mucosae, a narrow muscularis mucosae (consisting of longitudinal muscle fibers), and a well defined submucosa. The lamina propria mucosae increased in thickness from a minimum of forty-five microns on the 15th day to a maximum of ninety microns on the 20th day. The increase in thickness of the muscularis mucosae was less pronounced, i.e., it increased from twelve microns on the 16th day to a maximum of forty-five microns on the 20th day. The submucosa increased from a minimum with a range of sixty to ninety microns, to a maximum with a range of 150 to 200 microns, as the age of the fetus increased from 15 to 20 days.

The tunica muscularis externa was first identified in 13-day fetuses. It consisted of an inner circular muscle layer which was made up of short fibers. By the 19th day of incubation the length of the fibers in this region had increased and were well differentiated. Individual fibers were invested by connective tissue fibers. Longitudinal muscle fibers in the muscularis externa were first identified in 15-day fetuses and differentiation was completed in 20-day fetuses, i.e., bundles of muscle fibers were invested by small amounts of connective tissues. The thickness of the tunica muscularis externa on the 15th day of incubation was 165 microns and on the 17th day it decreased to sixty-five

microns. This decrease was attributed to an increase in length of individual muscle fibers. A similar variation in thickness of the muscular layer was observed between the 18th and 19th day of incubation.

The tunica adventitia, a loose connective tissue on the outer surface of the adult esophagus, was essentially the same in appearance as the underlying longitudinal layer of muscularis externa in 13-day fetuses, i.e., both consisted of mesenchyme. In 15-day fetuses the inner portion of the mesenchyme exhibited bundles of longitudinal smooth muscle fibers while the outer layer was less densely packed. The latter layer of tissue continued to loosen and in 19-day fetuses it was differentiated into a layer of loose connective tissue.

Auerbach's plexus was first identified between the circular and longitudinal layers of the tunica muscularis externa in 18-day fetuses.

It is evident from these observations that the submucosa was the most active growth center in the esophagus in fetuses ranging in age from 15 to 20 days.

Esophageal connective tissue (lamina propria mucosae, submucosa, and tunica adventitia) in all ages of fetuses (11-20 days) did not show elastic tissue with either Weigert's stain or Dempsey's et al (1952) modification of orcein method.

TABLE 4

MEASUREMENTS (IN MICRONS) OF TISSUES IN
THE ESOPHAGUS OF DEVELOPING FETUSES

Tissue	Days of Incubation									
	12	13	14	15	16	17	18	19	20	
Epithelium	12	15	15	13-30	20-35	20-50	30-60	30-60	60-100	
Lamina Propria	—	—	—	45	60	60	60	60	90	
Muscularis Mucosae	*30	*120	*60	—	12	15	30	30	45	
Submucosa	—	—	—	60-90	30	30	30-90	45-120	150-200	
Circular Muscle	30	75	60	120	50	45	90	120	60-110	
Longitudinal Muscle	—	—	30	45	20	20	30	45-60	45-90	

* The lamina propria and submucosa cannot be identified as separate layers and the muscularis mucosae is not a differentiated layer at this time.

Crop. It appeared as a swollen region in the posterior third of the esophagus in 11-day fetuses. At this stage in development the histological structure was essentially the same as the esophagus of a corresponding age, i.e., it consisted of pseudostratified columnar epithelium and undifferentiated mesenchyme. Mucous glands were restricted to the region adjacent to the esophagus in 20-day fetuses. The muscularis mucosae was narrow and was first identified in 20-day fetuses. Development of the external muscularis and tunica adventitia, parallels the development of these regions in the esophagus proper.

Proventriculus. Observation made in this study showed that cross sections of the proventriculus exhibited a mucosa with a small number of folds protruding into the lumen. The number of folds rapidly increased until the 15th day of incubation (fig. 13) and then they decreased but deepened through the 16th day. On the 20th day (fig. 15) the folds were wider and shallower.

The distal layer of cells in the stratified columnar epithelium of the proventriculus (from the 11th through the 16th day of incubation) showed a well developed layer of cilia on their distal surfaces. Between the 16th and 20th day the cilia disappeared and the epithelium itself was transformed from stratified columnar epithelium to simple columnar epithelium on the 20th day of incubation.

The lamina propria mucosae consisted of loosely arranged mesenchymal cells in 11-day fetuses (fig. 12). In 14-day fetuses a portion of the mesenchymal cells were differentiated into connective tissue and blood vessels. In 15 through 17-day fetuses the connective tissue underlying the epithelium consisted of closely packed cells and fibers. In the 18-day fetuses the lamina propria mucosae (fig. 14) consisted of loosely arranged cells and fibers and between 18 and 20 days the number of longitudinally arranged muscle fibers increased.

The muscularis mucosae was identified as a longitudinal layer, lateral to the deep glands and almost in contact with the circular layer of the tunica muscularis externa. This layer appeared on the 12th day of incubation and differentiation continued through the 20th day. Breaks were identified in the muscularis mucosae and at these sites the submucosa was confluent with the lamina propria mucosae.

The submucosa appeared on the 16th day of incubation in a few isolated areas between the muscularis mucosae and the tunica muscularis externa. In 20-day specimens the submucosa was very thin and often absent.

The circular muscle layer was identified in the proventriculus on the 11th day of incubation (fig. 12). From the 11th through the 16th day the circular muscle fibers remained short and an abundance of connective tissue elements

were seen between the fibers. The longitudinal muscle layer which differentiated on the 12th day was relatively thin and even absent in some areas.

The serosa existed as compact mesenchymal tissue between the 11th and 15th day of incubation. Blood vessels appeared within it on the 12th day. In 16-day fetuses it was thinner, less compact, and collagenous tissue was identified within it by means of Van Giesen's stain.

The proventricular glands were located in the lamina propria mucosae. They were first seen as simple-branched tubular glands in 11-day fetuses (fig. 12). The glands branched again and again to form complex glandular structures in 14-day fetuses. Development of the glands was almost completed in the 16-day fetus and secretory droplets were identified within the lumen of the glands of the 18-day fetus (fig. 14).

Gizzard. Stratified ciliated columnar epithelium lined the gizzard of the 11-day fetus (fig. 17). Between the 14th and 15th day of incubation the ciliated border of the epithelium was transformed into a striated border. The stratified epithelium in 15-day fetuses (fig. 18) developed to form crypts which marked the points where the gland ducts opened to the surface. Between the 15th and 20th day the stratified epithelium (fig. 20) was replaced by simple columnar epithelium. An exudate of the glands was found in the crypts and on the surface of the epithelium of 16-day fetuses.

The lamina propria mucosae was identified as loose connective tissue in which the glands were located. On the 11th day of incubation (fig. 16) this layer appeared as a compact mesenchymal tissue continuous with the submucosa. Many small vacuoles appeared in cells of the lamina propria mucosae in 12-day fetuses. These small vacuoles coalesced to form larger ones on the 14th day. Between the 14th and 16th day these vacuolated areas developed into glands which were surrounded by loose connective tissue of the lamina propria mucosae.

The muscularis mucosae was not identified in the gizzard of 11 to 20-day fetuses (fig. 20).

The submucosa appeared as compact mesenchymal tissue and was continuous with the lamina propria mucosae in 11-day fetuses. By the 15th day of incubation (fig. 18) the submucosa appeared to be loosely arranged connective tissue which stained positive for collagenous tissue with Van Gieson's stain. The submucosa had developed into a compact layer of connective tissue by the 20th day (fig. 20). On the ventral and dorsal surfaces of the gizzard, the tunica muscularis externa was absent and in these areas the submucosa came into direct contact with the fibrous connective tissue (aponeurosis) which covered the entire organ.

The tunica muscularis externa appeared as a large mass of loose, circular fibers which extended from one aponeurosis

to the other in the 11-day fetus. By the 15th day of incubation (fig. 18) the muscle bundles were invested with loose connective tissue fibers. From the 15th to the 20th day the muscle fibers lengthened and this accounted for the increase in the size of the gizzard. There were no longitudinal fibers in the gizzard of the 11 to 20-day fetuses.

The serosa was identified as a compact mesenchymal layer on the 11th day of incubation. This layer appeared very thick on the dorsal and ventral surfaces where the tunica muscularis externa was absent. This layer continued to develop and from the 16th to 20th day was compact and stained positive for collagenous fibers with Van Gieson's stain.

On the basis of the results obtained with Weigert's stain and the orcein procedure of Dempsey et al (1952) elastic tissue was not identified in the lamina propria mucosae, the submucosa, or the tunica adventitia of the gizzard in 11 to 20-day fetuses, inclusive.

Small Intestine. The microscopic structure of the duodenum and jejunum-ileum was essentially alike and from a morphological point of view the regions were undistinguishable.

The stratified columnar epithelium exhibited numerous folds in 11-day fetuses. These folds deepened and by the 13th day of incubation they were differentiated into villi. The epithelium was transformed into simple columnar tissue and it remained in this condition through the 20th day

(fig. 23). Goblet cells were first identified in epithelium of 17-day fetuses (fig. 22) and glands of Lieberkühn in 19-day fetuses.

The lamina propria mucosae first appeared as compact connective tissue in the villi of 17-day fetuses (fig. 22). This layer stained positive for collagenous fibers with Van Giesen's stain.

The muscularis mucosae of the small intestine consisted of an inner longitudinal layer and an outer circular layer. The former was identified as bundles of muscle fibers located at the base of the villi on the 17th day of incubation. This layer continued to develop through the 20th day (fig. 23) at which time bundles of muscles from this layer were identified in the villi. It was difficult to identify the circular layer of muscles from the circular layer of the tunica muscularis externa. The circular layer was identified in only a few areas of the small intestine of 20-day fetuses.

The submucosa was seen in only a few areas of the duodenum of the 20-day fetus. By use of selective staining (Mallory's, Masson's, and Van Giesen's), it was observed that there was connective tissue between the circular muscle layers of the muscularis mucosae and the lamina muscularis.

The tunica muscularis externa was made up of an inner circular layer and an outer longitudinal layer. The circular layer was identified as bundles of short fibers and was

located in the mesenchymal walls of 11-day fetuses. The outer longitudinal layer was first apparent on the 17th day of incubation.

The serosa from the 11th through the 16th day of incubation appeared as undifferentiated mesenchymal tissue. By the 17th day this layer was loosened and fibers were first identified. Differentiation of fibers continued through the 20th day and a positive reaction for collagenous fibers was obtained with Van Gieson's stain.

Large Intestine. The large intestine appeared morphologically similar to the small intestine in most respects, including the presence of villi and a muscularis mucosae which consisted of a circular and longitudinal layer.

The epithelium on the 11th day of incubation (fig. 24) was identified as a stratified columnar layer of tissue. By the 14th day folds appeared in the stratified columnar epithelium which deepened on the 15th day and were differentiated into villi by the 16th day. The epithelium at this time had been transformed from stratified columnar into simple columnar and remained in this condition through the 20th day (fig. 26). On the whole, the villi of the large intestine appeared to be shorter than those found in the small intestine on the 20th day.

The lamina propria mucosae was part of the undifferentiated mesenchymal tissue which was located between the

epithelial layer and the circular muscle fibers of the tunica muscularis externa of 11 through 15-day fetuses. By the 16th day of incubation this mesenchymal layer was divided into a compact area adjacent to the epithelium and a loose layer adjacent to the circular muscle layer of the tunica muscularis. The compact area adjacent to the epithelium sent fibers into the villi and by the 20th day it had differentiated into the lamina propria mucosae. The fibers of this compact layer were found to stain positive for collagenous tissue with Van Gieson's stain.

The muscularis mucosae consisted of an inner longitudinal and an outer circular layer. In 16-day fetuses, bundles of longitudinal muscle fibers first differentiated in the loose mesenchymal tissue medial to the circular muscle layers of the tunica muscularis externa. From the 17th to 20th day of incubation, differentiation of these bundles continued and a well developed layer of muscle fibers appeared in 20-day fetuses (fig. 26). The circular layer first appeared on the 18th day separate and distinct from the circular layer of the tunica muscularis externa.

The submucosa was identified as a very thin layer of collagenous tissue between the circular muscle layer of the muscularis mucosae and the circular layer of the tunica muscularis externa, on the 18th day of incubation. In 20-day fetuses the submucosa was very thin and in a few areas

it made contact with the lamina propria through spaces where the muscularis mucosae was not a complete layer. This tissue stained positive for collagenous fibers with Van Gieson's stain.

The tunica muscularis externa was identified as a circular muscle layer in the compact mesenchymal tissue, forming the wall of 11-day fetuses. On the 14th day of incubation (fig. 25) bundles of longitudinal muscle fibers were seen and by the 16th day these bundles had formed a complete layer lateral to the circular fibers. The short circular fibers continued to lengthen through the 20th day.

The very thin serosa which contained connective tissue and blood vessels, developed from a thick mesenchymal layer in the 11-day fetus. On the 16th day of incubation it began to differentiate into a thin white fibrous connective tissue and continued to differentiate through the 20th day.

Elastic tissue was not identified in the lamina propria mucosae, submucosa, and the serosa, by Weigert's or Dempsey's et al (1952) orcein modification stains.

5. STUDY OF METACHROMATIC STAINING ELEMENTS IN THE DIGESTIVE TRACT OF CHICK FETUSES BY THE USE OF TOLUIDINE BLUE "pH SIGNATURES", PROTEINS, AND RIBONUCLEASE.

Various regions in the digestive tract (esophagus, small intestine, and large intestine) of chick fetuses varying in age from 12 to 20 days, were used for study of metachromasia

in embryonic tissues. Tissues were stained with toluidine blue according to the procedures of French and Benditt (1953). To test the blocking of staining reactions with proteins, tissues were left in either albumin, globulin, or protamine solutions (1 mg./ml.) for two hours prior to staining with 0.1 percent toluidine blue. The hydrogen ion concentrations were controlled according to the procedures described in the Materials and Methods section. In control dye solutions, treatment with proteins was omitted but tissues were placed in aqueous buffer solutions prior to staining.

Estimation of basophilic materials was based on the intensity of the staining reaction, i.e., deep purple staining reactions were given a value of 3; moderately purple, a value of 2; and light blue reactions, a value of 1. The letter "G" was used for denoting blue-gray reactions and "P" was used for weak metachromatic reactions. The results obtained were presented in Table 5.

Staining intensity in sections of tissue from the digestive tract. It is evident in Table 5 that in all toluidine blue control solutions (pH 2.5 - 8.5 inclusive), intensity of staining reactions was inversely related to hydrogen ion concentration, i.e., as the hydrogen ion concentration decreased from pH 2.5 to 8.5, intensity of the staining reaction increased from about 1 to 3.

TABLE 5

The Influence of pH and Various Proteins Upon Staining by Toluidine Blue in Developing Chick Tissues

AREA	AGE*	CONTROL						ALBUMIN						GLOBULIN						PROTAMINE					
		pH						pH						pH						pH					
		2.5	3.5	5.5	8.5	2.5	3.5	5.5	8.5	2.5	3.5	5.5	8.5	2.5	3.5	5.5	8.5	2.5	3.5	5.5	8.5	2.5	3.5	5.5	8.5
Esophagus	20 day	1	1-2	2	3	1	G	3-P	3	1	G	3-P	3	1	G	3-P	3	1	1-2	2	2-3	1	1-2	2	2-3
Esophagus	16 day	1	1	2	3	G	G	2-P	3	1	G	3-P	3	1	G	3-P	3	1	1-2	2-G	3-P	1	1-2	2-G	3-P
Esophagus	12 day	1	1	1-P	3	G	G-P	3-P	3	1	G	3-P	3	1	G	3-P	3-P	1	1-G	2-G	3-P	1	1-G	2-G	3-P
Small Intestine	20 day	1	1	3	2	G	1-G	3-P	3-P	1	G	2-P	3	1	G	2-P	3	1	2-P	2-G	3-P	1	2-P	2-G	3-P
Small Intestine	16 day	1	1	3-P	3	1-2	1	3-P	3-P	1	G	3-P	2	1	G	3-P	2	1	2-G	2-G	3-P	1	2-G	2-G	3-P
Small Intestine	12 day	1	2	3-P	3-P	1-2	1	3-P	3-P	1	G	2	3-P	1	G	2	3-P	1	2	G	3	1	2	G	3
Large Intestine	20 day	1	2	3	3	2	1-G	3-P	3-P	1	G	2-G	2	1	G	2-G	2	1	2	G	3	1	2	G	3
Large Intestine	16 day	1	1	1	3	1-P	1-G	2-P	3	1	G	2	2	1	G	2	2	1	G	G	3	1	G	G	3

Estimates of staining intensity were based on the amount of stain in the total sections of tissue.

Intensity of Staining: 3 - deep purple, 2 - purple, 1 - blue, other color reactions: G - blue gray, P - pink.

* Days of incubation.

It is also evident that immersion of tissues in albumin solution prior to staining, influenced the intensity of the staining reaction. Staining reactions at a pH of 8.5, in tissues from all regions of the digestive tract, were essentially the same as the staining reactions in the corresponding control solution. On the other hand, tissues which were left in albumin prior to immersion in toluidine blue at pH 5.5 stained more intensely than tissues that were transferred directly to toluidine blue solution at pH 8.5.

Albumin-treated tissues when stained with toluidine blue at pH 3.5, stained less intensely than the tissues in toluidine blue control solutions. Tissues in the esophagus of 20-day fetuses which were left in albumin and subsequently stained in toluidine blue (pH 2.5) exhibited staining reactions essentially the same as those obtained in the toluidine blue control solution (pH 2.5). Immersion of esophageal tissues from 12 and 16-day fetuses and intestinal tissues from 20-day fetuses in albumin, decreased the staining reaction, i.e., in control solutions the tissues were stained light blue while albumin-treated tissues stained gray.

Exposure of tissue sections to globulin prior to staining with toluidine blue influenced the intensity of the staining reaction. At a hydrogen ion concentration of pH 8.5, staining intensity of globulin-treated tissue was similar to the untreated control tissues. However, globulin-treated

tissues at pH 5.5 stained more intensely than tissues from control solutions (pH 5.5).

It is evident that at pH 3.5 a very marked difference in staining intensity was observed between tissues treated with globulin and untreated control tissues. The globulin-treated tissues stained a light gray, while on the other hand, the tissues in control solution stained blue or light purple.

Tissues stained with toluidine blue subsequent to treatment with protamine sulfate also demonstrated that intensity of staining was affected by protein. At pH 8.5 the staining reactions in all tissues in the digestive tract were essentially the same as corresponding regions which were stained following control procedures. On the other hand, at pH 5.5 the staining was decreased in tissues treated with protamine in comparison to those of control treated tissues. Tissues which had been immersed in protamine prior to staining at pH 3.5, were somewhat variable. The intensity of the staining (pH 3.5) reaction in the esophagus of 12-day fetuses and the small intestine and large intestine of 16-day fetuses was decreased, while staining in the other tissues was similar to the controls. Tissues stained with toluidine blue (pH 2.5) following protamine sulfate treatment were similar to tissues stained following control treatment.

Intensity of staining reaction in specific layers of tissues of the digestive tract. The data presented in the preceding paragraphs summarized the over-all basophilic reactions obtained in tissues from various levels of the digestive tract with experimental and control solutions. Although the results presented in Table 5 are estimations of staining reactions in a given section of tissue, individual reactions in the various layers of tissue were subject to considerable variation. For example, tissues in control solution prior to staining at pH 8.5 stained the nuclei in all layers of tissue (mucosa, submucosa, and the muscular layers) and mucous glands deep purple. Muscle fibers stained light blue and the lamina propria mucosae, submucosa and tunica adventitia, stained metachromatically. Staining reactions at pH 5.5 were essentially the same as those obtained at pH 8.5.

At pH 3.5 the staining reaction in all layers of tissue was less intense. Secretions within the lumen of the mucous glands were stained light purple while the nuclei and cytoplasm of cells in the gland itself stained blue and light blue respectively. The staining intensity of all tissues in the esophagus, small intestine and large intestine were fairly constant with the exception of goblet cells and glands of Lieberkühn in both the large and small intestine. The former (goblet cells) stained metachromatically at pH 8.5

and 5.5, while at pH 3.5 and 2.5 these cells were colorless. The latter (glands of Lieberkühn) stained metachromatically at pH 8.5, 5.5, and 3.5 and blue at pH 2.5.

Staining reaction in individual layers of tissues in cross-sections of the digestive tract after immersion in albumin and subsequent to staining in toluidine blue differed from staining reactions obtained in the control solution. At pH 5.5 and 8.5, nuclei in all layers of tissue in the digestive tract stained deep purple. At the latter hydrogen ion concentration, cytoplasm in connective tissue cells stained metachromatically. At pH 5.5 the cytoplasm of connective tissue cells was less metachromatic.

When the hydrogen ion concentration of the staining solution was increased to pH 3.5, the intensity of staining of nuclei in the esophagus and large intestine decreased from about 3 (deep purple) to less than 1 (blue-gray). The cytoplasm of connective tissue cells was practically colorless and the color of the cytoplasm of muscle cells was gray. In 20-day fetuses the nuclei continued to stain blue even at pH 2.5 but in 12 and 16-day fetuses, the nuclei stained blue-gray. The small intestine in all ages of fetuses stained similarly to that of the controls.

Differences were observed in the staining intensity of individual layers of tissues stained in toluidine blue, subsequent to treatment with globulin, as compared with control

treated tissues. At pH 5.5 and 8.5, nuclei in all layers of tissues observed (the mucous glands of the esophagus, goblet cells and glands of Lieberkühn of the large and small intestine), stained deep purple. At these hydrogen ion concentrations, the cytoplasm of the muscle fibers stained metachromatically as did the cytoplasm of connective tissue elements.

At pH 3.5 the staining reaction was markedly reduced in all layers of tissue. The nuclei stained blue-gray; the connective tissue cytoplasm and muscle fibers stained gray; the mucous glands of the esophagus stained purple; and the goblet cells and the glands of Lieberkühn did not stain. At pH 2.5 all tissue elements of the digestive tract in all ages of fetuses stained similarly to that of the controls.

Sections of tissue which were treated with protamine sulfate prior to staining with toluidine blue were subject to considerable variation in staining reaction. At pH 8.5, 5.5, and 3.5, the staining which was observed appeared granular. In esophageal tissues of all fetuses, the nuclei stained purple at pH 3.5, 5.5, and 8.5, and were blue at 2.5; mucous glands were stained purple at pH 8.5 and 5.5, blue at pH 3.5 and pink at pH 2.5; the cytoplasm of the connective tissue (lamina propria mucosae, submucosa, the tunica adventitia in older fetuses, and the undifferentiated mesenchyme in young fetuses) stained pink (metachromatic) at pH 8.5, gray at pH 5.5 and 3.5 and colorless at pH 2.5. The nuclei

of the small intestine stained deep purple at pH 8.5, purple pH 5.5 and 3.5, and blue at pH 2.5; the cytoplasm of the connective tissues stained pink (metachromic) at pH 8.5, gray at pH 5.5 and 3.5 and colorless at pH 2.5; while muscle cytoplasm was blue at pH 8.5 and gray at pH 5.5, 3.5 and 2.5; the goblet cells stained deep purple at pH 5.5 and 8.5 and were unstained at pH 2.5 and 3.5; and the glands of Lieberkühn stained purple at pH 5.5 and 8.5 and were blue at pH 2.5 and 3.5. The large intestine followed a similar pattern as the small intestine except for the nuclei of all layers of tissues which stained deep purple at pH 8.5, gray at pH 5.5, purple at pH 3.5 and blue at pH 2.5.

The above observations indicate that staining of metachromatic components was dependent upon the pH of the stain. It is also evident from the above observations that staining these components with a basic dye (toluidine blue) can be modified by placing the tissues in the presence of protein at a specified hydrogen ion concentration. It was observed that albumin was most effective in blocking dye uptake at pH 2.5 and 3.5, globulin at pH 3.5 and protamine at pH 3.5 and 5.5.

The nucleic acids have a very strong affinity for basic dyes (Singer, 1954). In order to ascertain whether or not basophilia was due to ribonucleic acid, sections of the esophagus, small intestine and large intestine of 20, 16, and 12-day chick fetuses were treated for one and one-half

hours at 37° with ribonuclease at pH 5.5 prior to staining with toluidine blue (pH 5.5). The nuclei in all tissue layers (mucosa, submucosa, and muscular) stained deep blue. The cytoplasm of epithelial cells stained pink; the connective tissue elements stained deep pink; and muscle cytoplasm stained gray. The mucous glands of the esophagus, the goblet cells and glands of Lieberkühn of the small and large intestine were stained pink. By comparing the above results with the untreated control tissues which were stained at the same pH, it was observed that the nuclei appeared lighter and the mucous glands, goblet cells and the glands of Lieberkühn were stained pink instead of purple, and muscle cytoplasm was gray instead of pink. On the other hand, the connective tissues (lamina propria, submucosa and tunica adventitia) and undifferentiated mesenchyme continued to stain pink (metachromatic) in both experimentally treated tissues and in control tissues.

6. THE PERIODIC ACID-SCHIFF TEST APPLIED TO TISSUES OF THE DIGESTIVE TRACT OF CHICK FETUSES.

Sections of tissue were stained for polysaccharides by the Hotchkiss (1948) method. In some experiments periodic acid A (400 mg. periodic acid, 5 ml. M/5 acetate and 35 ml. of ethyl alcohol) was used as an oxidizing agent for soluble polysaccharides and in other experiments periodic acid B (400 mg. periodic acid, 5 ml. M/5 acetate and 45 ml. of

distilled water) was employed for oxidation of insoluble polysaccharides. Some tissues were fixed in Bouin's fixative for demonstration of insoluble polysaccharides. Soluble polysaccharides were demonstrated after fixation of tissues in acetic acid-alcohol-formalin.

Insoluble polysaccharides. The esophagus of 11 and 12-day fetuses were negative for polysaccharides while esophageal tissues in 13-day fetuses exhibited a strong acid-Schiff reaction. The epithelium bordering the lumen and the cytoplasm of undifferentiated connective tissue cells were stained uniformly light red. In 15-day fetuses the epithelial border, basement membrane and connective tissue between the muscle fibers of the external muscular layers stained light red. In fetuses from 18 to 20 days inclusive, the intensity of the staining reaction was increased in esophageal glands, i.e., they now stained deep red. The underlying lamina propria mucosae and also the submucosa stained less intensely, i.e., light pink.

The proventriculus of 11-day fetuses showed scattered Schiff-positive granules in the cytoplasm of epithelial cells. On the 12th day of incubation there was a thin layer of Schiff-positive material on the distal border of the epithelium in addition to the Schiff-positive cytoplasmic granules. From the 14th to the 20th day Schiff-positive materials were more extensive. The distal surface of the epithelium, the

lumen of the glands, the cytoplasm of cells and fibers in the lamina propria, the submucosa, the connective tissue investing muscle in the external muscle layers, and the serosa, stained light red.

On the 11th and 12th days of incubation the epithelial border and basement membrane of the gizzard exhibited Schiff-positive reactions. In 14-day fetuses the epithelial border, the lumen of the glands, submucosa, and serosa were Schiff-positive. While in 17 - 20-day fetuses the Schiff-positive reactions were more extensive, i.e., the lamina propria and connective tissues investing the fasciculi of the external muscular layers were also Schiff-positive.

The small intestine exhibited Schiff-positive granules in the cytoplasm of epithelial cells from the 11th through the 16th day of incubation. On the 15th day Schiff-positive materials were observed within the cytoplasm of connective tissue cells and fibers which surrounded the muscle bundles. By the 17th day the goblet cells and the connective tissues were positive and this condition continued through the 20th day.

Scattered granules of Schiff-positive material were seen in the cytoplasm of epithelial cells of the large intestine in chicks ranging from 11 to 18 days of incubation. The basement membrane was positive from the 11th through the 16th day. The epithelial border showed a thin layer of

Schiff-positive material from the 12th to the 20th day. Both the connective tissue fibers and the cytoplasm of connective tissue cells exhibited positive Schiff reactions in 14-day fetuses. In 19 and 20-day fetuses goblet cells, the epithelial border, lamina propria, submucosa and connective tissue elements around the muscle bundles were Schiff-positive. Schiff-positive materials were first observed in the serosa and goblet cells on the 18th day of incubation.

Soluble polysaccharides. The glands of the esophagus, proventriculus, and gizzard, the epithelial borders of all organs comprising the tract, and the goblet cells in the intestine, stained red. All other tissues stained pink. The connective tissue which surrounded the gland and serosa, stained deep pink. All tissues were Schiff-positive in the staining reaction.

Tissues stained for soluble polysaccharides and treated with ribonuclease. Red staining occurred in the epithelial border; in the lumen of the glands of the esophagus, proventriculus, and gizzard; and also in the goblet cells of the intestine. The other tissues stained pink. The serosa and connective tissue around the glands stained deep pink. All tissues were Schiff-positive in the staining reaction.

Tissues stained for soluble polysaccharides and treated with saliva. The connective tissue, epithelial border, lumen of the glands of the esophagus, proventriculus, gizzard, and

the goblet cells of the intestine stained light red after tissues were exposed to saliva for twenty minutes. The epithelial and muscle layers showed a marked decrease in staining intensity after exposure to saliva.

It can be seen in the above observations that only some tissues exhibited a Schiff-positive reaction when Bouin's solution was used as the fixative and periodic acid B was used as the oxidizing agent. When acetic acid-alcohol-formalin was used as a fixative and periodic acid A as the oxidizing agent, all stained positive with the Schiff reagent. The ribonuclease did not prevent the staining of the Schiff reagent in any of the tissues observed. Saliva did not abolish the staining although it did decrease the intensity of it.

DISCUSSION

1. GROWTH AND HISTOGENESIS OF THE DIGESTIVE TRACT.

It is evident from results obtained in this study that in fetuses of 11 to 20 days of incubation the geometric growth rate of the alimentary canal exceeded the growth rate of the fetus. These observations are in general agreement with the calculations of Latimer (1928) who used the data of Schmalhausen for estimation of prenatal growth rate.

Observations presented in preceding pages demonstrated that the pattern of differentiation of the various layers of tissue in the digestive tract was essentially the same in the foregut, midgut, and hindgut regions. Epithelium lining the tract differentiated first; circular muscle in the external muscular layer was next to appear; and lastly the lamina propria and submucosa differentiated.

Differentiation of the various layers of tissue in the esophagus was incomplete in 11-day fetuses but subsequent rate of development in this region of the tract exceeded differentiation in both the small and large intestine. Differentiation of the gizzard and proventriculus closely paralleled the differentiation of the esophagus.

It was shown in the preceding pages that the morphological features of the esophageal epithelium were profoundly

modified between the 11th and 20th day of incubation. In 11-day fetuses it was classified as pseudostratified columnar epithelium, in 16-day fetuses as stratified columnar and in 20-day fetuses it was differentiated into stratified squamous epithelium. Other regions of the digestive tract (proventriculus, gizzard, small and large intestine) exhibited stratified columnar epithelium lining the tract in 11-day fetuses which was transformed into simple columnar epithelium between the 16th and 20th day of incubation.

Cilia were identified on the free border of the epithelial cells lining all regions of the tract in 11-day fetuses. There was an inverse ratio in the number of cilia to the age of the fetus, i.e., as the age of the fetus increased the number of cilia decreased. By the 17th day of incubation cilia were no longer identifiable on the distal surfaces of the epithelial cells.

Glands were identified in the lamina propria of the proventriculus of 11-day fetuses while in the esophagus and gizzard, glands appeared on the 13th day of incubation. However, the glands of the esophagus and proventriculus developed into large compound glands, while those of the gizzard appeared as a compact layer of parallel simple tubular glands in the lamina propria mucosae.

The lamina propria mucosae, muscularis mucosae, and the submucosa of the tract developed from a compact

mesenchymal layer located between the epithelial border and the circular muscle bands of the tunica muscularis externa. The only exception to the above was in the gizzard where no muscularis mucosae was differentiated. This is in agreement with Calhoun (1954) who found no muscularis mucosae in the gizzard of adult chickens. The muscularis mucosae in both the small and large intestine consisted of an inner longitudinal layer and an outer circular layer, while that of the esophagus and proventriculus consisted only of longitudinally arranged fibers. Generally the lamina propria mucosae and submucosa differentiated before the muscularis mucosae was formed. The mesenchyme in the area where the lamina propria formed, loosened up prior to the formation of the muscularis mucosae and after the formation of the muscularis mucosae the lamina propria mucosae developed into a compact layer of collagenous tissue. On the other hand, the mesenchyme which developed into the submucosa remained compact and after formation of the muscularis mucosae the submucosa developed into a very loose layer of connective tissue in the esophagus; a rather compact layer in the gizzard; and a very thin loose layer in the proventriculus and the small and large intestine.

In all organs of the digestive tract with the exception of the esophagus, the circular layer of the tunica muscularis externa could be identified on the 11th day of incubation.

However, it was clearly differentiating in the esophagus by the 12th day. The tunica muscularis externa consisted of an inner circular layer and an outer longitudinal layer of muscle bundles in the esophagus, proventriculus, small intestine, and large intestine in 20-day fetuses. However, in the gizzard, only large amounts of circular muscle fibers were identified but no longitudinal fibers were present in 11 to 20-day fetuses. Calhoun (1954) also found that there were no lateral muscle layers in the tunica muscularis externa of the gizzard in adult chickens. The outer longitudinal layer of muscle developed from mesenchyme which lies lateral to the circular muscle layer.

The tunica adventitia of the esophagus and the serosa of the other organs developed from the mesenchyme which was located lateral to the circular muscle layer of the tunica muscularis externa. When fully differentiated it was found to be composed of loose collagenous tissue with an abundance of blood vessels.

2. HISTOCHEMICAL STUDIES OF THE CONNECTIVE TISSUES.

Wachstein (1949) and Leblond (1950) maintained with Hotchkiss (1948) and Mc Manus (1948) that periodic acid transforms polysaccharides into polyaldehydes. The latter were found to stain various shades of red with fuchsin-sulfurous acid reagent. Gibb and Stowell (1949) demonstrated that the Schiff reagent will stain glycogen, mucin, mucoprotein,

hyaluronic acid and chitin. Gomori (1952) included with the above mentioned Schiff-positive materials such substances as starch, cellulose, glycolipids and mucopolysaccharides.

Dempsey et.al. (1947) found that mucopolysaccharides could be identified on the basis of their acidic groups, i.e., sulfate-containing mucopolysaccharides. Wislocki and Sognnaes (1950) demonstrated that acid mucopolysaccharides were not only metachromatic but also reacted intensely with basic dyes at low pH.

Observations made in this study suggest that both acid mucopolysaccharides and "neutral" polysaccharides exist in the connective tissues of the embryonic digestive tract. At pH 8.5 and 5.5 both the lamina propria and submucosa stained metachromatically but when the pH of the dye solution was decreased to 3.5 and 2.5 the staining reaction in these tissues was weakly basophilic. The use of basic proteins such as albumin, globulin and protamine, support this view. Intensity of the toluidine blue staining reaction was decreased at pH 3.5 when tissues were treated with albumin and globulin prior to staining while the staining reaction at pH 5.5 in protamine treated tissues was less intense than it was in the control solution. French and Benditt (1953) found that basic proteins competed with the dye solution for the stainable groups in polysaccharides. Observations reported in the

preceding pages demonstrated that basophilia in the lamina propria and submucosa could not be attributed to ribonucleoprotein since immersion of tissues in ribonuclease did not abolish the reaction. Moog and Wenger (1952) maintain that polysaccharides containing sulfate groups (i.e., acid mucopolysaccharides) generally take on a reddish coloration when stained with toluidine blue. Observations reported in this study showed that the connective tissue of the developing digestive tract stained pink with toluidine blue at pH 5.5 and 8.5.

It was also observed that the periodic acid-Schiff reaction was not completely abolished although the reaction was decreased upon immersion of tissues in saliva. This observation suggests that glycogen exists in combination with other mucopolysaccharides. Wislocki et. al. (1947) observed that a large amount of glycogen was present in fetal tissues of mammals.

The intensity of the staining reaction with the periodic acid-Schiff procedure for soluble polysaccharides and toluidine blue remained fairly constant in the digestive tract of fetuses between 11 and 20 days of incubation. On the other hand, the periodic acid-Schiff procedure for insoluble polysaccharides did not produce a positive reaction in undifferentiated mesenchyme but was positive in differentiated tissues of the lamina propria, submucosa and serosa. The

intensity of staining with Schiff reagent for insoluble polysaccharides was not diminished by exposing tissues to saliva before staining. Jorpes et. al. (1948) maintained that the periodic acid-Schiff procedure will not stain polysaccharides containing chondroitin sulfuric acid. This would suggest that from the results observed in this study the material stained by the Schiff procedure was a "neutral" polysaccharide.

Meyer (1950) found that both hyaluronic acid and chondroitin sulfate were present in mesenchymal tissues. Bunting (1950) maintained that acid mucopolysaccharides when stained with toluidine blue stained metachromatically at low hydrogen ion concentrations. Further, he maintained that at low pH values, tissues containing chondroitin sulfate exhibit basophilia.

Evidence obtained in this study would indicate that the fetal connective tissues were composed of acid mucopolysaccharides and "neutral" polysaccharides. The former were present in undifferentiated mesenchyme while the latter were not present until differentiation had taken place. The acid mucopolysaccharides were demonstrated by staining with toluidine blue at various hydrogen ion concentrations. The "neutral" polysaccharides were demonstrated by use of the periodic acid-Schiff reagent for insoluble polysaccharides (Hotchkiss, 1948). These Schiff-positive materials were found to be resistant to digestion by saliva.

SUMMARY

1. Fetuses were obtained from single-comb white leghorn hatching eggs. These were used for studies of the histogenesis of the digestive tract and the histochemistry of the connective tissues found in the digestive tract.

2. Weights of fetuses of various ages were taken subsequent to removal from the egg. The digestive tract of various ages of chick fetuses was weighed and a comparison of the geometric growth rates of the fetus and the digestive tract was made. In this study it was found that the geometric rate of growth in the digestive tract was greater than the geometric rate of growth in the fetus from the 11th to the 20th day of incubation.

3. Tissues stained with hematoxylin and eosin, Mallory's triple stain, Masson's trichrome stain, Weigert's elastic tissue stain, Dempsey's orcein procedure, and Van Gieson's stain for collagenous tissue were used in the study of the histogenesis of the digestive tract in chick fetuses.

4. It was observed in this study that the different tissue layers which comprise the digestive tract differentiated in an orderly sequence in all organs of the alimentary canal. The layers differentiated in the following sequence: first the epithelial layer which was followed by the circular

muscle fibers of the external muscle layer, then the lamina propria and submucosa, and finally the muscularis mucosae.

5. When toluidine blue was used as a stain it was observed that connective tissues stained either basophilic or metachromatically depending upon the pH of the dye solution. The staining intensity of the connective tissues could be altered if the tissue sections were treated with basic proteins prior to staining. Albumin, globulin and protamine were the basic proteins used in this study.

6. It was possible with the use of toluidine blue and the basic proteins, to demonstrate that the undifferentiated mesenchymal connective tissues and the differentiated connective tissues contain acid mucopolysaccharides.

7. The periodic acid-Schiff procedures of Mc Manus (1946) and Hotchkiss (1948) were used to study glycogen and insoluble polysaccharides of the fetal tissues comprising the digestive tract.

8. Observations made following the use of the Schiff reagent demonstrated that glycogen was found in all tissues of the digestive tract in chick fetuses of 11 to 20 days of age. It was further observed that the differentiated connective tissues of the alimentary canal were Schiff-positive and this was not soluble in saliva.

9. The conclusions reached in this study of the connective tissue indicate that they contained an acid

mucopolysaccharide and a "neutral" polysaccharide. The former were identified by staining with toluidine blue at various hydrogen ion concentrations. These acid mucopolysaccharides were found in both undifferentiated mesenchyme and in the differentiated connective tissues of the alimentary canal. The "neutral" polysaccharides were observed by staining with the Schiff reagent, were insoluble in saliva, and were identified only in differentiated connective tissues and not in the undifferentiated mesenchyme.

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PLATE I

Fig. 1. Total fetuses (including yolk sac) ranging in age from 15 to 20 days of incubation.

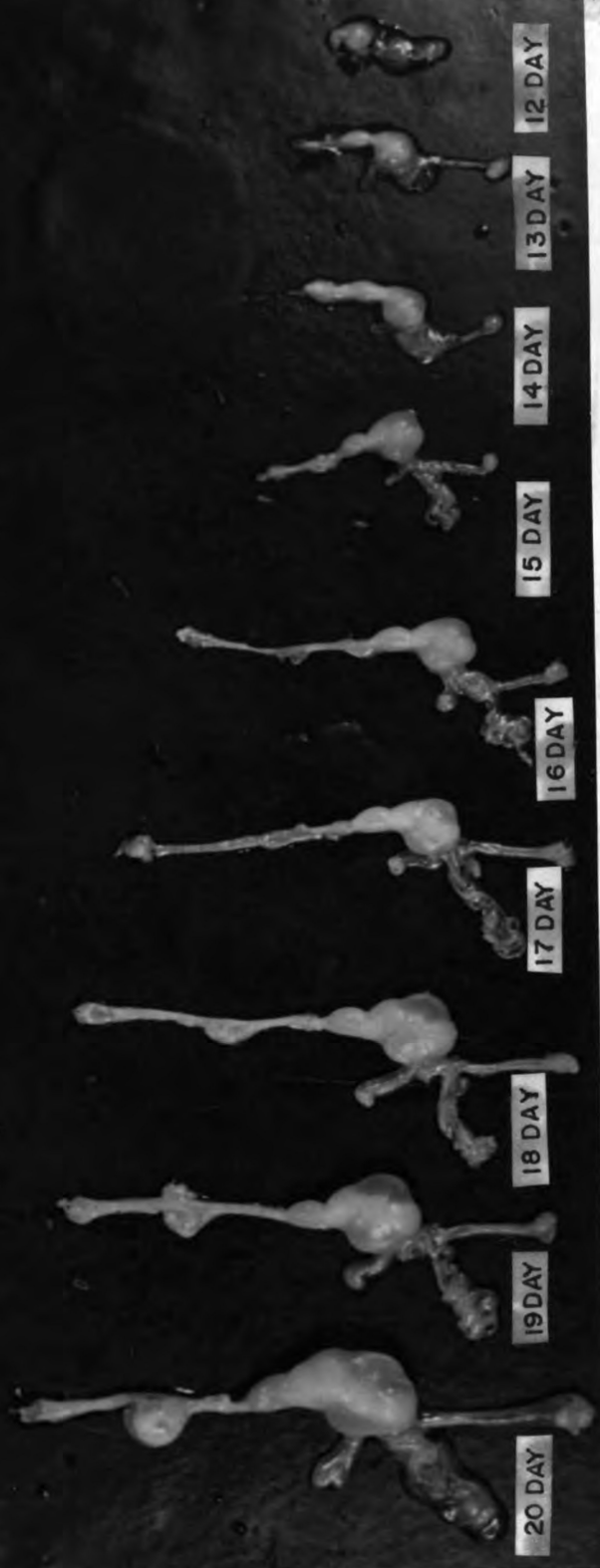
Fig. 2. Total fetuses (including yolk sac) ranging in age from 11 to 14 days of incubation.



PLATE II

Fig. 3. Alimentary canals removed from fetuses ranging in age from 12 to 20 days of incubation.

3



12 DAY

13 DAY

14 DAY

15 DAY

16 DAY

17 DAY

18 DAY

19 DAY

20 DAY

PLATE III

Fig. 4. Entire alimentary canal of a 20-day fetus with the jejunum-ileum uncoiled.

- A. Esophagus
- B. Crop
- C. Proventriculus
- D. Gizzard
- E. Duodenum
- F. Jejunum-ileum
- G. Large intestine
- H. Cloaca
- I. Bursa Fabricius

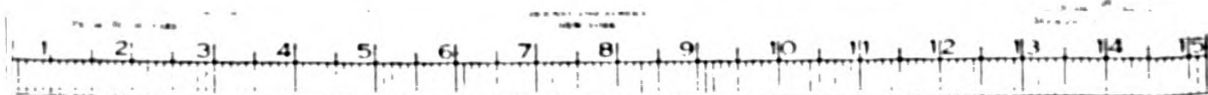


PLATE IV

- Fig. 5. Cross section through the esophagus of an 11-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Undifferentiated mesenchyme.
- Fig. 6. Cross section through the esophagus of an 11-day fetus. Hematoxylin-eosin. Magnification X520.
A. Epithelium; B. Mesenchyme.
- Fig. 7. Cross section through the esophagus of a 16-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Lamina propria; C. Gland;
D. Muscularis mucosae; E. Submucosa;
F. Tunica muscularis externa; G. Tunica adventitia.
- Fig. 8. Cross section through the esophagus of an 18-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Lamina propria; D. Muscularis mucosae; E. Submucosa; F. Tunica muscularis externa.
- Fig. 9. Cross section through esophagus of a 20-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Lamina propria; C. Gland;
D. Muscularis mucosae; E. Submucosa; F. Tunica muscularis; G. Tunica adventitia.
- Fig. 10. Cross section of esophagus of a 19-day fetus using Masson's stain. Magnification X170.

Micrometer scale insert: 1 space= 0.01 mm.

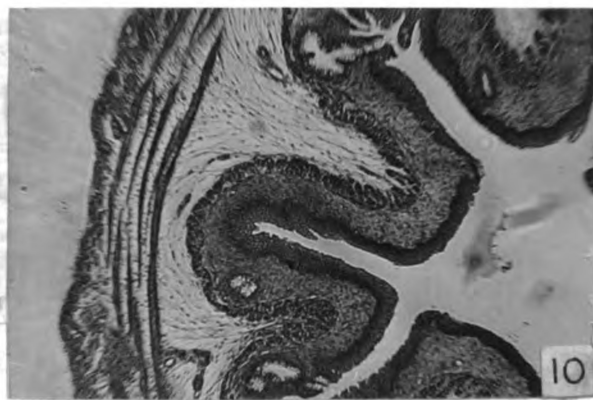
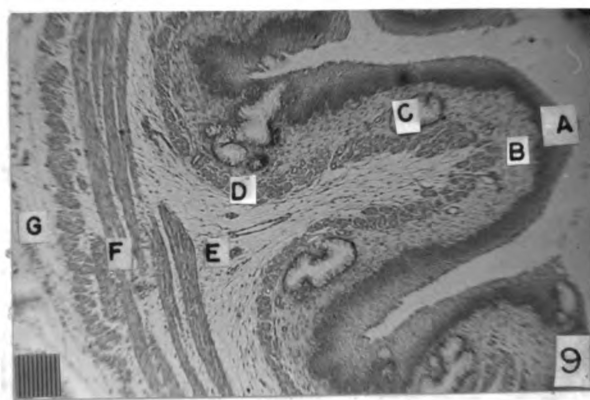
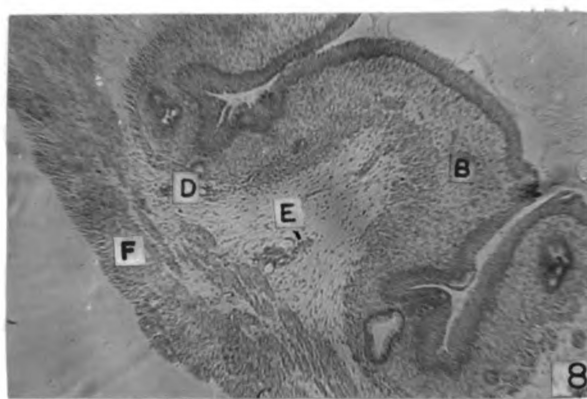
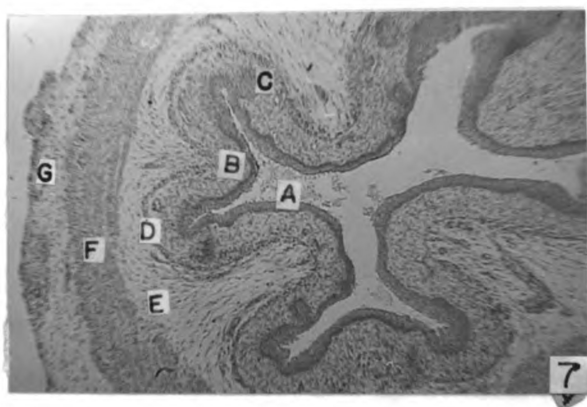
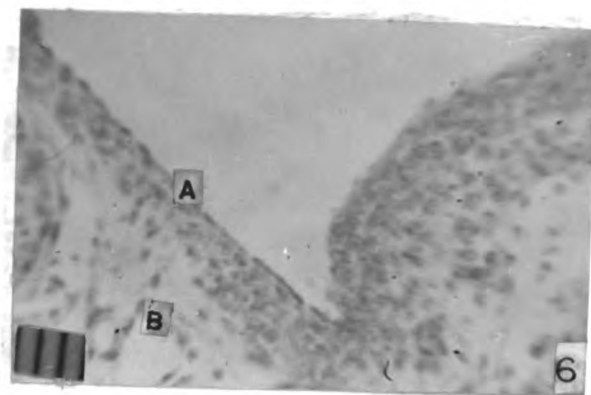
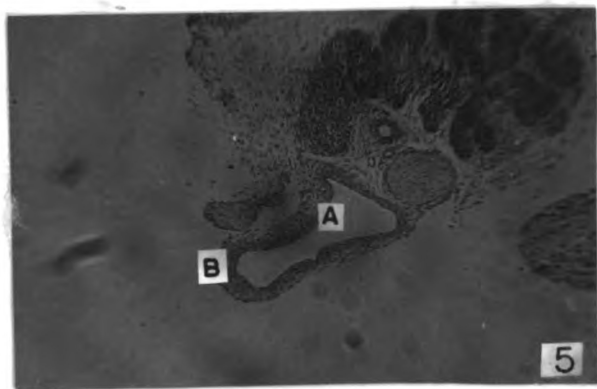


PLATE V

- Fig. 11. Cross section through the esophagus of a 20-day fetus. Stained with toluidine blue at pH 8.5. Magnification X170.
- Fig. 12. Cross section through proventriculus of an 11-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Gland; C. Undifferentiated mesenchyme; E. Circular muscle layer;
F. Serosa.
- Fig. 13. Cross section of proventriculus of a 15-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Gland; C. Lamina propria;
E. Tunica muscularis externa.
- Fig. 14. Cross section of proventriculus of an 18-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Gland; C. Lamina propria;
D. Muscularis mucosae; E. Tunica muscularis.
- Fig. 15. Cross section through the proventriculus of a 20-day fetus. Hematoxylin-eosin. Magnification X170. (note opening of large gland into the lumen of the proventriculus)
A. Epithelium; B. Gland; C. Lamina propria.
- Fig. 16. Cross section through the gizzard of an 11-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; C. Undifferentiated mesenchyme;
E. Tunica muscularis externa.

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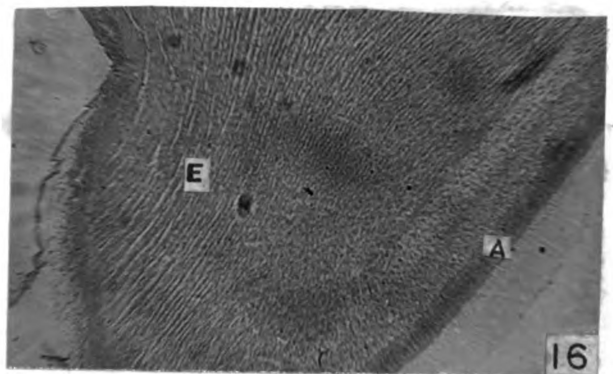
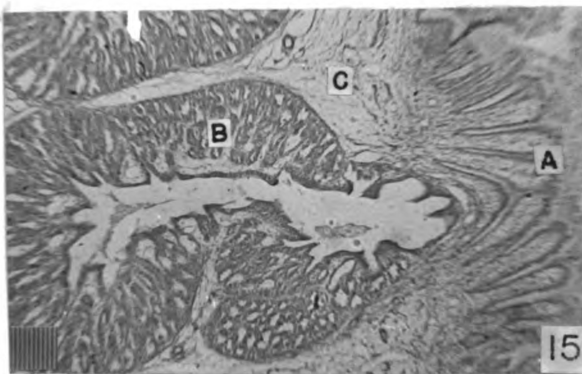
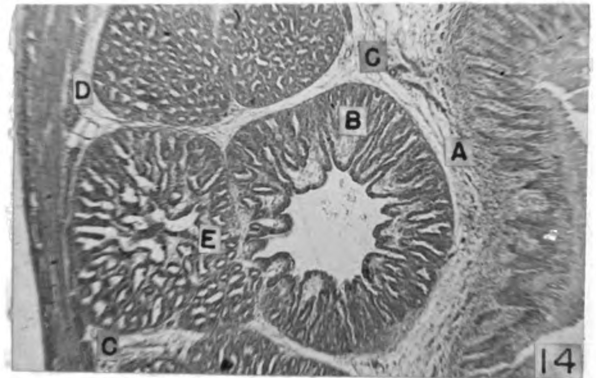
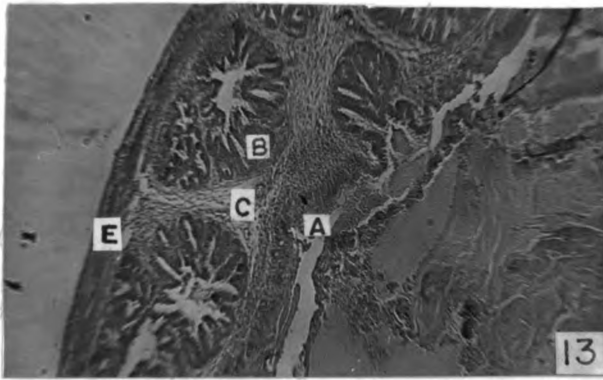
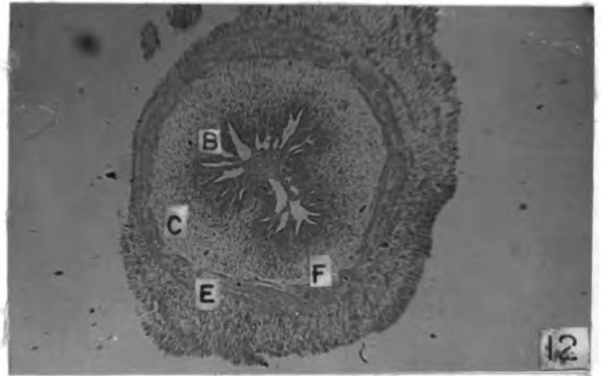


PLATE VI

- Fig. 17. Cross section through the gizzard of an 11-day fetus (showing cilia). Hematoxylin-eosin. Magnification X520.
A. Epithelium; B. Undifferentiated mesenchyme.
- Fig. 18. Cross section through the gizzard of a 15-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Glands; E. Tunica muscularis.
- Fig. 19. Cross section through the gizzard of an 18-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Glands; C. Submucosa; E. Tunica muscularis; F. Serosa.
- Fig. 20. Cross section through the gizzard of a 20-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; B. Glands; C. Submucosa; E. Tunica muscularis; F. Serosa.
- Fig. 21. Cross section through the small intestine of a 12-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; C. Undifferentiated mesenchyme; E. Circular muscles of tunica muscularis.
- Fig. 22. Cross section through the small intestine of a 17-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; C. Lamina propria; E. Tunica muscularis.

Micrometer scale insert: 1 space = 0.01 mm.

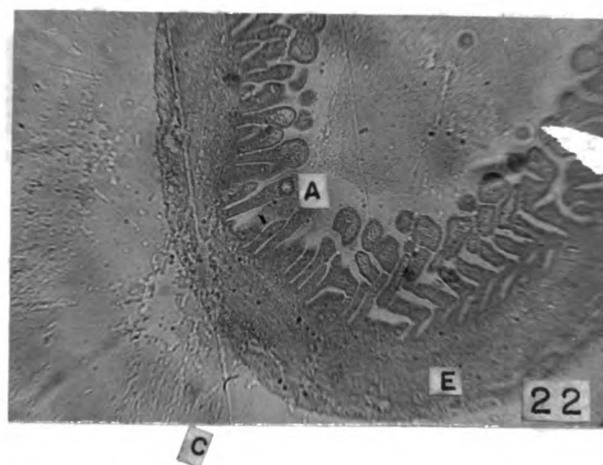
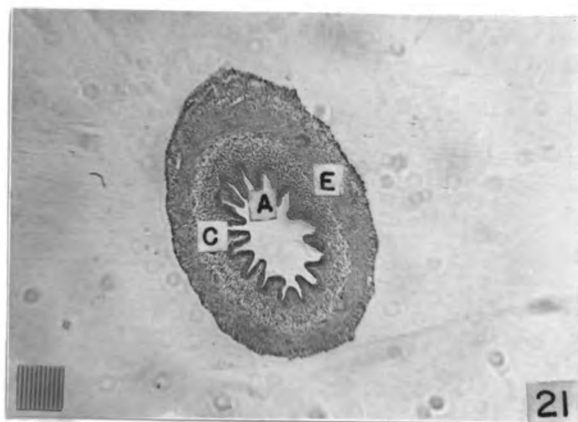
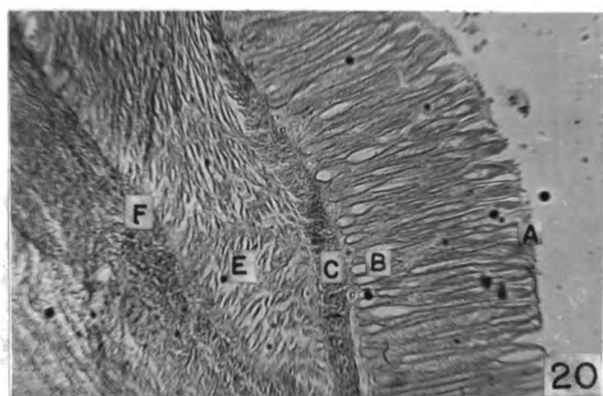
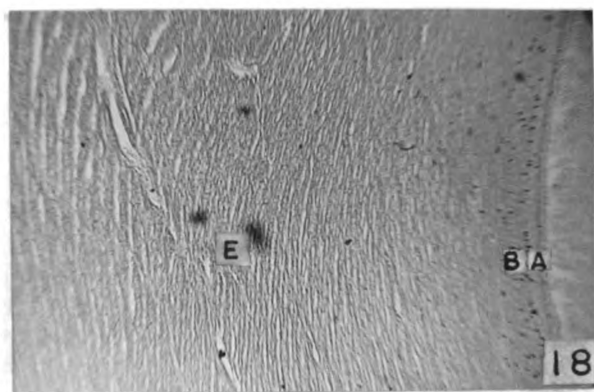
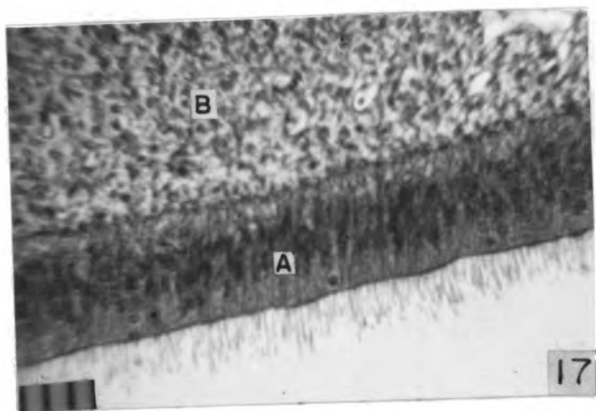
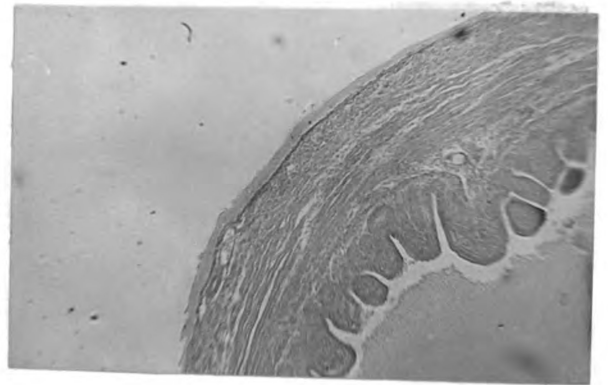
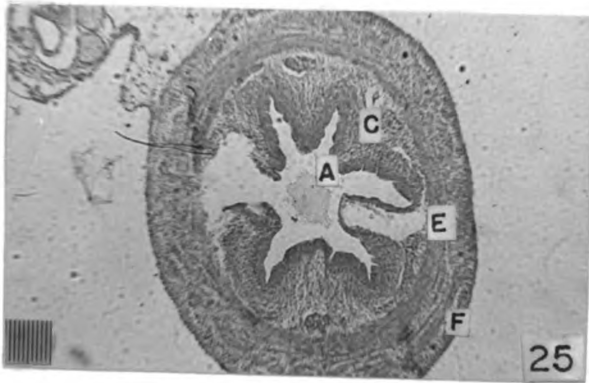
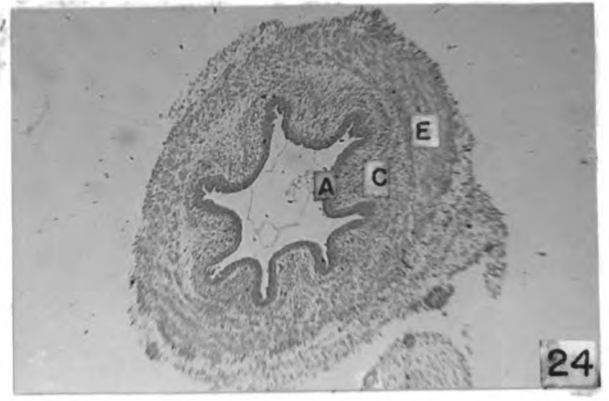
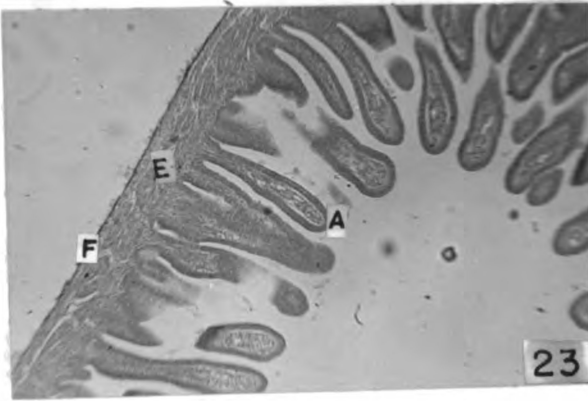


PLATE VII

- Fig. 23. Cross section through the small intestine of a 20-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; E. Tunica muscularis;
F. Serosa.
- Fig. 24. Cross section through the large intestine of an 11-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; C. Undifferentiated mesenchyme;
E. Tunica muscularis.
- Fig. 25. Cross section through the large intestine of a 14-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; C. Undifferentiated mesenchyme;
E. Tunica muscularis; F. Undifferentiated mesenchyme.
- Fig. 26. Cross section through the large intestine of a 20-day fetus. Hematoxylin-eosin. Magnification X170.
A. Epithelium; C. Lamina propria; E. Tunica muscularis.

Micrometer scale insert: 1 space = 0.01 mm.



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