TECHNIQUE APPRAISAL FOR STUDYING COTTONTAIL RABBIT COCCIDIOSIS

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ABSTRACT

TECHNIQUE APPRAISAL FOR STUDYING
COTTONTAIL RABBIT COCCIDIOSIS

by

Theodore Lopushinsky

Intestinal coccidiosis may influence cottontail rabbit (Sylvilagus floridanus) populations to a greater extent than present mortality figures indicate. This study is an exploration of techniques that might aid in evaluating effects of sublethal coccidiosis. Most of the animals used for these explorations were of known age from a successful breeding colony established in outdoor pens. In addition, wild animals were examined and an attempt was made to rear germfree cottontails.

The determinations of enzyme concentrations present in the cottontails' tissue and serum were the primary techniques employed to assess effects of the parasite. Changes in patterns of enzyme concentrations may ultimately prove useful in evaluating sublethal effects of coccidiosis. In the present study, uninfected animals were too few to permit a valid comparison of enzyme systems for infected and non-infected animals. Enzyme patterns of concentration are described for the population at hand and may provide a point of comparison for future studies.

The enzymes studied were alkaline phosphatase, glucose-6-phosphatase, succinic dehydrogenase and non-specific esterase. Each enzyme system displayed a gradient of concentration within the gastro-intestinal tract. Alkaline phosphatase and glucose-6-phosphatase were most concentrated in the upper small intestine while the greatest levels of succinic dehydrogenase and esterase occurred in the cecum.

Both adult and non-adult cottontails had similar tissue enzyme patterns in the gut. Areas of tissue enzyme concentration were also determined for the kidney, liver, spleen, lung, adrenal gland, ovary and testis.

A second method for measuring sublethal intestinal damage involved the determination of serum levels of alkaline phosphatase because damage to the intestinal mucosa releases intracellular alkaline phosphatase into the blood serum.

Normal serum alkaline phosphatase levels were found to decrease with age. But serum contains a mixture of several molecular varieties (isoenzymes) of alkaline phosphatase. Identification of activity due to the intestinal isoenzyme may be made by use of specific inhibitors of the isoenzyme. One such inhibitor, L-phenylalanine, reduced normal serum alkaline phosphatase activity approximately 75 percent. As a control, parallel serum samples were tested using D-phenylalanine, a form considered to be an inhibitor of all alkaline phosphatase isoenzymes except that of the intestine. But, when used, the D-form inhibited normal serum values almost 45 percent. It was not determined whether this discrepancy between the L- and D-phenylalanine inhibitions was due to the former inhibiting non-intestinal isoenzyme activity or whether the latter partially inhibited intestinal isoenzyme activity.

Eighty-seven percent of 83 wild cottontails had coccidia present. In only one instance, that of a 13-day old animal, was coccidiosis suggested as a cause of death. Diagnosis and estimation of fecal coccidial numbers were undertaken by sugar flotation in glass wells.

No false determinations of positive infections occurred using this technique, as occasionally occurred using fecal smears or intestinal scrapings. The youngest pen-born animal found to be infected was three days old, suggesting initial infection at day one or two. This last diagnosis was based upon histological examination of the intestine instead of the usual fecal analysis.

Coccidial species identified were: Eimeria maior, E. environ, E. media, E. neoleporis and E. sylvilagi. Patterns of coccidial elimination by naturally infected adult cottontails included periods of elevated oocyst numbers (showers) that lasted 4-6 days. The highest numbers of oocysts counted during a shower was 640,000 oocysts per gram of feces. Coccidial development within the intestinal tissue was photographed. Cottontails were successfully infected with prepared coccidial solutions.

The breeding colony permitted easy access to a wide spectrum of age classes. Penned adult females, placed in pens with either one or two males, were kept there for two days and were then removed to their own pens. From 64 matings, 20 litters resulted. Over a three-year period, the highest percentage of successful matings occurred in May. Mean gestation time was 29.1 days. The average litter size was 4.9.

In an attempt to obtain coccidia-free cottontails, newborn were removed from the nest and reared indoors. But, even when force-fed they refused to nurse and subsequently died. Insufficient quantity rather than quality of diet was assumed to be the probable cause of this failure. Stomach intubations of prepared formula gave growth rates

comparable to those resulting from nursing on the natural mother. These intubations, however, eventually caused esophageal perforations and killed the animals. Foster-mother trials employing lactating domestic does also failed to enable such young to survive. Nursing took place but the cottontails could not or did not obtain sufficient milk.

Three litters were surgically removed from does under sterile conditions and transferred to an isolator for germfree rearing. Feeding difficulties again occurred and all died. Two animals which lived 16 days germfree were stunted in growth.

Growth rates of nestlings were determined and the occurrences of various external features were noted. Weight differences as high as 45% existed between the largest and smallest nestlings in some litters. The period spent in the nest averaged 11.2 days. Thirty-one percent of 167 young used in the study died from unknown causes.

Penned adult females demonstrated annual fluctuations in body weight, the lowest weights occurring during the November-February period of the year. Females lost from 0.4-8.4 percent body weight 75% of the time when paired with either one or two males for two days. Daily food and water consumption for adult cottontails, caged indoors, averaged 7% and 16% of body weight, respectively, regardless of the animal's weight. Daily defecation averaged 5% of body weight, regardless of the size of the animal.

Anatomic differences were noted between adults and non-adults. Extramedullary hematopoietic centers were usually present in cottontails

less than a week old, and varied in their time of disappearance. The oldest animal found with these centers was 74 days old. Within the small intestine, juveniles always had fewer migrating lymphocytes in the lamina propia and epithelium than did adults. Spermatogenesis was found in a 14-day old cottontail. Eye lens weights were established for known-age cottontails less than a month old for use as an aid in age determinations. Three-day old nestlings were found with plant material already present in their intestines.

Supplemental findings showed total red and white blood cell counts, as determined from cardiac arterial blood, to increase with age. Differential leukocyte values were similar for rabbits of all ages. Adult differentials derived from cardiac blood were similar to those obtained from peripheral blood of the ear vein except for lower heterophil values. Similar serum and plasma protein fraction percentages were noted in juveniles, subadults and adults except for lower alpha-1 values in juveniles.

Nestling mortality from Wohlfahrtia vigil was observed. Larval migrations of Taenia pisiformis were tentatively blamed for gross hepatic lesions. Additional pathological findings included gastric ulcers and various neoplasms.

TECHNIQUE APPRAISAL FOR STUDYING COTTONTAIL RABBIT COCCIDIOSIS

By

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INTRODUCTION

A knowledge of the role of wildlife disease and parasitism in regulating animal populations is essential to sound game management, as well as to an understanding of the relationships between wild species, domestic animals and man. Referring to studies of wildlife parasites, Herman (1959) stated that ". . . there is an almost complete lack of information concerning incidence, pathogenicity, immunity or significance in regard to host survival".

The importance of coccidia as intracellular protozoan parasites of domestic animals is well documented (Davies et al., 1963; Pellerdy, 1965). There is considerable evidence, too, though less well documented, that coccidia may be of pathogenic significance to wildlife in general. Kiris (1962) concluded that coccidiosis was the most significant disease of squirrels in the USSR. These parasites are also believed to be significant population-regulatory factors for the wild rabbit Oryctolagus cuniculus (Wodzocki, 1950; Bull, 1953; Tyndale-Biscoe and Williams, 1955), the snowshoe hare Lepus americanus (Boughton, 1932; Erickson, 1944) and the jackrabbit Lepus californicus (Lechleitner, 1959).

The original objective of the thesis was to determine what lethal and sub-lethal effects were induced by coccidia (Eimeria spp.) in cottontail rabbits (Sylvilagus floridanus mearnsii). These common parasites of the cottontail may be of significance in regulating cottontail numbers. Although this goal became unattainable due to unforeseen difficulties, and no comparisons were made between infected

and non-infected cottontails, standard values were established for tissue and serum enzymological techniques selected to measure coccidial pathogenicity.

Enzyme analysis of host pathology measures the damage from a pathogen in terms of biochemical alterations. Such physiological modifications are demonstrable even in the absence of usual gross and microscopic lesions. A sensitive method is thus available for quantitating sub-lethal pathology which must be considered in any investigation of the effects of coccidia on a host population under field conditions (Joyner and Davies, 1960).

LITERATURE REVIEW

Cottontail Coccidiosis

The cottontail rabbit, considered to be the most hunted game mammal in the United States, suffers an unexplained high juvenile mortality. This annual mortality rate ranges from 60 to 85 percent (Carson and Cantner, 1963; Lord, 1963). The more obvious causes of death such as hunting (Geis, 1956), shortage of winter food (Lord and Casteel, 1960), predation (Hickie, 1940; Haugen, 1942; Kirkpatrick, 1950), and adverse weather (Kaczynski, 1958) account for the losses in only a few cases. Parasitic disease remains as a factor which may exert a greater constraint on cottontail populations than is presently believed.

All cottontail populations harbor a wide variety of parasites (Clancey et al., 1940; Morgan and Waller, 1940; Bell and Chalgren, 1943; Ecke and Yeatter, 1956; Dorney, 1962; Stringer, 1966). Among the most prevalent are the intestinal coccidia (Eimeria spp.). These protozoan parasites (Appendix A) undergo intracellular reproduction in the host tissues, with enclosed zygotes (oocysts) passed in the host's feces. Sufficient time under appropriate conditions of warmth and moisture renders the oocysts infective so that, if ingested by cottontails, are capable of infecting the rabbit.

Although coccidia were the first microscopic parasites discovered (by Van Leeuwenhoek in 1674), their pathological significance is still unknown for many wild species, including the cottontail. Yuill (1964), however, implicated coccidia as a significant control in cottontail

populations. Through medication of penned cottontails, he caused coccidial infection levels to be halved with the number of young produced being doubled in the treated group as compared to the untreated control group. Dorney (1962) also has shown that a huge reservoir of coccidial infection is available to nestling cottontails. He recorded oocyst counts above 200,000 per gram feces and found over one-third of the oocysts in feces collected in the field throughout the winter to be capable of infection.

Although few wild cottontails are found soon enough after death to allow valid post-mortem diagnoses, reports exist of adult (McGinnes, 1958) and juvenile (Haugen, 1942; Ecke and Yeatter, 1956) deaths from coccidiosis.

Enzymology

A living organism has been described as an ". . . orderly integrated succession of enzyme reactions markedly disturbed in inborn or acquired disease" (Kugelmass, 1964). Disease may thus be characterized as a disturbance in enzyme reactions.

Trauma to the intestinal mucosa, such as might occur in intestinal coccidiosis, produces a loss of intracellular enzymes (Lawrence, 1964). This loss is considered to be the result of damage to the selective permeability of the cell membrane (Hess, 1963). Loss of enzymatic activity within the intestine has been demonstrated in baby pigs with transmissible gastroenteritis (Maronpot and Whitehair, 1967) and in guinea pigs suffering from coccidiosis (Jervis et al., 1966). Decrease in alkaline phosphatase, succinic dehydrogenase and glucose-6-phosphatase

activities were found in the latter study. Even though the guinea pigs were not outwardly affected, the parasitism caused the absorptive capacity of the small intestine to decrease.

The enzyme systems examined in this study (Appendix B), alkaline phosphatase, succinic dehydrogenase, glucose-6-phosphatase and non-specific esterase, are concerned with energy transfers from absorbed food. Sub-lethal intestinal damage to cottontails from coccidiosis, therefore, may be manifested as a malabsorption of food with a corresponding decrease in measurable enzyme concentration. Alkaline phosphatase received major emphasis in this study because, in mammals, the small intestine is the primary site of its concentration (Innerfield, 1960) as well as the area of host damage from the intestinal coccidia.

The enzyme analysis of tissues referred to above required killing the animal. A second technique for quantitating host pathology measured enzymatic activity in blood serum and did not require killing the animal. As previously mentioned, cellular damage releases intracellular enzymes into the intercellular spaces which ultimately reach the blood. But, for any particular enzyme, the blood is a 'pool' of different molecular varieties (isoenzymes) of that enzyme which has originated in different organs (Hess, 1963). Thus, the total serum activity of an enzyme is a mosaic of various isoenzyme fractions.

Isoenzymes (Appendix C) are normally present in serum because of necrobiosis, whereas in certain diseases the damaged organs release their specific isoenzymes into the blood resulting in significantly elevated isoenzyme concentrations (Lawrence, 1964). In man, damage to

the intestinal mucosa releases an above-normal amount of the intestinal isoenzyme of alkaline phosphatase into the blood (Lawrence, 1964). Since a minimum of eight alkaline phosphatase isoenzymes have been found in normal human sera (Tasewell and Jeffers, 1963), identification of the intestinal isoenzyme is required before it may be determined whether the isoenzyme is present in amounts indicative of intestinal damage. Through the use of organ-specific inhibitory and sparing (non-inhibitory) compounds, isoenzymes are identifiable in serum. In both human and rat sera, the amino acid L-phenylalanine specifically inhibits the activity of the intestinal isoenzyme of alkaline phosphatase, but has no inhibitory effect on isoenzymes of liver, bone, lung, kidney, bile and spleen (Fishman et al., 1963). Hugon and Borgers (1966) used L-phenylalanine to inhibit alkaline phosphatase in tissue sections of mouse intestine.

Gnotobiology

Since the younger age classes of the host are most stressed by coccidial infection (Pellerdy, 1965), an outdoor breeding colony was established to insure a ready supply of young cottontails. The colony also provided pregnant females for germfree research (Appendix D). Other workers (Sheffer, 1957a; Rongstad, 1966a; Forcum, 1968) had successfully bred cottontails in captivity, but in larger pens than those used here.

Ultimate proof of the pathological significance of cottontail coccidiosis must rely on experimentation using animals as free as

possible of other contaminating organisms. Since rabbits harbor a wide variety of protozoa, bacteria, fungi and various worm and arthropod groups, an attempt was made to obtain germfree cottontails. The development of modern germfree techniques (Reyniers, 1959) enables the production of germfree animals which are the most valid for determining the effects of a specific pathogen (Glimstead, 1959).

The only previous reports of germfree research on lagomorphs applied to domestic rabbits. Pleasants (1959) and Wostmann and Pleasants (1959) hand-reared germfree cesarean-derived rabbits. Surgically-removed domestic rabbits have been also reared coccidia-free (Hills and MacDonald, 1956). The germfree research on cottontails by Lopushinsky and Youatt (1968), as far as is known, is the first report of this kind for a North American wild mammal.

MATERIALS AND METHODS

Cottontail Age Categories

To insure an understanding of terms: adults were animals more than one year old; subadults were young of the year which were capable of breeding; young which had left the nest but were incapable of reproduction were called juveniles; juveniles too young to leave the nest were called nestlings.

The only other report separating young cottontails into juveniles and subadults, the latter capable of reproduction, used pelage molts (Negus, 1958). But in this study, juvenile and subadulthood status were based upon body weight. The minimum weight for adults was arbitrarily set at 1000 grams since all adults studied weighed over 1000 grams. No question existed of these being adults since they had been captive for at least 1.5 years. Cottontails less than 1000 grams but more than 500 grams were classified as subadult while juveniles weighed less than 500 grams. Some physiological basis exists for selecting 500 grams as the lower limit for subadulthood. Negus (1958) found that animals beginning their subadult molt weighed about 500 grams. This molt, furthermore, began at 11 weeks of age which agreed with reports of first breeding at 3-6 months for both male and female cottontails (Conaway and Wight, 1963). Beule and Studholme (1942), Lord (1963) and Rongstad (1966a) all indicated three-month old cottontails to weigh between 500-550 grams. One 2.5-month old breeder has also been reported (Negus, 1959). Petrides (1961: Fig. 7), furthermore, estimated 2.5-month old cottontails to weigh 500 grams.

Experimental Animals

Wild adult cottontails were trapped on the Michigan State University farms and housed throughout the year in pens 50 feet long, 6 feet wide and 6 feet high at the Rose Lake Wildlife Research Center near East Lansing, Michigan. The pens were covered with 1-inch mesh poultry wire, and bordered with ¼-inch mesh hardware cloth from two feet above to one foot below the ground. Water and pelleted rabbit food (Farm Bureau Rabbit Ration) were fed ad libitum. Straw piles covered the food bins and provided shelter, as did scattered brush piles. Straw and brush were re-located every few months because of fecal accumulations under them. When indoors, rabbits were housed in 3 x 2 x 1.5-foot animal cages with bottom screening which permitted wastes to fall through into a collecting pan.

Penned rabbits were easily caught with a dip net (6-foot pole, 2-foot diameter rim with ¼-inch mesh seining net). A dish towel was placed in the bottom of the net to buffer the rabbit against impact. Less than one minute was generally required for capture. In removing rabbits from indoor cages, a 3 x 2-foot grain sack placed over the rabbit prevented it from damaging itself as well as allowing for a rapid capture.

Adult domestic rabbits (Oryctolagus cuniculus) were used in germfree surgical trials. The Dutch Belt and Florida Albino varieties employed were always housed indoors.

Two systems for breeding were utilized. The first kept the sexes apart. Females, housed 2-3 per pen, were individually placed with a

male for two days and then removed to separate pens. Occasionally, an excess of males required placing the female in a pen with two males. The second breeding procedure kept a male and female constantly together.

Weekly weighings and palpations of females were made to determine pregnancies. Nestlings born in the pens were found usually no later than 15 hours after birth. Daily weight and hind foot measurements of nestlings were recorded for at least the first two weeks. The hind foot was compressed against the rule and measured from heel to the end of the extended toes.

Techniques Used in Feeding Unweaned Cottontails

Young cottontails were obtained both from the breeding pens and from the Michigan Department of Natural Resources which yearly receives numerous orphaned nestlings. Two formulas were used to hand-feed the young. The first 'rough' formula consisted of a 1:1 evaporated milk and water mixture, to 50 ml of which a tablespoon of Karo syrup was added. This was warmed and fed every two hours, but young rabbits had to weigh at least 50-60 grams in order to be successfully reared with this diet. Fresh green clover or lettuce was provided once their eyes opened.

A second formula (Table 1) was used to feed newborn rabbits and was essentially that used by Pleasants et al., (1963) for rearing germfree domestic rabbits. Difficulties in preparation involved the solubilities of tocopherol, vitamin K1 and the A and B salt mixtures. Too much powdered skim milk in solution caused it to turn brown and to

Table 1. Formula for hand-feeding newborn cottontail rabbits (based on Pleasants et al., 1963). Rose Lake Wildlife Research Center.

Mixture A: 55 ml distilled water with 15 g powdered skim milk dissolved in it.

20 ml whipping cream.

25 ml evaporated milk.

Mixture B: .14 g DL-methionine (General Biochemicals, Laboratory Park, Chalgrin Falls, Ohio).

.05 g L-trytophane (General Biochemicals).

5.0 ml 1% calcium ascorbate (General Biochemicals).

0.3 ml Vi-synerol* (U.S. Vitamin Corporation, Casmir Funk Laboratories, N.Y., N.Y.) with one drop of DL-alpha tocopherol (General Biochemicals) and one drop of Vitamin-K-1** (General Biochemicals).

3.0 ml of B-mix 103*** (in triple distilled water), (General Biochemicals).

Mixture C:

A Salts	B Salts	
176 mg KH_2PO_4	14 mg $MgSO_4$	5.2 mg $ZnSO_4 \cdot H_2O$
180 mg NA_2HPO_4	4 mg $MnCl_2 \cdot 4\ H_2O$	0.8 mg $CoCl_2 \cdot 6\ H_2O$
0.4 mg Kl	2.4 mg $CaCl_2$	24 mg Ferric Ammonium Citrate.

Mixture A, after sterilization at 121° C for 15 minutes, had mixtures B and C (sterilized together) added to it.

* Each 0.3 ml contained 2500 USP units Vit. A; 500 USP units Vit. D; 25 mg Ascorbic Acid; 0.5 mg Thiamine HCl; 0.2 mg Riboflavin; 0.15 mg Pyridoxine HCl; 0.25 mg Niacinamide; 1.0 mg Pantothenic acid.

** Dissolved in 2 drops of Wesson Oil.

*** 3 ml B-mix 103 contains; 3.0 mg Thiamine HCl; 1.0 mg Riboflavin; 1.0 mg Pyridoxine HCl; 12.5 mg CaPantothenate; 2.5 mg Niacinamide; 0.25 mg Folic Acid; 0.025 mg Biotin; 0.025 mg Cyanocobalamin; 100 mg i-Inositol; 100 mg Choline Dihydrogen Citrate.

solidify upon autoclaving. The 15 grams per 100 ml of Mixture A was near the maximum allowable concentration and did not solidify.

Three methods were used for feeding the unweaned young: a) an eye dropper or syringe with or without a prepared latex nipple (Appendix D); b) stomach intubation via plastic tubing; c) lactating domestic does. Once the latter had dropped her litter, Vicks Vapo-rub was placed on the doe's nose and on the heads of the young in order to destroy the mother's ability to identify her young by their scent. A short time later, the doe was removed from the cage for at least one hour. When she was returned, newborn cottontails, with Vicks on their heads, had replaced the domestic young. But, when left overnight, the cottontails had not nursed. Further trials always kept a single domestic nestling with the cottontails so as to insure continual lactation by the doe. An improved nursing reflex occurred if the initial hand-feeding was done the day after removal. Daily measurements were taken prior to the first morning feedings.

Surgical Removal (Cesarean-derivation) of Young

Pregnant cottontails selected for cesarean delivery remained in their pens until two days before the projected date of birth and were then placed indoors. Surgery was timed hopefully to occur on the expected day of birth.

Successful trial surgery on pregnant domestic rabbits resulted in the following operational techniques and required two people. Before killing the doe, her abdomen was washed with disinfectant (MEDIC: MSU Industries, Ionia, Michigan). The doe was killed by a sharp blow at

the base of the skull to avoid possible intra-uterine death from surgical anesthesia (Casteel and Edwards, 1965). Death was instantaneous but the heart beat for a few minutes more. The abdomen was then skinned and a two-inch incision was cut through the abdominal muscles directly above and along a uterine horn. Internal pressure forced part of the horn through the incision making it easy to pull out the entire uterus.

After cutting through the uterine walls, each young was removed from its fetal sac by cutting through the sac under the chin of the fetus. This enabled the head to pop out and breathing to start. The whole fetus was then gently squeezed out of the sac, care being taken not to tear out the umbilical cord. Once pinched with forceps, however, the cord could be cut without damage to the animal. The young were wiped dry, weighed, measured and placed in a plastic laboratory mouse box.

A heating pad under the box maintained a 37^{o} C temperature at the floor of the box. When young were surgically delivered inside the isolator for germfree rearing (see beyond), the heating pad was placed under the isolator. The mouse box, within the isolator, was set on the heating pad as were flasks with water for wetting paper towels. Since insufficient humidity caused the young to die of dessication, wet towels were placed as a cover over the mouse box as well as on the floor of the box. All towels required 2-3 wettings per day.

Description of Germfree Isolator

The flexible-film isolator (G-F Supply Division, Standard Safety Equipment Co., 431 North Quentin Road, Palatine, Illinois) measured 6 x 2 x 2 feet. Access to the isolator was through a transfer chamber located at one end, or through a ventral trap door (Fig. 1). Both the transfer chamber and trap door had inner and outer covers. At the end opposite the transfer chamber, air was blown into the isolator and sterilized by passage through fiber glass filters. The air exited at the same end by bubbling through an outlet trap filled with mineral oil. A pair of gloves permanently attached to each side of the isolator allowed two workers, facing each other, to work simultaneously within the isolator.

The empty isolator was sterilized by spraying with a 2% peracetic acid solution (Becco Chemical Division, Food Machine and Chemical Corp., 161 East 42nd Street, New York 17, New York). Spraying of the isolator was done 24 hours before young were to be placed inside. Non-living materials for entrance into the sterile isolator were double-wrapped in brown wrapping paper, taped closed and autoclaved at 121^{o} C for 15 minutes. These packages were quickly placed within the transfer chamber, the outer cover placed on and the interior of the transfer chamber sprayed through a sealable nozzle. If animals were inside the isolator, at least three hours were required to clear the transfer chamber of peracetic acid. The inner door of the transfer chamber was then removed and the packages placed inside the isolator.

Figure 1. The germfree isolator used in the study with the transfer chamber at the left and ventral trap door (arrow) at the lower left.

The materials required for surgery and maintenance of young within the isolator are listed in Appendix D.

Two methods were used to introduce the young into the isolator under germfree conditions: 1) A plastic sleeve was made by attaching two 10" x 8" x 24" polyethelene bags (Triquet Paper Co., Lansing, Michigan) end to end. One end was attached to the trap door (inner cover intact, outer cover removed) with the other end sealed. Before sealing the distal end, peracetic acid was sprayed inside the sleeve to insure sterility since removal of the trap door's inner cover made the interior of the sterile isolator continuous with the interior of the sleeve.

The bared stomach of the doe was placed against the sleeve which projected from the bottom of the isolator. It was necessary to partially decompress the isolator in order to insert the doe up and into the chamber. Decompression was accomplished by leaning on the isolator forcing out excess air. Removal of young proceeded as previously outlined with the addition of Kelly hemostatic forceps attached along the abdominal incision to maintain air-tight conditions between the plastic sleeve and stomach muscles.

2) Alternatively, the uterus was removed outside the isolator with the uterine ends clamped off with hemostatic forceps. The uterus was submerged in a pail of disinfectant (MEDIC) which contained the distal half of the plastic sleeve. From inside the isolator, the second worker cut through the submerged section of the sleeve and pulled the uterus by one of the forceps through the slit into the isolator. The

cut section of sleeve was weighted to keep it submerged at all times. With either of the above methods, the time required from killing the doe to removal of all fetuses from the fetal sacs was about three minutes. In one instance, five minutes elapsed before the fetuses were taken from the uterus, but all were alive.

Each entrance into the isolator had cotton-swab smears made of the inner walls and equipment to insure that no contamination occurred. These were incubated in thioglycollate broth. Prior to surgery, additional smears were plated on blood agar. Both broth and agar were incubated at 25^{o}, 37^{o} and 55^{o} C under aerobic and anaerobic conditions to ascertain the presence or absence of contaminating micro-organisms.

Examination for Coccidia

All cottontails were examined for coccidia. These were recorded as present or absent as based on fecal smears and on smears of ingesta from various intestinal locations. A series of indoor studies quantitated daily coccidial elimination as evidenced by fecal oocyst numbers.

Cottontails produce both hard and soft fecal pellets (reviewed by Heisinger, 1962), with the latter probably being reingested directly from the anus. Occasionally, a group of these soft pellets was found in the waste pans. These were easily distinguished from the more prevalent hard pellets by their putty-like consistency and their adherence to each other. Soft pellets were not used for coccidial counts because: 1) such pellets were an unusual finding, since most were probably reingested; and 2) these soft pellets had significantly

higher oocyst numbers than hard pellets produced during the same period. The reason for the latter condition is not known.

Determinations of oocyst numbers per gram of feces were made using a method modified from Clark and Conner (1959). Four grams of feces were estimated by a volumetric displacement. This was done by dropping fecal material into a volumetric flask, filled with water to the 96 ml mark, until the 100 ml mark was reached yielding a 1:25 fecal dilution. Before being added to the water, fecal pellets were first placed on paper towels to remove excess urine and then crushed. Actual weighings of the fecal material showed this displacement method to slightly underestimate the assumed four gram fecal sample. Any bias, however, was considered to be constant for all samples.

After thorough mixing, 1 ml of the fecal solution was diluted with 1 ml of concentrated sugar solution and thoroughly mixed. This last dilution resulted in a final 1:50 fecal dilution. One ml of this mixture was then divided between two glass-well counting chambers made from ½-inch glass tubing cut into 3/8-inch sections and glued two per microscope slide. Additional sugar solution was added to each well to form a positive meniscus which was carefully covered with a coverslip.

After 20-30 minutes, the total areas under both coverslips were examined under low power. No oocysts were ever found in the sugar solution after 30 minutes, thus each oocyst counted represented 50 oocysts per gram of feces. Oocysts too numerous to count under low power were tallied in a sample of 20 high power (X440) fields per well

with the total multiplied by a factor of 38.2 (756 fields per well ÷ 20). Identification of coccidial species was made from glass-well solutions examined under oil immersion. Coccidia species-percentages for any solution were recorded per 100 oocysts counted using the taxonomic classification of Carvalho (1943).

Preparation of Experimental Oocyst Solutions

Stock solutions of sporulated (infective) oocysts for experimental infections were prepared from feces exhibiting high oocyst numbers. Ten to twenty grams of feces were crushed in water and allowed to stand one day. The supernatant was poured off with the sediment resuspended in water and filtered through cheese cloth.

The solution was then mixed with a 2.5% potassium dichromate solution and aerated by a vacuum pump. Oocyst sporulation was essentially completed in four days, at which time the solution was cleared of dichromate by repeated washings and centrifugations. The solution of oocysts was then diluted 1:1 with sodium hypochlorite (full strength Chlorox) for one minute to remove tissue debris. Repeated washing and centrifuging cleared the solution of the Chlorox. The oocyst solution was then diluted 2:1 with 2% peracetic acid for 30 minutes for sterilization against other micro-organisms. Subsequent washings used sterile water with all centrifuging done in capped vials flamed before and after all transfers.

Oocyst counts of this final solution were made and the solution was stored at 4^{o} C in 10 ml aliquots. Prior to use in experimental infections the aliquots were recounted. Prepared infective oocyst

solutions were administered to larger-sized rabbits by stomach intubation, while smaller animals were infected orally with an eyedropper.

Tissue Histochemistry

For enzyme determinations, tissues that could be incubated within one hour were fresh frozen at -30° C in a cryostat (International Equipment Company, Needham Heights, Massachusetts). Fresh tissues, not able to be incubated within one hour, were rapidly frozen by immersion in isopentane (Matheson Scientific Company) containing dry ice and stored at -20° C. Occasionally, fresh tissues were also fixed in a cold calcium-formol solution* for 24-48 hours before incubation for alkaline phosphatase activity.

All tissues used for histochemical staining were sectioned in the cryostat at 10 microns between -20 and -30° C. Microscope slides which were at room temperature and coated with an adhesive (Tissue-Tac; Dade Reagents, Inc., Miami, Florida), were held just above the frozen sections. This resulted in the sections being attached to the slide. The adhesive kept the sections on the slides when placed in the incubation medium. Adhesion was best when the Tissue-Tac was wet.

At least two types of tissues, i.e., lung and liver, were placed on each slide. All enzyme incubations had negative and positive control sections incubated at the same time as the test sections (see Appendix B). The slides remained inside the cryostat until incubated. Within 20 minutes, the slides were removed from the cryostat, brought

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* 75 ml 40% formaldehyde, 5.51 g $CaCl_2$; 425 ml distilled H_2O.

to room temperature (about one minute) and placed in the appropriate incubation medium contained in coplin jars. After the required incubation time, the slides were washed in water, air dried and coverslips were added using a glycerol-gelatin (Sigma Chemical Co.). This gelatin had to be applied to completely dried slides, otherwise its water solubility resulted in numbers of air bubbles.

Alkaline Phosphatase: The incubation solution contained 2 ml naphthyl AS-MX phosphate, 48 ml distilled water (dist. H_2O) and 25 mg of either Fast Blue RR or Fast Red TR diazonium Salt (Sigma Kit 85L-1). After 20 minutes of incubation, the slides were washed in running distilled water and air dried. Even if kept in the refrigerator, the medium could not be used a second day. Calcium-formol fixed tissues gave results as good as those from fresh or isopentane-frozen tissues.

Mode of action: The alkaline phosphatase splits off the naphthyl AS-MX which combines with the diazonium salt to form an insoluble azo dye. A deep blue color develops at the site of enzyme activity if the Fast Blue RR is used, while a cherry red is seen using Fast Red TR.

Succinic Dehydrogenase (Nitro-BT [N-BT] method of Nacklas *et al.*; in Pearse [1960], p. 910): The medium contained 10 ml of a stock succinate solution* and 10 ml (1 mg/ml) aqueous solution of N-BT (Dajac Laboratories). A coplin jar containing the medium was placed in

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* Stock succinate solution: equal volumes of (a) 0.2 M phosphate buffer at pH 7.6 (Pearse, 1960: 780); 26.83 g/500 ml dist. H_2O of 0.2 M $NA_2HPO_4 \cdot 7\ H_2O$ buffered with 13.61 g/500 ml dist. H_2O of 0.2 M KH_2PO_4, and (b) 0.2 M Na succinate (Nutritional Biochemical Co., Cleveland, Ohio).

a water bath at 40° C and protected from light. Slides were incubated for 20 minutes, washed in saline, rinsed for 5 minutes in 15% alcohol and air dried.

Mode of action: Succinic dehydrogenase releases H+ from the succinate which is accepted by the N-BT, forming an insoluble diformazan. Sites of enzyme activity appeared blue. If refrigerated, and protected from light, the medium was usable a second day. The enzyme was destroyed by calcium-formol fixation.

Glucose-6-Phosphatase (the method of Wachstein and Meisel; [in Pearse, 1960: 880]): Twenty ml of a 125 mg percent solution of potassium glucose-6-phosphate (Sigma Chemical Co.), 20 ml of 0.2 M tris buffer at pH 6.7*, 3 ml 2% lead acetate and 7 ml distilled water were stirred in coplin jars and then placed in a water bath at 40° C. Incubation was for 8 minutes. The slides were then washed in distilled water, developed in dilute yellow ammonium sulfide (3 drops/50 ml dist. H_2O) for two minutes, again washed in distilled water and dried. The medium, if refrigerated, was good for at least two days. Calcium-formol fixed tissues gave results comparable to fresh frozen tissues. Sites of enzyme activity became brown-black.

Mode of action: Glucose-6-phosphatase dephosphorylates glucose-6-phosphate which reacts with the lead acetate and forms an insoluble lead phosphate. The addition of ammonium sulfide changes the lead phosphate to lead sulfide.

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* 24.2 g Tris (hydroxymethyl) - aminomethane (Sigma Chemical Co.)/ liter dist. H_2O and buffered to pH 6.7 with 0.1 N HCl.

Esterase (Pearse 1960: 886 - substituting alpha naphthyl acetate with alpha naphthyl proprionate): The medium contained 10 mg alpha naphthyl proprionate dissolved in 0.25 ml acetone, 20 ml 0.1 M phosphate buffer at pH 7.4* and 75 mg of Fast Blue RR Salt. This mixture was stirred and filtered through No. 40 filter paper onto the slides. The slides were incubated at room temperature for 15 minutes, washed in distilled water for 2 minutes and dried. Blue-black sites indicated sites of enzyme activity. If refrigerated, the medium was usable a second day.

Mode of action: Esterase splits the alpha naphthyl proprionate releasing the alpha naphthyl. The latter combines with the diazonium salt to form an insoluble azo dye. The technique does not differentiate between different types of esterases (see Appendix B).

Photomicrographs of these enzyme reactions were taken the same day as the incubations using a Leitz Ortholux Microscope with micro-camera attachment (MIKAS).

Serum Enzyme Analysis

Whole blood was collected by cardiac puncture with a one-inch 22-gauge hypodermic needle attached to a 6-cc syringe. Cardiac blood, if left at room temperature (25° C) formed "chicken-fat" clots which greatly reduced serum production. To prevent such clot formation, the blood vial was placed in a water bath at 37° C for 15 minutes and then placed in the refrigerator (4° C) for one hour. A highly contracted clot with high serum yields was obtained. No decrease in

* See succinic dehydrogenase method.

alkaline phosphatase activity seemed to occur from this procedure. Serum not used immediately for alkaline phosphatase determinations was frozen at -20° C for later analysis.

At first, serum alkaline phosphatase determinations used the method of Klein, Read and Babson (1960) (Phosphatabs-Alkaline Quantitative: General Diagnostics Division, Warner-Chilcott, Morris Plains, New Jersey). This procedure was replaced by an improved test (Phosphastrate TM Alkaline: Warner-Chilcott) whose advantages included zero order kinetics (allowing straight line calibration curves) and stable colored end products with final values read as International Units (Appendix C).

Alkaline phosphatase determinations were controlled by the use of Versatol-EN (standard-in-serum, normal enzyme: General Diagnostics). The standard curve used in determining alkaline phosphatase activity levels for the unknowns was calibrated employing varying mixtures of Versatol-EN and Versatol-E (standard-in-serum, elevated enzyme).

All serum alkaline phosphatase determinations were run in duplicate. When a serum sample was tested using the organ specific inhibitory L-phenylalanine and organ specific sparer D-phenylalanine (Appendix C) the following procedure was used:

Tube 1 Control blank	Tube 2 Regular test	Tube 3 Inhibitor test	Tube 4 Sparer test
serum	serum	serum	serum
dist. H_2O	dist. H_2O	L-phenylalanine	D-phenylalanine
	substrate	substrate	substrate

Because serum enzyme activities should be reported as International Units per liter serum (Appendix C), the Klein-Read-Babson (KRB) units reported for the initial method were converted by a multiplication factor of 10.4667 derived as follows:

$$\text{One KRB Unit} \times \frac{2}{} = \text{no. micrograms substrate transformed/0.2 ml serum} \div \frac{318.3}{} =$$

$$\text{no. micromoles substrate transformed/.02 ml serum} \times \frac{50{,}000}{} = \text{no. micromoles substrate transformed/liter serum}$$

$$\div \frac{30}{} = \text{no. micromoles substrate transformed/liter/minute} = \text{One International Unit.}$$

Tissue Histology

Tissues for routine histological examination were fixed in 10% formalin, processed in an Autotechnicon (Model 2A, Technicon Co., Chauncey, N.Y.), sectioned at 7 microns, and stained with hematoxylin and eosin employing standard procedures (Benjamin, 1961). Special stains also used included Prussian Blue, Congo Red and Periodic Acid Schiff (PAS).

Eye Lens Weights

All necropsied cottontails had an eye removed for age determination by lens weight (Lord, 1961). The eye was fixed in 10% formalin for at least seven days. The lens was then removed and dried in a vacuum oven at 80° C for 24 hours, weighed, re-dried for 24 hours, and re-weighed to obtain the final lens weight. Insignificant lens weight losses during additional drying periods indicated that these did not warrant the extra effort involved. It was deemed unnecessary to

record lens weights beyond whole milligrams because of the lens weight overlaps for different age classes.

RESULTS AND DISCUSSIONS

Normal Tissue Enzymology

The sites of concentration for the intracellular enzymes alkaline phosphatase, glucose-6-phosphatase, succinic dehydrogenase and non-specific esterase were determined within the gastrointestinal tract. Whenever possible, other organs also received examination.

Eight adult and 27 non-adult cottontails were studied, the youngest was 3 days of age. No gastrointestinal enzyme differences in site concentration or location existed between adults and non-adults.

Alkaline Phosphatase (AP)

The walls of blood vessels always displayed AP-activity but this was not considered as evidence of alkaline phosphatase in the particular organ being studied.

Stomach: The pyloric area never exhibited AP-activity. In the fundic portion, the enzyme was confined to the deeper regions of the gastric glands (Fig. 2) within unidentified cells. The enzyme was not uniformly distributed throughout the gastric glands.

Duodenum: Regardless of the enzyme system studied, the epithelium displayed an irregular pattern of enzyme distribution (Fig. 3). This pattern of alkaline phosphatase concentration, always present in the upper duodenum, became a solid continuous pattern from mid-duodenum on. The enzyme occurred mainly within the apical ends of the epithelial cells. This intracellular location also has been demonstrated in duodenal cells of mice (Hugon and Borgers, 1966). No AP-activity was

observed in the Brunner's glands, a finding also reported in domestic rabbits (Jervis, 1963).

Mesenteric Small Intestine: No specific jejunum or ilium sections of small intestine were designated. Instead, the portion of small intestine beyond the duodenum which was tightly coiled by mesentery, was termed the mesenteric small intestine. In the mesenteric intestine, the enzyme was primarily concentrated in the distal half of the epithelial cells and showed a solid, continuous band of concentration along the periphery of the villus (Fig. 4).

A section of tapeworm with AP-activity in its outer cuticle (Fig. 5) showed granular sites of activity situated just beneath the cuticle. This location suggested enzyme formation by the worm and not merely absorption from the intestinal lumen of the rabbit.

Terminal Small Intestine: This section of intestine identified histologically by an increase in epithelial mucous cells, comprised the lower one-quarter of the small intestine. Enzymatic concentration was similar to that present in the mesenteric small intestine except for breaks in the continuity of epithelial activity caused by the mucous cells. Peyer's patches consistently demonstrated alkaline phosphatase because of the characteristic accumulation of lymphocytes which always contained the enzyme.

Cecum: Alkaline phosphatase occurred mainly in the distal half of the epithelial cells of the outer portions of the villi (Fig. 6). A decreased concentration also occurred in the crypts.

Colon: The portion of colon leaving the cecum, the sacculus rotundus, displayed some enzymatic activity but none was present distal to the mid-colon (Fig. 7).

Kidney: No activity was present in the medulla. Intense sites of AP-concentration occurred only in various proximal convoluted tubules (Fig. 8). Intracellular localization of the enzyme was in the apical end of the cell. These tubular sites of AP-activity occurred throughout the renal cortex but never within the nephrogenic zone which consists of enzymatically inactive immature glomeruli and tubules (Wachstein and Bradshaw, 1965). Only scattered glomerular AP-activity was observed in the kidneys of young cottontails. Wachstein and Bradshaw (1965) demonstrated a similar AP-pattern in the kidneys of neonatal domestic rabbits which they considered occurring in mature nephrons. Though it was not determined when the cottontail kidney became fully matured, the adult kidney had AP-activity within all proximal convoluted tubules.

Spleen: Alkaline phosphatase was confined to the aggregations of lymphocytes which make up the white and red pulp of this organ.

Adrenal gland: Alkaline phosphatase activity was observed in the cortex but not the medulla, which agreed with the findings of Naik and George (1964).

Testis: Alkaline phosphatase in the epididymis of a breeding male was concentrated in the apical area of the cells, but only the stroma about the seminiferous tubules had enzyme present (Figs. 9 and 10).

The epididymis of non-breeding adults (Fig. 11) demonstrated greatly reduced enzyme concentrations.

Ovary: Sites of AP-activity were most evident in the thecal linings of the developing follicle (Fig. 12).

Liver: No activity.

Lung: No activity.

Figures 2-12 demonstrate sites of alkaline phosphatase (AP) in cottontail rabbit tissues.

Fig. 2: AP-activity in deeper glands of the stomach. No reaction is evident in the submucosa at the upper left. X188.

Fig. 3: AP-activity in the duodenum demonstrating the irregular pattern of distribution. The enzyme was present primarily at the apical border of the cell with limited reaction at the lower border (arrow). X188.

Fig. 4: AP-activity in mesenteric small intestine demonstrating the continuous band of enzyme concentration in the distal half of epithelial cells. X188.

Fig. 5: AP-activity in the outer cuticle of a tapeworm (T) located in the small intestine (I). X188.

Fig. 6: AP-activity in the cecum showing a decreased enzyme concentration in the crypts. X188.

Fig. 7: Section of lower colon showing no AP-activity. X188.

Fig. 8: AP-activity in epithelial cells of proximal convoluted tubules. X750.

Fig. 9: AP-activity in epithelium of the epididymal ducts. Breeding condition. X750.

Fig. 10: AP-activity in seminiferous tubules showing no activity in gametocytes but intense activity in the surrounding stroma. Breeding condition. X188.

Fig. 11: AP-activity in epithelium of the epididymal ducts. The arrow indicates the most intense site found in the epididymis. Non-breeding condition. X188.

Fig. 12: AP-activity of ovary, primarily in the thecal layers of the follicles. X188.

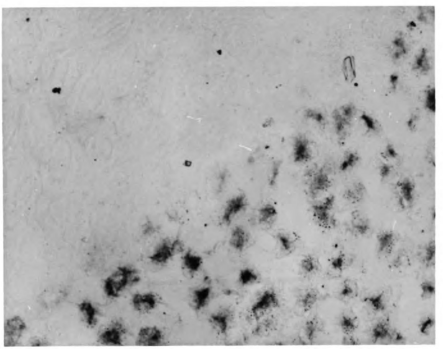


FIGURE 2.

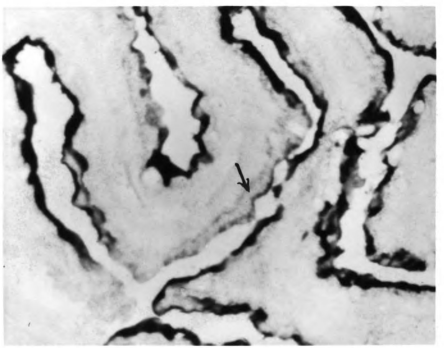


FIGURE 3.

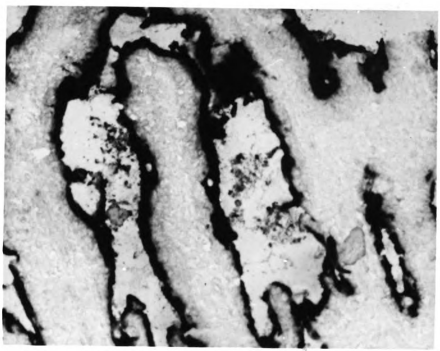


FIGURE 4.

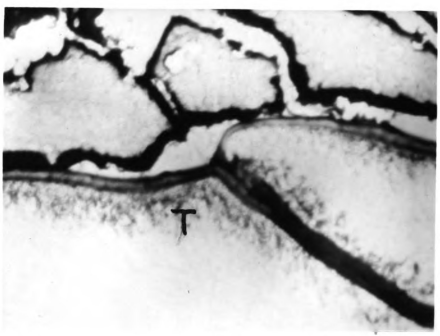
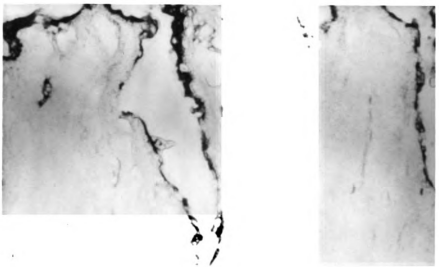


FIGURE 5.



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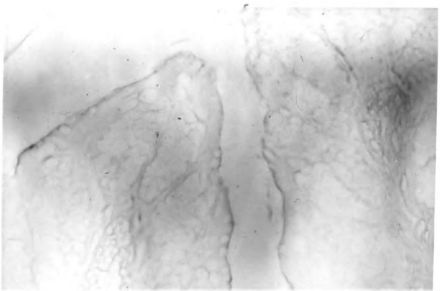

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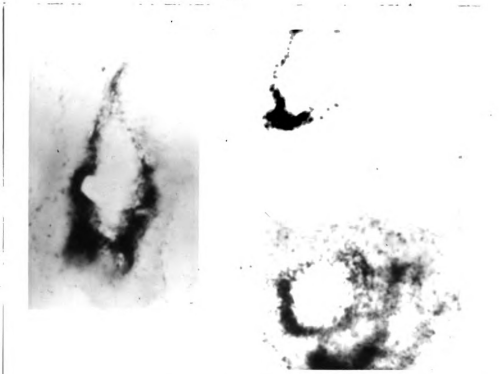

FIGURE 8.

FIGURE 9.

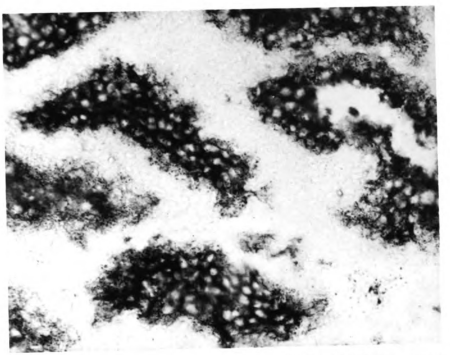

FIGURE 10.

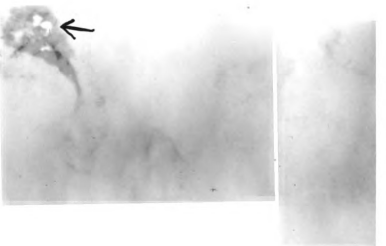

FIGURE 11.

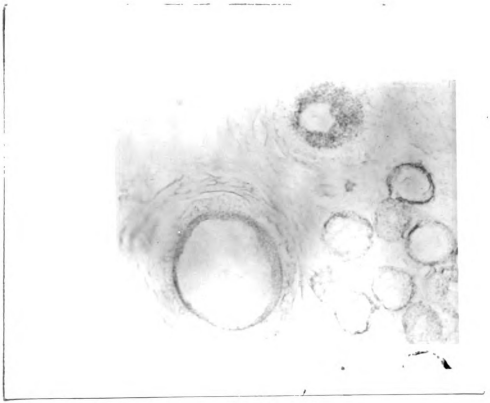

FIGURE 12.

Glucose-6-Phosphatase (G6P)

Stomach: No G6P-activity was observed.

Duodenum: The irregular, non-continuous pattern of distribution was again present for this enzyme (Fig. 13). The greatest concentration of glucose-6-phosphatase relative to the gastrointestinal tract occurred here. The enzyme was concentrated in the distal half of epithelial cells.

Mesenteric Small Intestine: Cellular enzymatic activity varied in distribution but was primarily contained at the apical edge of epithelial cells. Figure 14 demonstrates this pattern of activity and also illustrates fluke (probably Hasstelesia tricolor) eggs with G6P-activity in the shell.

Terminal Small Intestine: G6P-activity in the villi was greatly reduced and present only in isolated outer portions of the villi (Fig. 15).

Cecum: No activity.

Colon: No activity.

Kidney: Glucose-6-phosphatase was concentrated mainly in the proximal convoluted tubules, agreeing with results for domestic rabbits (Wachstein, 1955). Activity was concentrated at the apical portions of the cells.

Testis: Aside from a few scattered areas of activity in the tunica albuginea of an animal in breeding condition (Fig. 16), no sites of activity were found.

Lung: No activity.

Figures 13-16 demonstrate sites of glucose-6-phosphatase (G6P) in cottontail rabbit tissues.

Fig. 13: Glucose-6-phosphatase in duodenum demonstrating the irregular pattern of enzyme distribution. X188.

Fig. 14: G6P-activity in mesenteric small intestine. Fluke eggs (arrow) also stained positive. X188.

Fig. 15: G6P-activity in terminal small intestine showing a greatly reduced enzyme concentration. X188.

Fig. 16: Limited concentrations of glucose-6-phosphatase in the tunica albuginea of a breeding male. No other G6P-activity was observed in the testis. X188.

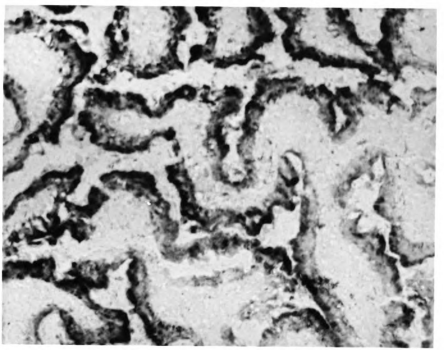

FIGURE 13.

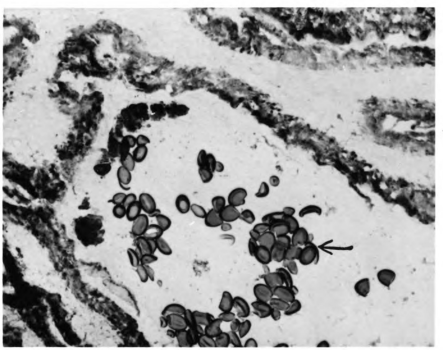

FIGURE 14.

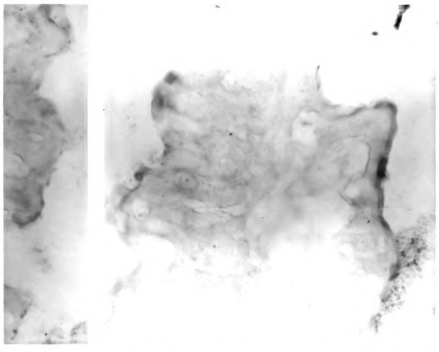

FIGURE 15.

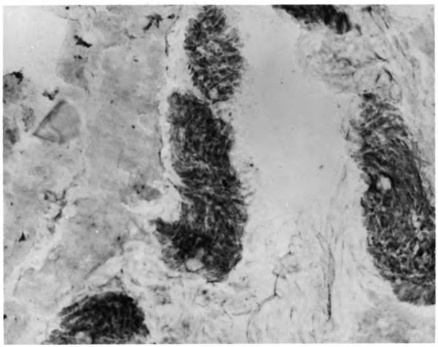

FIGURE 16.

Succinic Dehydrogenase (SD)

Stomach: The deeper gastric glands had the greatest concentration of succinic dehydrogenase. Figure 17 shows SD-activity in what are presumed to be parietal cells. The enzyme was evenly distributed throughout these cells (Fig. 18). Nachlaus et al. (1957) found the parietal cells of dogs and rats to contain succinic dehydrogenase.

Duodenum: The apical portion of the epithelial cells had the greatest concentration of what appeared to be discrete areas of enzyme concentration (Fig. 19). Smaller amounts of enzyme were located at the basal section of the cell. Nachlaus et al. (1957) found this double border of enzyme to be present in dogs. These workers found some SD-activity in the Brunner's glands which was also observed in this study.

Mesenteric and Terminal Small Intestine: The greatest concentration of succinic dehydrogenase was in the apical portion of the cell with fewer areas of enzyme present at the basal edge (Fig. 20). The crypts contained less enzyme than the villi, a finding also observed by Nachlaus et al. (1957) in dogs and rats.

Cecum: This organ had the greatest concentration of succinic dehydrogenase of all regions of the intestine. Within the cells, there was a double border of enzyme (Fig. 21). A distinct concentration of enzyme at the basal edge of the cell was not always present. Sometimes, the enzyme was uniformly distributed throughout the cell.

Colon: The double border of enzyme concentration occurred in the apical and basal parts of the epithelium (Fig. 22), but some areas

of colon had enzyme activity throughout the cell. The enzyme was present at least to mid-colon.

Testis: A faint reaction occurred but only in the apical border of the cells lining the ducts of the epididymis.

Figures 17-22 demonstrate sites of succinic dehydrogenase (SD) in cottontail rabbit tissues.

Fig. 17: Succinic dehydrogenase present in deeper gastric glands. X188.

Fig. 18: Higher magnification of cells of the gastric glands containing succinic dehydrogenase. X750.

Fig. 19: SD-activity in duodenum. Distinct concentrations of enzyme occurred primarily at the apical edge of epithelial cells. Less numerous concentrations of enzyme were present at the basal edge of the cells. X188.

Fig. 20: SD-activity in mesenteric small intestine demonstrating the double border of enzyme at the apical and basal portions of the cell. X188.

Fig. 21: SD-activity of the epithelium of the cecum. X750.

Fig. 22: SD-activity of the colon. X188.

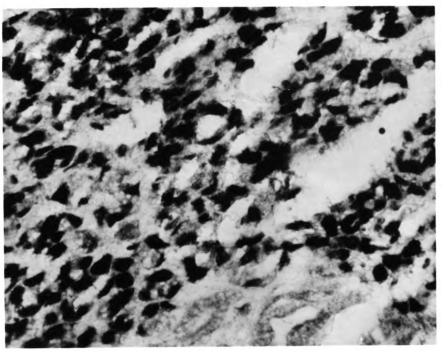

FIGURE 17.

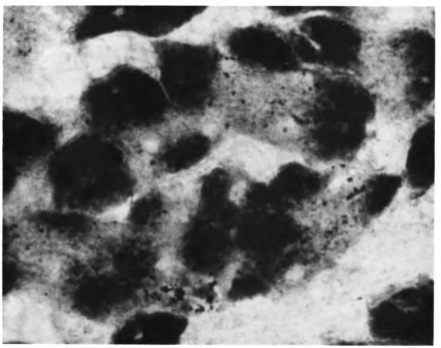

FIGURE 18.

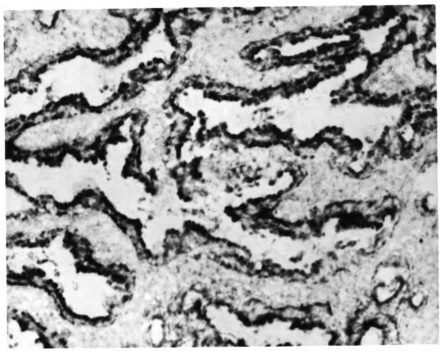

FIGURE 19.

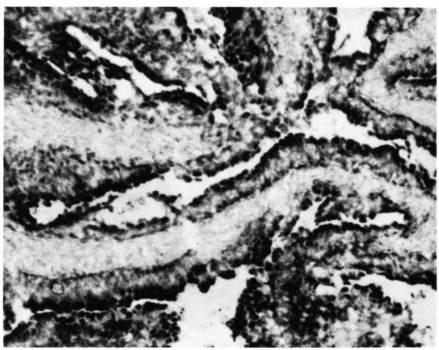

FIGURE 20.

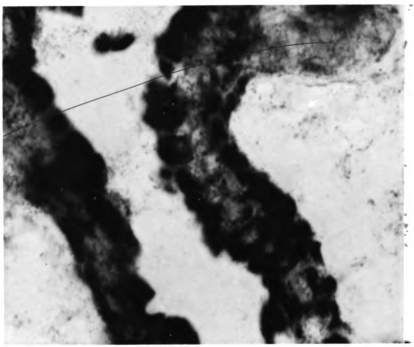

FIGURE 21.

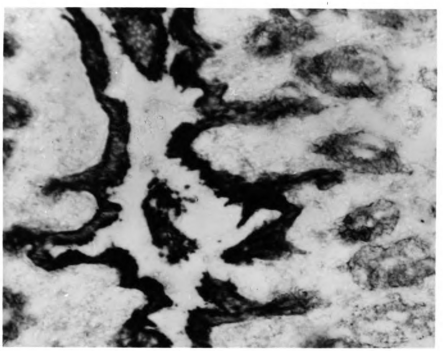

FIGURE 22.

Non-specific Esterase (E)

Stomach: Sites of esterase were scattered (Fig. 23). The greatest E-activity occurred within the deeper pits of the gastric glands just above the muscularis mucosa.

Duodenum: Esterase was concentrated within distinct areas primarily at the apical edge of the epithelial cells. Smaller concentrations of enzyme occurred at the basal edge of the cell (Fig. 24). In Brunner's glands, esterase was scattered (Fig. 25).

Mesenteric and Terminal Small Intestine: A reduced enzyme concentration was observed in the epithelium with some areas showing none at all.

Cecum: Distinct areas of E-activity were primarily located in the apical portion of the epithelial cells (Fig. 26).

Colon: An intense reaction was present in the epithelial cells. Some areas had epithelial cells with the double border of enzyme while other areas did not (Fig. 27).

Kidney: The proximal convoluted tubules were the primary sites of kidney E-activity, a finding also reported in domestic rabbits (Wachstein and Bradshaw, 1965).

Testis: Small amounts of esterase were present in the epithelial cells of the epididymal ducts.

The above results for esterase apply only to samples incubated in media using alpha naphthyl proprionate as substrate. Changes in substrate can alter the sites of location of non-specific esterases (Friedman et al., 1966).

Figures 23-27 demonstrate sites of non-specific esterase (E) in cottontail rabbit tissues.

Fig. 23: Esterase present in deeper glands of the stomach. X188.

Fig. 24: E-activity in duodenum. Distinct areas of enzyme concentration occurred at the apical end and basal edges of the epithelial cells. X469.

Fig. 25: Scattered sites of esterase in Brunner's glands of the duodenum. X188.

Fig. 26: Esterase in the cecum confined mainly to the apical edge of the epithelial cells. X469.

Fig. 27: E-activity of the colon. X469.

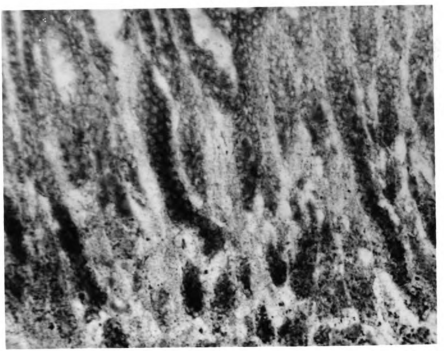

FIGURE 23.

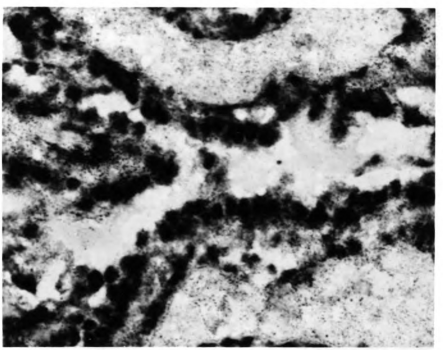

FIGURE 24.

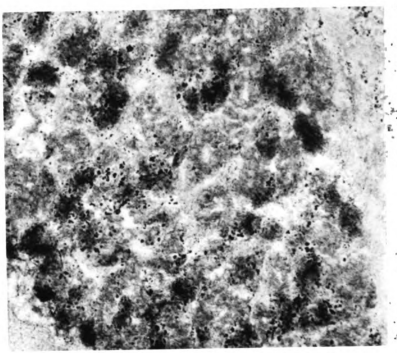

FIGURE 25.

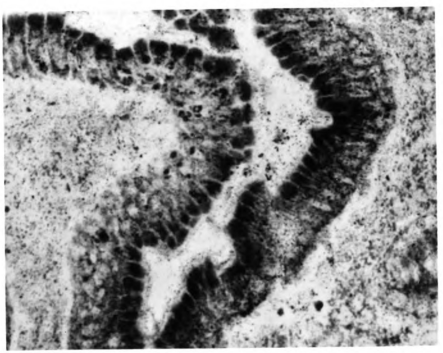

FIGURE 26.

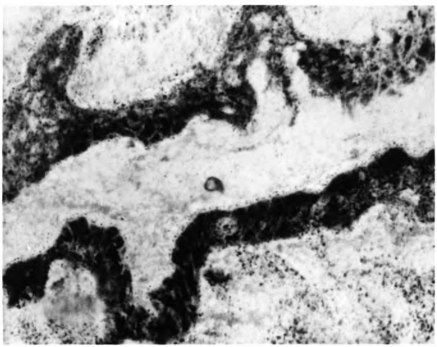

FIGURE 27.

When the sites and concentrations of the enzyme systems in the cottontail were compared (Table 2), differences in patterns of distribution were noted. Both alkaline phosphatase and glucose-6-phosphatase had their greatest activity in the small intestine. Succinic dehydrogenase and non-specific esterase were most prevalent in the cecum with the latter equally common in the duodenum. Jervis (1963) found a similar gradient of alkaline phosphatase concentration in domestic rabbits.

Table 2. Relative concentrations and sites of intracellular enzymes within various cottontail rabbit organs.

Organ	Enzyme System			
	Alkaline phosphatase	Glucose-6-phosphatase	Succinic dehydrogenase	Non-specific esterase
Stomach	(+)	-	+	+
Duodenum	+	++	+	++
Mesenteric small intestine	++	+	+	(+),-
Terminal small intestine	+	(+)	+	(+),-
Cecum	(+)	-	++	++
Colon	(+),-	-	+	+
Kidney cortex	+	+		+
Spleen (lymphocytes)	+			
Liver	-			
Lung	-	-		
Testis (breeding)				
Epididymis	+	-	(+)	(+)
Seminal vesicles	+	-		
Ovarian follicles	+			
Adrenal cortex	(+)			

++ = greatest relative activity
+ = intense activity
(+) = faint or scattered activity
- = negligible activity or none.

Normal Serum Enzymology

A decrease with age in serum alkaline phosphatase concentration was noted when serum enzyme concentrations in adult, subadult and juvenile cottontails were compared (Tables 3, 4 and 5, respectively). Mean serum values for adults and subadults were reduced from the juvenile mean by 67 and 25 percent, respectively. This decrease in enzyme concentration with age may be due to an increased rate of cell turnover in younger animals, releasing greater amounts of intracellular enzyme to the blood.

Adult cottontails had serum alkaline phosphatase determinations also made using the newer Phosphastrate technique (Table 6), and resulted in a mean enzyme concentration identical with that indicated by the Klein-Read-Babson technique (Table 3). The similar accuracy, ease, and rapidity of the Phosphastrate method recommends it as the method of choice for serum alkaline phosphatase determinations.

Inhibition of Serum Alkaline Phosphatase: Parallel serum samples from thirty rabbits were run using the L- and D-stereoisomers of phenylalanine (Tables 7 and 8). The L-form always gave the greatest reduction in enzyme activity. Furthermore, regardless of cottontail age, a similar decrease from normal alkaline phosphatase levels of activity occurred. The reductions from normal enzyme levels for adults, subadults and juveniles averaged 73, 75 and 74 percent, respectively. These large reductions indicate that most of the serum alkaline phosphatase originates in the small intestine because L-phenylalanine is considered to be a specific inhibitor of the intestinal isoenzyme.

D-phenylalanine caused mean reductions of 44, 46 and 49 percent from normal enzyme values for adults, subadults and juveniles, respectively. These were of interest in that the D-form, not considered to be an inhibitor of the intestinal isoenzyme, is considered to be an inhibitor of other alkaline phosphatase isoenzymes (Fishman et al., 1963). But when the percent inhibitions of L- and D-phenylalanine were summed, they totaled over 100 percent, indicating that either one or the other of the phenylalanine forms did not react to the alkaline phosphatase isoenzymes of cottontails as was expected. This suggested that lagomorph enzymology may differ from that of rats and humans which were used by Fishman et al. (1963) in their D- and L-phenylalanine investigations.

Table 3. Normal serum alkaline phosphatase levels for 41 adult cottontail rabbits.

Rabbit weight (grams)	Serum levels (in KRB Units)	Serum levels (in International Units/liter serum)
1020	2.8	29
1035	.8	8
1070	8.6	90
1090	2.1	22
1100	1.3	14
1120	1.3	14
1120	1.7	18
1150	3.4	36
1158	8.3	87
1160	1.2	13
1160	2.1	22
1220	2.2	23
1220	2.1	22
1228	2.4	25
1241	.8	8
1246	.5	5
1253	1.2	13
1257	1.1	12
1264	.4	4
1266	1.4	15
1272	1.6	17
1274	5.7	60
1344	1.1	12
1350	3.4	36
1356	.9	9
1372	4.7	49
1374	1.1	12
1378	1.4	15
1406	1.3	14
1420	4.6	48
1427	3.9	41
1430	.5	5
1447	2.2	23
1450	7.4	77
1452	1.2	13
1460	2.8	29
1480	5.2	54
1485	.5	5
1488	3.3	35
1710	2.0	21
1753	.6	6
Range:	(.4-8.6)	(4-90)
Mean:	2.4	25

Table 4. Normal serum alkaline phosphatase levels for 11 subadult cottontail rabbits.

Rabbit weight (grams)	Serum levels (in KRB Units)	Serum levels (in International Units/liter serum)
520	3.0	31
525	3.3	35
532	12.8	140
589	9.6	100
620	5.9	62
628	6.2	65
663	14.7	154
808	1.6	17
970	2.1	22
980	.5	5
980	1.3	14
Range:	(.5-14.7)	(5-154)
Mean:	5.5	58

Table 5. Normal serum alkaline phosphatase levels for 15 juvenile cottontail rabbits.

Rabbit weight (grams)	Serum levels (in KRB Units)	Serum levels (in International Units/liter serum)
53	9.1	95
113	4.8	50
120	5.9	62
165	5.0	52
172	9.3	97
178	8.1	85
178	8.0	84
264	12.8	140
281	5.7	60
300	5.0	52
313	8.1	85
365	8.3	87
397	7.7	81
415	4.2	44
436	7.7	81
Range:	(4.2-12.8)	(44-140)
Mean:	7.3	76

Table 6. Normal serum alkaline phosphatase levels for 11 adult cottontail rabbits using a newer technique.

Rabbit weight (grams)	Serum levels (in Phosphastrate International Units)
1500	1.2
1385	1.6
1160	1.0
1317	.8
1307	6.7
1300	1.0
1260	1.4
1078	2.4
1585	.9
1090	3.3
1325	6.8
Range:	(.8-6.8)
Mean:	2.4

Table 7. Inhibition of normal serum alkaline phosphatase levels by D- and L-phenylalanine for 13 adult cottontails.

Rabbit weight (grams)	Normal serum levels (in KRB Units)	Serum levels when D-phenylalanine present (in KRB Units)	Serum levels when L-phenylalanine present (in KRB Units)
1100	1.3	.8	.1
1228	2.4	1.2	.6
1257	1.1	.7	.1
1272	1.6	.9	.6
1344	1.1	1.0	.8
1356	.9	.6	.5
1378	1.4	.6	.1
1406	1.3	.9	.6
1427	3.9	1.4	--
1450	7.4	3.5	.5
1460	2.8	.8	.2
1710	2.0	.9	.4
1753	.6	.4	.2

Table 8. Inhibition of normal serum alkaline phosphatase levels by D- and L-phenylalanine for eight subadult and eleven juvenile cottontails.

Rabbit weight (grams)	Normal serum levels (in KRB Units)	Serum levels when D-phenylalanine present (in KRB Units)	Serum levels when L-phenylalanine present (in KRB Units)
Subadult			
520	3.0	1.5	.9
532	12.8	7.3	3.6
589	9.6	5.3	2.0
620	5.9	1.6	.8
628	6.2	1.9	.8
663	14.7	13.1	5.2
808	1.6	.8	.6
970	2.1	1.5	.5
Juveniles			
172	9.3	6.3	--
178	8.1	4.6	2.6
178	8.0	5.0	1.9
264	12.8	8.1	3.5
281	5.7	3.3	1.8
300	5.0	2.0	1.0
313	8.1	4.2	1.9
365	8.3	5.1	2.6
397	7.7	3.5	1.4
415	4.2	1.8	1.2
436	7.7	4.8	1.5

Coccidial Infections

Seventy-two of 83 (87%) living, wild cottontails were found to be infected with Eimeria spp. This percentage was considered to be an underestimate because the majority of the non-infected animals were nestlings with less opportunity for infection due to their limited mobility.

Although all the adult and numerous non-adult rabbits found dead had coccidia present, only one animal was believed to have died from coccidiosis. This was a 13-day old cottontail and it had small intestinal ulcers containing coccidial forms. No other lesions were found and an examination for pathogenic micro-organisms yielded negative results.

Only three references to cottontail mortality from coccidiosis were found in the literature. McGinnes (1958) recorded an adult death, and Hagen (1942) and Ecke and Yeatter (1956) each reported one juvenile death. The latter authors reported "diarrhea, rapid loss of weight and partial paralysis" in a 13-day old animal. Morgan and Waller (1940) did not report coccidiosis mortalities, but found a "severe acute catarrhal enteritis" present in six cottontails which were infected with unidentified coccidia. A mild case of coccidiosis in an immature cottontail was described by Smith and Cheatum (1944).

Although flotation of ingesta in glass-wells was previously described (see Materials and Methods) as the most valid method for finding the oocysts of coccidia, ingesta samples from various intestinal sites in several necropsied cottontails were examined for coccidia by

direct smears (Table 9). The direct smear is the fastest and most practical technique available for diagnosing coccidial infection. Since it has been and will continue to be widely used in coccidial investigations, regardless of the host species involved, I felt additional study of the direct smear was justified.

The first two animals in Table 9 did not have direct smears made of their ingesta. These animals were found to be infected when their intestines were histologically examined. They were included here to demonstrate that the early stages of coccidiosis can be diagnosed only by histological examination of the intestine. The three-day old cottontail was the youngest found to be infected with coccidia (Fig. 28). In the second animal, mature oocysts were already present in the lumen of the small intestine. Since it requires at least six days for oocyst formation (Carvalho, 1943), this eight-day old animal must have become infected on the second or third day of life. This rabbit, furthermore, was the youngest found with fecal pellets in the stomach indicative of coprophagy.

Assuming production of oocysts, fecal pellets offered the best possibility of finding coccidia by direct smear. No false (0) results were recorded for the feces as occurred in both small intestinal and cecal samples. These false results were designated as such only after an ingesta sample, shown by direct smear not to contain oocysts, was proven positive for coccidia by glass-well flotation. Most of the false findings occurred in the small intestine where intracellular pre-oocyst forms predominate, and in unstained smears, can be taken for

Table 9. Relative oocyst numbers present in cottontail ingesta samples taken from the small intestine, cecum and fecal pellets within the colon (direct smear).

Animal number	Age in days: based on known date of birth	Small intestine	Cecum	Fecal pellet in colon
6611-0	3	Present*	-	None
672-LR	8	Present*	-	None
6517-UL	22	+++	++++	-
6518-LR	23	++	++++	-
668-0	26	-	++++	++++
6610-UL	27	-	+++	++
664-LR	28	-	++++	+++
658-LR	29	-	++++	++++
6517-LR	29	+++	+++	-
6517-0	29	+++	++	-
658-0	30	++++	++	-
655-BT	31	+++	+++	++++
6518-UL	38	0	0	++
658-UR	40	++++	++++	-
655-0	45	+	++	-
6511-UR	49	-	0	+++
665-0	51	+	++	-
6516-0	52	0	+++	-
6517-LL	55	++	++++	-
6514-UR	57	++++	+++	-
656-LR	59	+	+	-
656-UR	59	++++	++++	-
656-0	62	0	+	-
656-BL	69	++++	++++	-
657-UL	80	++++	+++	-
657-LL	85	++	+	-
658-UL	91	0	++	-
658-BT	91	++	++	-
657-0	100	++++	++++	-
655-UL	121	++++	++	-
665-UL	123	++	++	-
04	Adult	-	+	+
19	Adult	-	++	+

* = histological examination.
0 = no oocysts; a false diagnosis of the sample shown to be positive by glass-well flotation.
\+ = 10 or less oocysts/low power (X100) field.
++ = 11-25 oocysts/low power (X100) field.
+++ = 26-50 oocysts/low power (X100) field.
++++ = high power (X440) samples required.
\- = not examined.

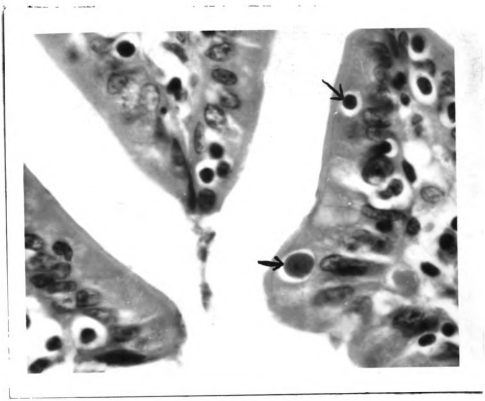

Figure 28. Intracellular coccidial forms (arrows) present in the small intestine of a three-day old cottontail. HE. X750.

epithelial cells. Intestinal sections cut an inch apart, furthermore, had numerous coccidia in one section, but were void of the parasite in the other. False cecal findings were believed to be due to inherent error present in the direct-smear technique.

When other reports of cottontail coccidial infections were examined, the highest percentages of infection were in those studies employing flotation of ingesta within some type of flotation chamber. The present study, however, was the only one using the wells previously described. Thus, Morgan and Waller (1940), Herman and Jankiewicz (1943), Ecke and Yeatter (1956) and Dorney (1962) reported, for populations of S. f. mearnsii, coccidial infections of 100, 98, 91 and 94 percent, respectively. Dorney's 94% was the mean of seven separate samples of 20-25 rabbits each, with four samples having infection rates of 100 percent.

Clancey et al. (1940) found 63% of their cottontails infected. Their samples were obtained from the end of a glass rod dipped into a centrifuged solution of concentrated sugar and feces. Although their technique could be considered a flotation of sorts, it was not here so considered. In the present study, separate trials using the technique employed by Clancey et al. (1940) resulted in several false (0) findings. Hensler (1959) reported a 51% infection rate, but used direct smears of small intestinal and cecal contents, a method judged here to result in occasional false reports for infected animals (Table 9).

Daily Fluctuations in Fecal Oocyst Numbers

Seven adult cottontails, caged indoors, had daily fecal counts made by glass-well flotation. The results for three of these animals

are diagrammed (Fig. 29). Data for Nos. 09 and 65 demonstrated the pattern of oocyst elimination found in six of the seven animals with normal oocyst levels of elimination periodically disrupted by periods of extreme oocyst elimination (showers). Rabbit No. 10 never demonstrated a shower period, but gradually eliminated its coccidia, no oocysts being found in its last 22 fecal samples. Because no Eimeria patency period exceeds three weeks (Carvalho, 1943), a continuous re-infection must have occurred in the other six cottontails since their infections were continuous beyond 70 days.

The degree of oocyst elimination fluctuated (Table 10). Oocyst counts were separated into a pre-shower, shower and post-shower sequence (insert, Fig. 29). A pre-shower count referred to the lowest oocyst tally just prior to a shower period. The post-shower period began with a return of oocyst numbers to approximate pre-shower levels or lower. The most complete sequences were found for Nos. 09 and 02. The latter had a shower period in each of three successive months which varied in their intensities. The 640,000 oocysts/gram feces of No. 02 was the highest recorded for any animal, almost 50 times its previous day's total. Oocyst numbers of over 240,000 per gram feces were recorded by Dorney (1962). The relatively high pre- and post-shower counts of No. 06 are an incomplete sequence, but were the only figures available for the animal.

The largest increase from a pre-shower oocyst level to a subsequent shower count was found for No. 64 (December) where the shower count was almost 115 times greater. The termination of showers also

Figure 29. Daily fecal oocyst counts for three adult cottontails caged indoors (glass-well flotation).

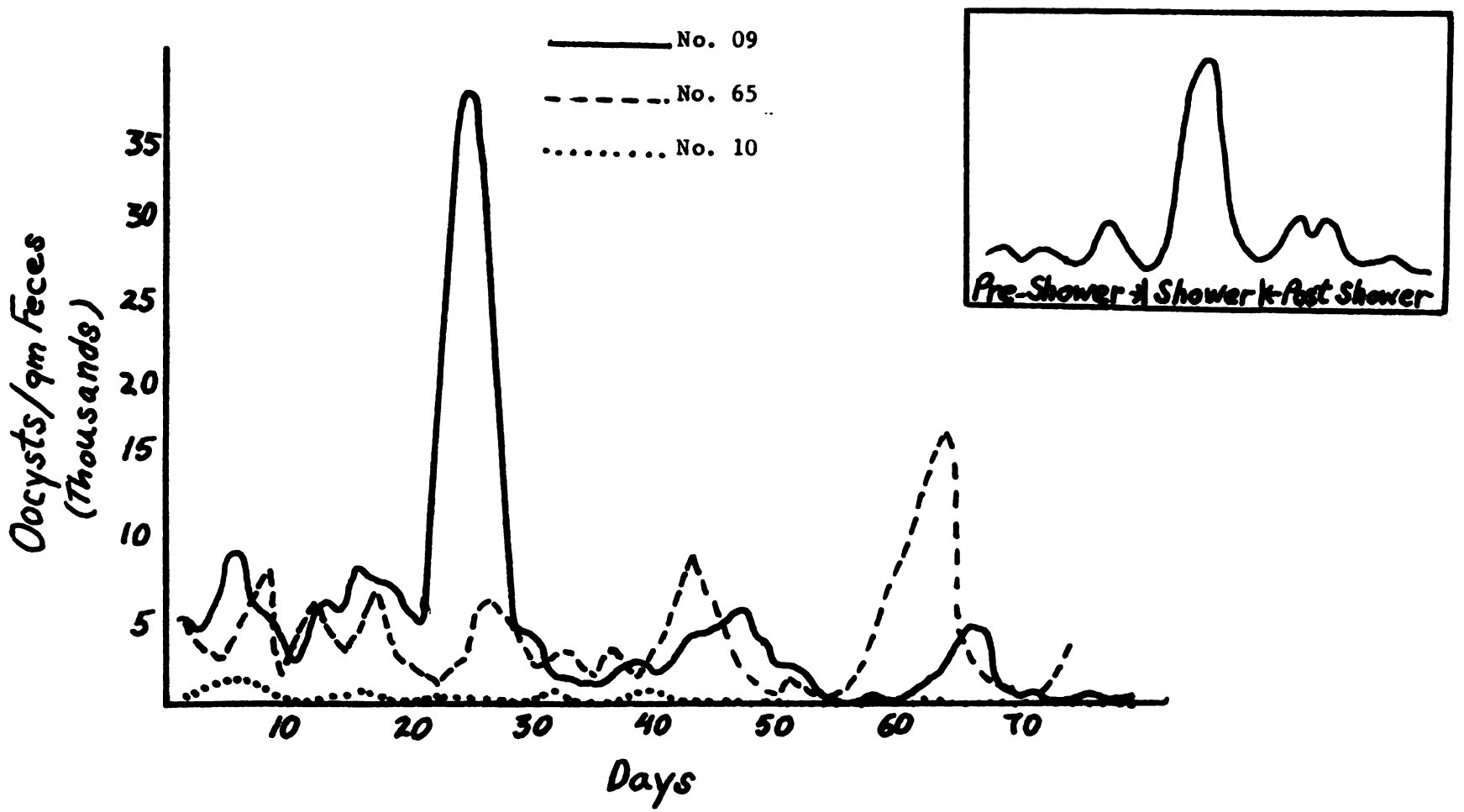

Table 10. Duration and intensity of oocyst shower periods for adult cottontails caged indoors (glass-well flotation).

Rabbit number	Weight (g)	Month of sample	Pre-shower count (oocyst nos/g feces x 10^3)	Days after pre-shower count (oocyst nos/g feces x 10^3)											
				1	2	3	4	5	6	7	8	9	10	11	12
02	950	July	14	640	314	156	106	101	19*	--	0.8				
		August	3	13	26	14	7	2*							
	1200	September	6	117	--	--	--	176	13	23	15	--	--	2*	
04	1380	October	0.8	13	--	24	3*								
06	1020	August	13	58	--	--	--	103	76	36*					
64	1450	November	0.8	--	--	11	34	--	37	--	--	--	2*		
		December	2	--	--	--	--	231	--	183	--	172	--	--	1*
09	1150	February	7	13	25	33	35	16	18	3*					

* initial post-shower count.
-- no data available.

occurred rapidly. In three days, the oocyst count of No. 64 dropped from 170,000 to 1,000.

Using only the fairly complete sequences of Nos. 02 (July and August), 04 and 09, the duration of oocyst showers averaged five days (Table 10). The December sequence of No. 64 also suggested a shower length of at least five days, while the shower period of No. 06 may have lasted seven or more days. Oocyst showers occurred throughout the year, a finding supported by Dorney (1962).

The significance of oocyst showers is not understood, but these should be included in any sampling of coccidia in cottontails. Weekly estimates of the level of coccidiosis in cottontails have been made based on fecal oocyst counts (Yuill, 1964). But weekly samplings can miss the shower periods. The daily pattern of oocyst elimination for a single cottontail was plotted and had superimposed on it the graphs which would have resulted had fecal samples for that specific cottontail been taken only every fifth or seventh day (Fig. 30). Fecal samples taken just once a week would have missed the shower period demonstrated by either the daily or every-fifth day samplings. Because oocyst showers last at least five days, fecal samplings of oocyst numbers should not be separated by more than this period of time if the shower period is to be demonstrated.

Identification of Coccidia

Eimeria species identification was based on the work of Carvalho (1943). In Table 11, his original tables are reduced to include only those characteristics which I found most useful. Even with the

Figure 30. Coccidial levels of infection for a single cottontail as determined from daily fecal samples. Every-fifth day and weekly graphs derived from the daily counts (glass-well flotation).

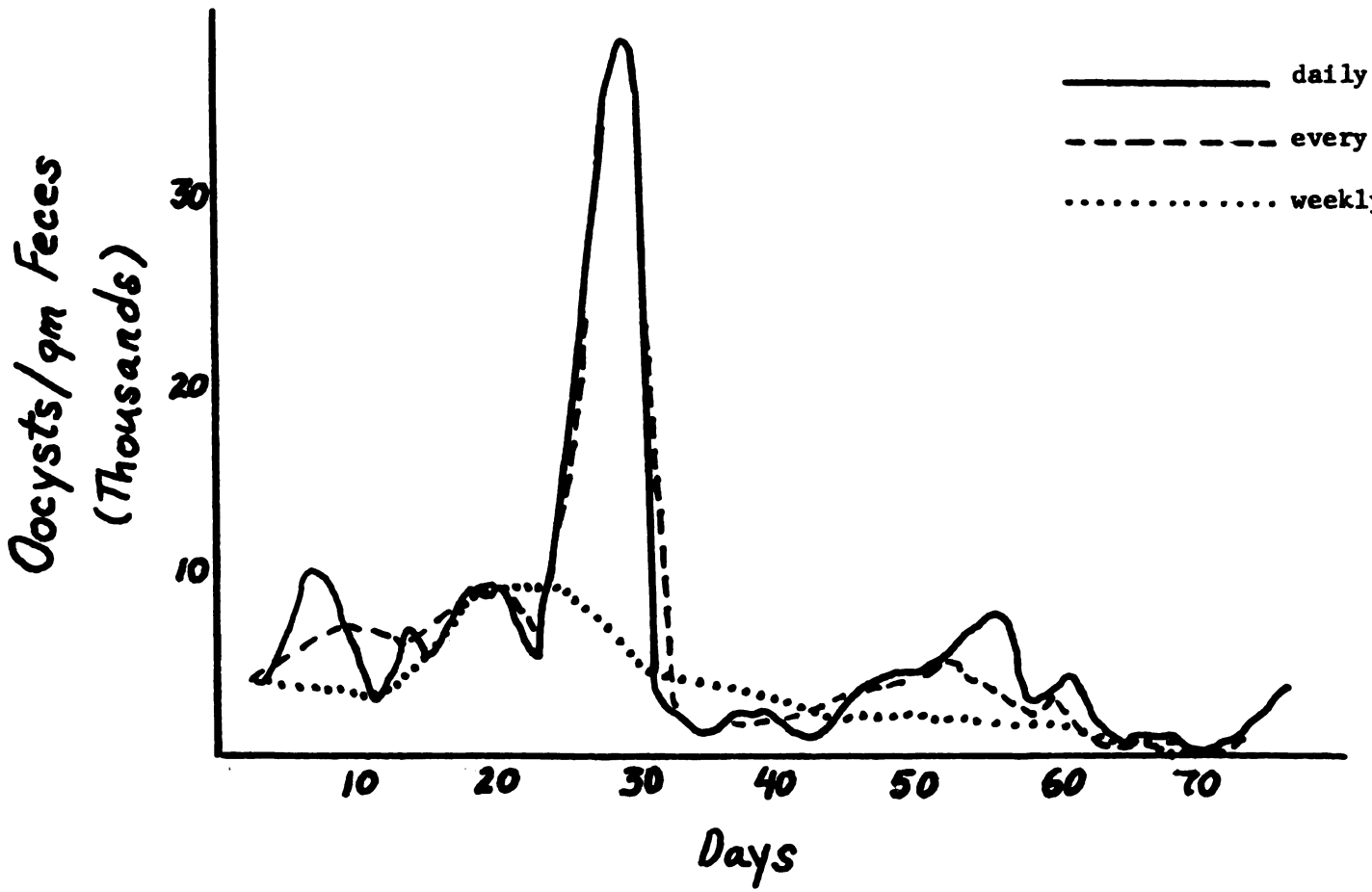

Table 11. Taxonomic characteristics found useful in the identification of cottontail Eimeria (based on Carvalho, 1943).

Characteristic	E. environ	E. media	E. neoleporis	E. sylvilagi	E. maior
Shape index (L/W)	1.38*	1.60	1.95	1.60	1.60
Length (in microns)	20-33 mean: (26)	27-36 (31)	33-44 (39)	23-31 (29)	44-57* (48)
Width (in microns)	14-23 (19)	15-22 (19)	16-23 (20)	15-20 (18)	25-35* (30)
Oocyst residual body	none	small, distinct*	none	none	present
Sporocyst residual body	none	small, sometimes granular	large, one-third of sporocyst*	granular	small
Micropyle	present with protruding cap*	present, extends far back	present	present, wide*	small, shrunken into wall.
Oocyst wall	smooth, uniform thickness	thinner at micropyle	thickened at micropyle	thickened at micropyle	brownish,* striated

* considered outstanding characteristics by this author.

combination of characteristics available, problems of species identification did require an occasional subjective choice to be made. Eimeria maior need not have been included in the table since its large size and unique cell wall (Fig. 31) identifies the species without oocyst sporulation. The characteristics in Table 11 are listed in what was considered to be a declining order of importance, the shape index offering the quickest initial species grouping.

In order to determine what difficulties were involved in the identification of cottontail Eimeria, the feces from five rabbits were prepared for oocyst sporulation and the oocysts identified. Forty percent of the oocysts were E. sylvilagi, 37% were E. media, and 23% were E. environ. Although not found in the above samples, E. neoleporis was also identified from other fecal samples.

Some idea of this problem of Eimeria identification is presented (Table 12) and lists three species of coccidia common to this study and Carvalho's (1943). The oocyst measurements of E. sylvilagi and E. media were similar in both studies, but the oocysts of E. environ were not. Thus, the coccidia listed here as E. environ while not completely agreeing with Carvalho's (1943) descriptions had to be so identified since these oocysts could not have been any other known species of Eimeria.

The only Eimeria species sought in all sampled rabbits was E. maior because sporulation was not required for its identification. This species is generally considered uncommon (Dorney, 1962), but Carvalho (1943) found 13% of his rabbits so infected. Hensler (1959)

Figure 31. The striated oocyst wall unique to Eimeria maior. Unstained, in sugar solution. X1875.

Table 12. *Eimeria* oocyst measurements for coccidia species reported both in this study and by Carvalho (1943). All measurements based upon a minimum of 50 samples.

Species	This Study					Carvalho				
	Length* Range (microns)		Width* Range (microns)		Shape index	Length* Range (microns)		Width* Range (microns)		Shape index
Eimeria sylvilagi	27	(22-32)	16.5	(14-19)	1.65	29	(23-31)	18	(15-20)	1.60
Eimeria media	29	(21-32)	17	(13-19)	1.70	31	(27-36)	19	(15-22)	1.60
Eimeria environ	23	(16-26)	15	(12-19)	1.45	26	(20-33)	19	(14-23)	1.38

* mean values.

for Michigan and Stringer (1966) for North Carolina, while not specifically identifying E. maior, recorded oocyst sizes and descriptions that could be no other known species. Morgan and Waller (1940) for Iowa and Herman and Jankiewicz (1943) for California also described the species.

E. maior was found in 18% of all cottontails examined in this study. Four adults and six orphaned nestlings were found infected with E. maior with a pure, heavy infection occurring in a month old juvenile. Two of the adults produced three litters totaling 11 young, seven of which developed E. maior infection suggesting a maternal source for an animal's initial exposure to Eimeria. Not all nestlings, however, obtained their initial coccidia from the mother. One E. maior-infected nestling was from a doe in which E. maior was not identified from over 50 fecal samples over a two year period.

Another problem involving identification of cottontail coccidia concerns the status of Eimeria steidae. This coccidial species which invades the liver is not considered natural to cottontails (Carvalho, 1943), but various reports exist of its presence in cottontails. Hensler (1959) for Michigan and Stringer (1966) and Harkema (1936) for North Carolina, reported E. steidae infections, but identified the species from hepatic lesions in the rabbit. But, the only valid technique for Eimeria identification depends upon oocyst morphology. To date, only one confirmed case of E. steidae has been reported which used oocyst morphology for identification (Honess, 1939 and pers. comm.). Since artificial cross-infections of E. steidae from domestic rabbits to cottontails have been successful (Jankiewicz, 1941; Herman

and Jankiewicz, 1943), it is possible for cottontails to have natural infections of E. steidae. But, care should be taken not to report the presence of this coccidial species in cottontails unless oocyst morphology was used to identify the species.

Intracellular Identification of Coccidial Forms

Over 40 coccidial forms per intestinal villus were counted (Fig. 32). Mature oocysts were found adjacent to younger sexual forms. Coccidia were occasionally found as deep as the crypts but, in most instances, were observed in the distal half of the villi.

Figures 33-39 record the intracellular development of coccidia beginning with the schizont stage (see Appendix A), on through the sexual phase and ending at final maturation of the oocyst. Most notable in the development was the change in oocyst wall structure from an initial wall of large, peripheral, eosinophilic granules, to one of a smooth homogeneous structure.

While examining the intracellular coccidial forms stained with a routine hematoxylin and eosin stain, a property unique to E. maior was discovered. In all Eimeria species, except E. maior, the schizont, microgamete and macrogamete stages and oocysts were always basophilic. Whenever E. maior was present intracellularly (as determined from fecal diagnosis), its oocysts, even when immature, were eosinophilic. Thus, of the five Eimeria species identified in this study, E. maior was identifiable intracellularly even when present in pre-oocyst form. This finding suggests the possibility that specific staining properties may exist for all Eimeria species and bears further investigation.

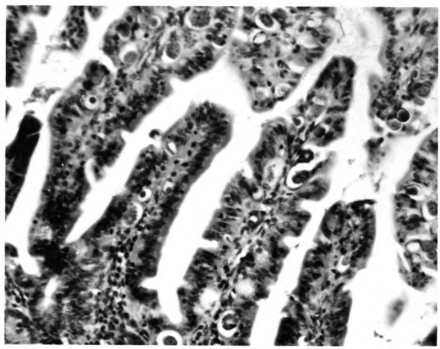

Figure 32. A section of cottontail small intestine containing large numbers of coccidia in various stages of development. HE. X188.

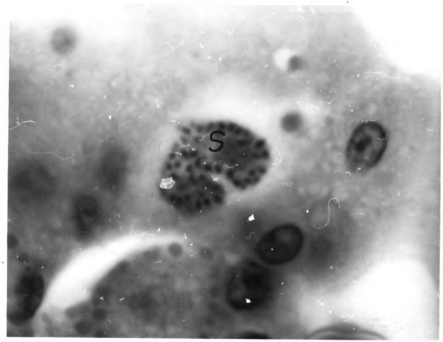

Figure 33. A schizont (S) located in a small intestinal villus. HE. X1875.

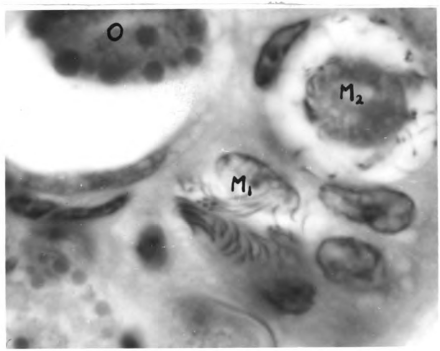

Figure 34. Tissue section of the small intestine showing: a maturing oocyst (O); microgametes (M_1); a possible macrogamete (M_2) surrounded by microgametes. HE. X1875.

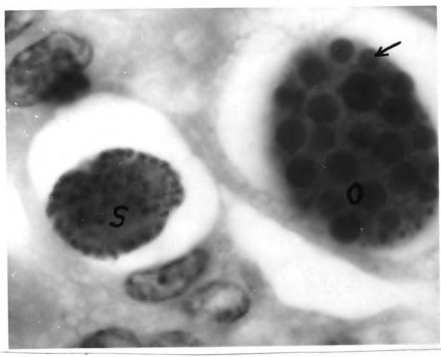

Figure 35. A schizont (S) adjacent to an early developing oocyst (O). The eosinophilic granules which make up the oocyst wall are evident (arrow). HE. X1875.

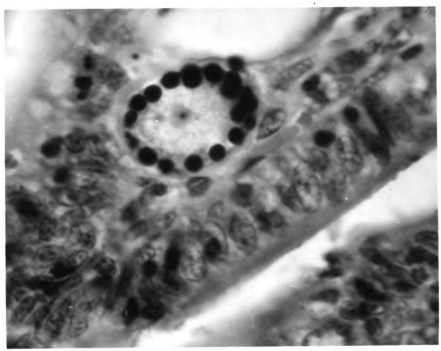

Figure 36. A developing oocyst demonstrating the peripheral arrangement of the eosinophilic granules of the cell wall. HE. X1875.

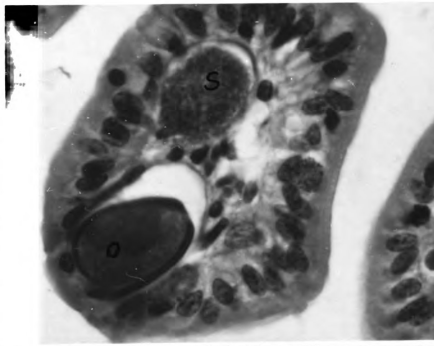

Figure 37. Small intestinal villus containing a schizont (S) and nearly-mature oocyst (O). The latter's cell wall is more developed and does not show granules. HE. X1875.

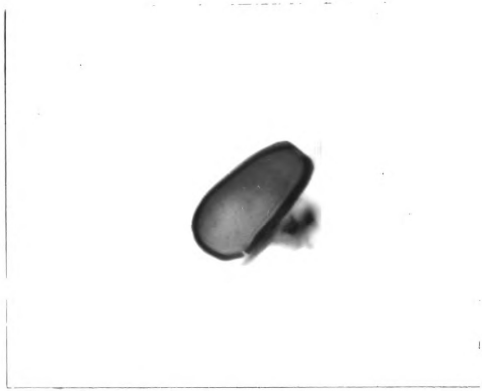

Figure 38. A mature oocyst extruding from the tissue. HE. X750.

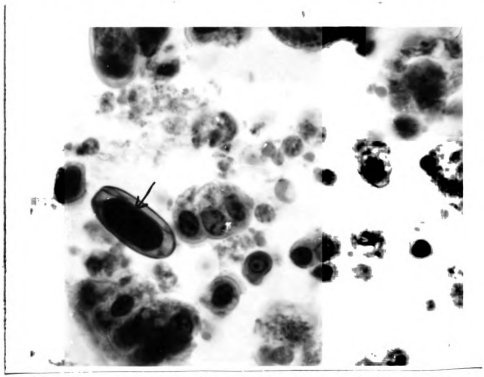

Figure 39. An oocyst lying free in the lumen of the small intestine. The centrally located zygote (arrow) is clearly seen. Iron Hematoxylin. X750.

Should such be the case, the aid to coccidial studies would be invaluable.

Experimental Infections

To determine whether cottontails can be artificially infected with coccidia, rabbits of different ages were infected with stock solutions of sporulated oocysts. Stomach intubation successfully infected older animals, but oral innoculation via eye-dropper had to be substituted for nestlings. Otherwise, the small tubing required for nestlings invariably caused esophageal perforations. Successful infections were determined by fecal oocyst counts from the time of infection to eight days later. Since all animals used had very low levels of coccidia, an oocyst shower on the sixth through eighth day with subsequently higher coccidial numbers was considered evidence of a successful infection.

Additional Observations of Parasites: In addition to unidentified ticks and fleas, ectoparasites found included warbles (Cuterebra spp.) and the flesh fly (Wohlfahrtia vigil). Endoparasites observed, grossly or microscopically, included the intestinal fluke (Hasstilesia tricolor; Trematoda), tapeworms (Cittotaenia spp.; Cestoda), the bladderworm Taenia pisiformis (Cestoda) within the abdominal cavity and the stomach worm Obeliscoides cuniculi (Nematoda). Fecal flotations revealed the eggs of the pin worm Passalurus ambiguus (Nematoda), nematode eggs which could have been either a Trichostrongylidae spp. or Obeliscoides cuniculi, and tapeworms of the family Anoplocephalidae.

Penned Cottontails

Breeding in Captivity

Since young rabbits were required for experimental Eimeria infections, a breeding colony of eleven females and seven males was established. Diagnosis of pregnancy by palpation of the female's abdomen was not possible until the end of the second week of gestation although Casteel (1967) was able to do so 8 days post-coitus.

Most of the litters produced from the yearly pairings of the rabbits (Table 13) were due to matings of a single male, No. 36, and two females, Nos. 04 and 05. These three rabbits were captive since 1964 and became acclimated to captive conditions. But female No. 65, also captive since 1964, had only limited breeding success. Although almost 30% of all pairings were successful, the fact that most female cottontails are bred right after parturition (Conaway, Wight and Sadler, 1963; Casteel, 1966) was not taken into account. Thus, the breeding system used here did not allow normal social interactions to occur. This could have accounted for most of the breeding failures since captive conditions per se are not believed to influence normal breeding patterns (Marsden and Conaway, 1963). The relative higher percentage of breeding success in 1967 was due to the fact that the pairings were made only during the early part of the breeding season. Allen (1938), Beule (1940) and Ecke (1955), all found that the earlier part of the breeding season produced the greatest number of litters.

The mean gestation time for 13 litters was 29.1 days. The unknown gestation periods were the results of not being able to locate

Table 13. Reproduction in penned cottontails. Females remained for two days with the male(s) during each pairing.

Year	Rabbit No. Female	Male	No. times paired	Number litters	Number young	Gestation (days)
1965	04	36	3	2	6	28
					6	29
	05	12	2	1	6	?
		36	2	0		
	09	36	2	0		
	65	12	4	1	3	29
		36	1	0		
	66	36	4	1	8	29
	Sub-totals		18	5	29	
	Percent breeding success: 28					
1966	04	36	3	2	6	28
					4	28
		13	3	1	6	31
	05	36	4	2	4	29
					4	30
		13	2	1	at least 1	30
	09	36	2	0		
		13	5	1	5	28
	16	36	2	1	4	30
		13	2	0		
	17	36	1	1	5	
		13	2	0		
	65	36	4	1	aborted	?
		13	2	0		
	66	36	4	1	at least 3	29
	Sub-totals		37	11	42	
	Percent breeding success: 29					
1967	04	36, 25	2	0		
	20	18, 13	2	0		
	22	36, 25	2	1	3	?
	27	48, 49	2	2	5	?
					5 (aborted)	?
	46	49	1	1	4	?
	Sub-totals		9	4	17	
	Percent breeding success: 44					
	TOTALS:		64	20	88	

the newborn. Even though each pen was only 300 square feet, occasionally and even with the assistance of several helpers, nests were missed. Young known to have been born were not found until a few days later.

In 17 litters with known numbers, the mean young per litter was 4.9. The breeding data of this study are listed (Table 14) with other reports of cottontail gestation, litter size and numbers of litters per year. In general, the yearly number of litters is larger in more southern states. A reduced litter size with decreasing latitude, as theorized by Lord (1960) and Barkalow (1962) is evident except for this study and Rongstad's (1966a). Both of these latter reports counted nestlings instead of deriving litter size from embryo counts as did the others, thus accounting for the smaller figures. In any case, comparisons of litter size should heed the suggestions of Conaway, Wight and Sadler (1963) who concluded that the age of the females and chronological sequence of litters influence litter size. In regard to litter sequence, the largest numbers of young are found in the first two litters of the year (Sheffer, 1957a; Rongstad, 1966a).

Months with the greatest numbers of litters and young were April through July, May being the peak month (Table 15). Earliest conceptions occurred in March, confirming the Michigan data of Haugen (1942) and Wisconsin findings of Rongstad (1966a). These observations when combined with the early February conceptions reported in Missouri (Marsden and Conaway, 1963; Conaway, Wight and Sadler, 1963), indicate a north-south gradient for initial conception date for cottontail rabbits.

Table 14. Listing of reports on cottontail rabbit gestation, litter size and number of litters per year according to latitude.

Location	Authorities	Gestation (days)	Litter size	No. litters per year
Wisconsin:	Hines (1956)	-	6.0	-
	Rongstad (1966a)	-	4.9	2.9
Michigan:	Allen (1938)	about 1 mo.	5.1	2.3
	Haugen (1942)	29-30	5.4	4
	Present study (1969)	28-31 (29.1)	4.9	4*
Pennsylvania:	Beule (1940)	-	5.4	-
Illinois:	Ecke (1955)	about 30	5.6	3-5
	Lord (1963)	about 1 mo.	5.3	-
Maryland:	Sheffer (1957a)	-	4.8	5**
Missouri:	Marsden & Conaway (1963)	26-28	-	-
	Conaway, Wight & Sadler (1963)	-	5	7-8

* the maximum number possible based on first conception date of mid-March and last conception data of mid-September.

** my calculation, based on Sheffer's data of 4.8 young/litter and a mean of 24 young per female.

Table 15. Monthly ratios of successful pairings to total numbers of pairings for penned female cottontails.

Month*	Female Specimens 04	05	09	16	17	20	22	27	46	65	66	Monthly ratios	Percentage success
February	0/1	0/1								0/1		0/3	0
March	0/2	1/1	0/2							0/1	0/1	1/7	14
April	1/2	1/1	0/1			0/1				0/1	0/1	2/7	29
May	1/2	0/1	1/1	1/1			1/2			2/2	1/1	7/10	70
June	1/1	2/3	0/1	0/1	1/1	0/1				0/2	1/1	5/11	45
July	2/2	0/1	0/1	0/1	0/1	0/1		2/2	1/1	0/2	0/2	5/14	36
August	0/1	0/1	0/2	0/1	0/1					0/2	0/1	0/9	0
September		0/1	0/1								0/1	0/3	0
Totals for individual females	5/11	4/10	1/9	1/4	1/3	0/3	1/2	2/2	1/1	2/11	2/8		
Percentage success	45	40	11	25	33	0	50	100	100	18	25		

* refers to conception date.

Breeding by the captive adults stopped in August which agreed with the findings of Lord (1958) and Negus (1959) that late summer litters of wild cottontails are due to subadult breeding. Lord (1958) considered subadult breeding to account for 20% of the annual cottontail population in Illinois, and Negus (1959) found 42% of subadult cottontails in Ohio to be reproductively active. The lack of adult breeding in pens after August indicates that late summer breeding in Michigan may be done by young of the year.

The latest wild litter of cottontails previously reported in Michigan was found for early September (Haugen, 1942). But, a letter received by the Department of Natural Resources (Rose Lake Pathology Laboratory, 1967) reported several wild litters found for early October in southeastern Michigan. Last litters were reported by Carson and Cantor (1963) for early October in West Virginia, for mid-August in Missouri by Marsden and Conaway (1963) and for early September in Wisconsin by Rongstad (1966a).

Nestling Development

Weights and hind foot lengths were measured in ninety-four young from 23 litters (Table 16). Thirty-seven were born in captivity and were of known age. The remaining young were acquired as orphans during their first few days of life. The data derived from the known-age penned nestlings were used as a basis for determining the age of these orphans. Largest sample sizes were in the age classes less than two weeks old because after this time the nestlings were used in other experiments. The sample

Table 16. Weight and hindfoot measurements for cottontail nestlings.

Age (days)	Number of young	Mean weight (grams)	Weight range	Mean hindfoot length (mm)	Hindfoot range
1	24	28	(23-34)	20	(18-22)
2	22	40	(31-46)	22	(20-24)
3-4	23	53	(34-66)	26	(21-31)
6-7	6	64	(55-67)	31	(29-32)
8-10	30	76	(51-96)	36	(30-40)
14-15	15	96	(76-115)	44	(39-50)
30	7	219	(200-257)	58	(52-65)
60	2	448	(381-515)	78	(75-80)

size totals over 94 because some of the litters were used in more than one age class.

Body weight (Table 17) was not judged to be the best criterion of nestling age because of overlaps between classes. Instead, the length of the hind foot offered a more constant and distinct measurement. The observed daily increase in foot length of 2 mm was also reported by Beule and Studholme (1942). Ecke (1955), however, found the hind foot to gain about 1 mm per day. For ease of comparison, this study's measurements were listed with similar data from other reports (Table 17). Once again, hind foot measurements for all reports had less overlap of values between the age classes than did body weights.

Further evidence that body weight is a poor measurement of nestling age was shown by data of consecutive daily weight gains for naturally-weaned nestling littermates (Table 18). Values from day 17 on are for weaned juveniles. The wide variation of daily weight gained for the same animal and between animals was evident, even for weaned animals. But, these variations could have been due to the stress induced by daily handling of the animals. A more uniform weight gain between nestling littermates resulted when the measurements were made for a single 24-hour interval (Table 19). It would be of interest to know whether these weight differences between nestling littermates are indicative of their relative adult weights.

Littermate weight differences are examined further (Table 20) where the differences in weight between the smallest and largest

Table 17. Mean and extreme measurements of nestling cottontails in this and other studies.

	Body weight (gm)	
	Present study	Other studies*
Day 1	28 (23-34)	35-45[3]; 33[7]; 26[4]; 25[2]
Day 2	40 (31-46)	46[1]
Day 6-7	64 (55-67)	73[1]; 63-58[4]
Day 30	219 (200-257)	159[4]; 200[5]; about 300[6]
Day 60	448 (381-515)	400[5]; over 500[6]; 530[4]**

	Hindfoot size (mm)	
	Present study	Other studies*
Day 1	20 (18-22)	20[2]; 22[3]
Day 2	22 (20-24)	23[2]
Day 3-4	26 (21-31)	25-27[2]
Day 6-7	31 (29-32)	31-33[2]

* 1 Allen (1938)
2 Beule & Studholme (1942)
3 Ecke (1955)
4 Lord (1963)
5 Petrides (1951)
6 Rongstad (1966a)
7 Trippensee (1936)

** my average based on Lord's (1963) day 59 and 61 weights.

Table 18. Daily weight gains (in grams) for a known-age litter of six nestling cottontails weaned by the mother.

Nestling	Cottontail age (days) 2	3	4	5	6	7	8	17	18	19	20
A	3 (7)*	6 (12)	3 (5)	9 (16)	3 (5)	19 (27)	11 (12)	14 (10)	5 (3)	14 (9)	13 (8)
B	11 (26)	7 (13)	6 (10)	1 (2)	0 (0)	20 (30)	no weights	6 (6)	13 (12)	5 (4)	15 (12)
C	13 (42)	6 (14)	2 (4)	3 (6)	5 (8)	11 (16)	no weights	15 (13)	5 (8)	13 (16)	6 (4)
D	13 (31)	5 (10)	8 (14)	2 (3)	1 (2)	killed					
E	10 (25)	5 (10)	7 (13)	4 (7)	4 (6)	killed					
F	12 (29)	4 (7)	4 (7)	4 (6)	killed						

()* = percentage weight gain from previous day.

Table 19. Weight gains (in grams) during a 24-hour interval for nestling littermates from five different litters.

Litter:	A	B	C	D	E
Age (days) at end of 24-hour interval	2	3	4	8	31
	9 (22)*	17 (35)	14 (27)	32 (31)	10 (7)
	7 (18)	15 (32)	12 (22)	22 (25)	11 (8)
	9 (22)	10 (15)	14 (21)	32 (37)	
	9 (23)	11 (18)	13 (23)		
	7 (18)				

()* = percentage weight gain from previous day.

Table 20. Weight differences (in grams) between the smallest and largest individuals within nine different litters.

	Age of litter (days)					
	1	2	3-4	8-10	14	30
Litter: A	25-27 (7)*	40-45 (6)	53-66 (5)	65-75 (5)	88-115 (3)	220-257 (3)
B		31-46 (6)	34-50 (6)	65-75 (5)		
C	27-36 (4)	35-42 (4)				
D			36-65 (7)	60-93 (9)		
E		39-41 (6)		51-67 (4)		
F	23-33 (4)					
G	30-34 (5)					
H	23-28 (4)					
I				80-93 (5)		
Greatest differences between littermates of the same age	20%	33%	45%	35%	23%	14%

()* = number of young in litter.

members of nine litters are listed. Reading across for any litter, the largest percentage difference observed for that litter at a particular age is listed below that age. The decreasing litter size with age was due to the removal of young for other experiments. The largest difference observed between littermates was 45%. Rongstad (1966a) also investigated littermate weight variation, and found it to average 15-20% with the largest difference being 25 percent. It was not determined whether the observed decrease in weight differentials between littermates after 3-4 days of age was due to aging or to the reduced numbers. These data again indicated that body weight alone is not a good criterion for age determination of young cottontails.

External Developmental Processes: Various physiological processes in nestlings were fairly constant in their initial appearance (Table 21). Nestlings opened their eyes by day 7 or 8, usually one eye at a time, but one animal did not have its eyes opened until day 11. Allen (1938) and Beule and Studholme (1942) reported similar results, while Ecke (1955) found a range of 5-7 days. The umbilical scab was visible and protruded through the fur until day 7. The scab was present until day 9 or 10, but was covered by the fur and not visible. Newborn rabbits were born with a naked forehead which became furred by day 4 or 5.

Not all young left the nest at the same time. A litter of five took three days before all were gone. Prior to a final departure, the nestlings explored the surrounding area, but returned to the nest. Even young cottontails with their eyes still closed were found crawling

Table 21. Visible physiological nestling processes and time of first appearance.

Process	Sample size	Mean age (days)	Range (days)
Opening of eyes	31	7.7	(7-11)
Disappearance of umbilical scab	10	8.3	(6-10)
Complete furring of forehead bald spot	7	4.5	(4-6)
Leaving the nest	3 litters observed	11.2	(9-13)

in the vicinity of the nest and then returning. Such trial excursions from the nest had been reported by Trippensee (1936) and Haugen (1942). Nests were deserted by the young on an average of 11.3 days (9-13), in contrast to 16 days reported by Allen (1938), and 13-17 days determined by Haugen (1942).

Nestlings have been assumed to begin nibbling at vegetation at about two weeks of age (Trippensee, 1936; Ecke, 1955). But, in this study, plant material was found in the intestines of three-day old nestlings. Such early nibbling would be conducive to the ingestion of parasites at an early age.

The largest litter born in the pens contained eight young. Rongstad (1966a) had three litters of nine produced within his pens. Other similarities of nestling behavior between this study and Rongstad's included: tolerance of handling by the doe and newborn; difficulty of the observer in finding the nest; the fact that dead nestlings were not removed from the nest by the doe; and that young born on the bare ground and deserted by the doe lived 2-4 days before dying.

Nestling distress calls first described by Haugen (1942) were also heard in this study. Rongstad's (1966a) suggestion that this cry elicits an escape response in other nestlings was corroborated in this study when a litter of five was brought into the laboratory. While measuring one nestling, it emitted a piercing cry which caused the remaining nestlings to scatter all over the room.

Physiological Observations of Juveniles

Hepatic Observations: In pre-natal life, the liver functions as an organ of hematopoiesis. This function may continue into post-natal life, and is indicated by the presence of extramedullary hematopoietic centers.

Two types of such centers were seen in cottontail livers. The first consisted of small, dark, circular cells presumed to be the proerythroblasts and erythroblasts of the erythrocytic series. The second hematopoietic type, which was the more common, had larger cells that contained large eosinophilic granules (Fig. 40). Whether these cell types are a primitive type concerned with granulopoiesis was not determined. Megakaryocytes were frequent in both types of hematopoietic centers (Fig. 41).

Cottontails one week of age or younger usually had one or the other type of hematopoietic center present in the liver. The time of disappearance of these hematopoietic centers varied. Such centers were present in 28-, 38-, 57- and 74-day old cottontails, but not in other animals of 12, 28, 31 and 50 days of age.

Also present in younger cottontails was a hepatic paravascular lymphocytic cuffing of various blood vessels. This cuffing was also present in the kidney, especially about veins.

Intestinal Observations: A rather constant histological difference was noticed in the small intestines of adult and juvenile cottontails. Adult animals always had numerous migrating leukocytes, presumed to be

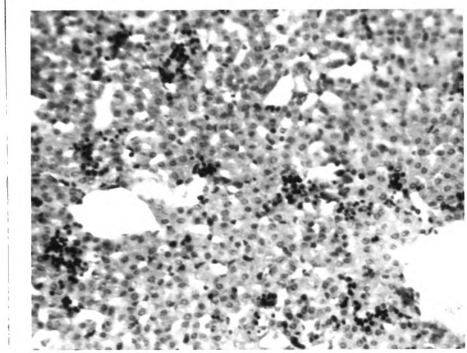

Figure 40. Hepatic extramedullary hematopoietic centers in a three-day old cottontail. HE. X188.

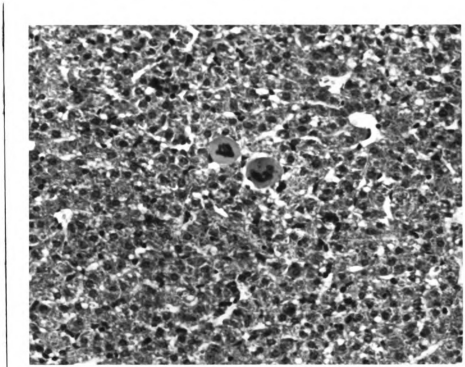

Figure 41. Megakaryocytes in the liver of a 12-day old cottontail. HE. X188.

lymphocytes, present in the epithelial cells (Fig. 42). Although varying in number, the number of such lymphocytes in adults was always greater than that found for younger cottontails (Fig. 43). Both in the epithelial cells and lamina propria, a greatly reduced number of lymphocytes was evident in juveniles. From over 40 individuals examined, the oldest juvenile found with no migrating lymphocytes was 70 days old, while the first indication of such migrating lymphocytes occurred in a 16-day old animal. In general, however, migrating lymphocytes were not observed in the small intestine until cottontails were over three weeks of age.

Eye Lens Weights: Cottontails have had their ages determined by eye lens weights (Lord, 1959; Rongstad, 1966b). The majority of published data was for animals over one month of age. In this study, lens weights were appraised for 38 juveniles of known ages less than a month old (Table 22). When these data were plotted along with additional data from 14 other juveniles less than two months of age, the resulting curve, fitted visually, was a straight line (Fig. 44). A downward extension indicated an eye lens weight of about 5 mg for newborn cottontails. Lord (1959) reported a range in weight of 6.6-8.4 mg for newborn eye lenses. From the curve derived by Rongstad (1966b) relating eye lens weights to cottontail ages, the weights for specific age classes were similar up to 40 days of age to those obtained in this study (Table 23).

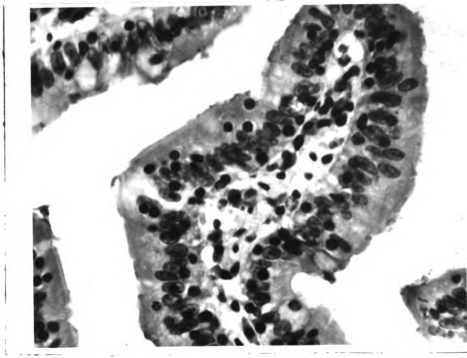

Figure 42. Small intestine of an adult cottontail demonstrating large numbers of migrating lymphocytes in the lamina propria and epithelium. HE. X750.

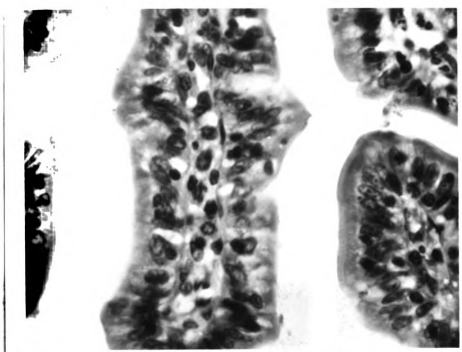

Figure 43. Small intestine of a 30-day old cottontail demonstrating few lymphocytes in the lamina propria and epithelium. HE. X750.

Table 22. Dry eye-lens weights from known-age cottontail rabbits reared in captivity.

Age (days)	Lens weight (mg)	Age (days)	Lens weight (mg)
3	9	9	18
3	9	9	18
3	11	9	17
4	6	10	14
4	6	10	18
4	11	11	20
4	10	11	23
4	6	12	22
4	8	12	18
5	9	13	24
5	13	16	40
7	14	16	28
8	15	17	21
8	14	17	20
8	20	18	34
8	15	20	41
8	14	20	43
9	13	21	35
9	13	21	35

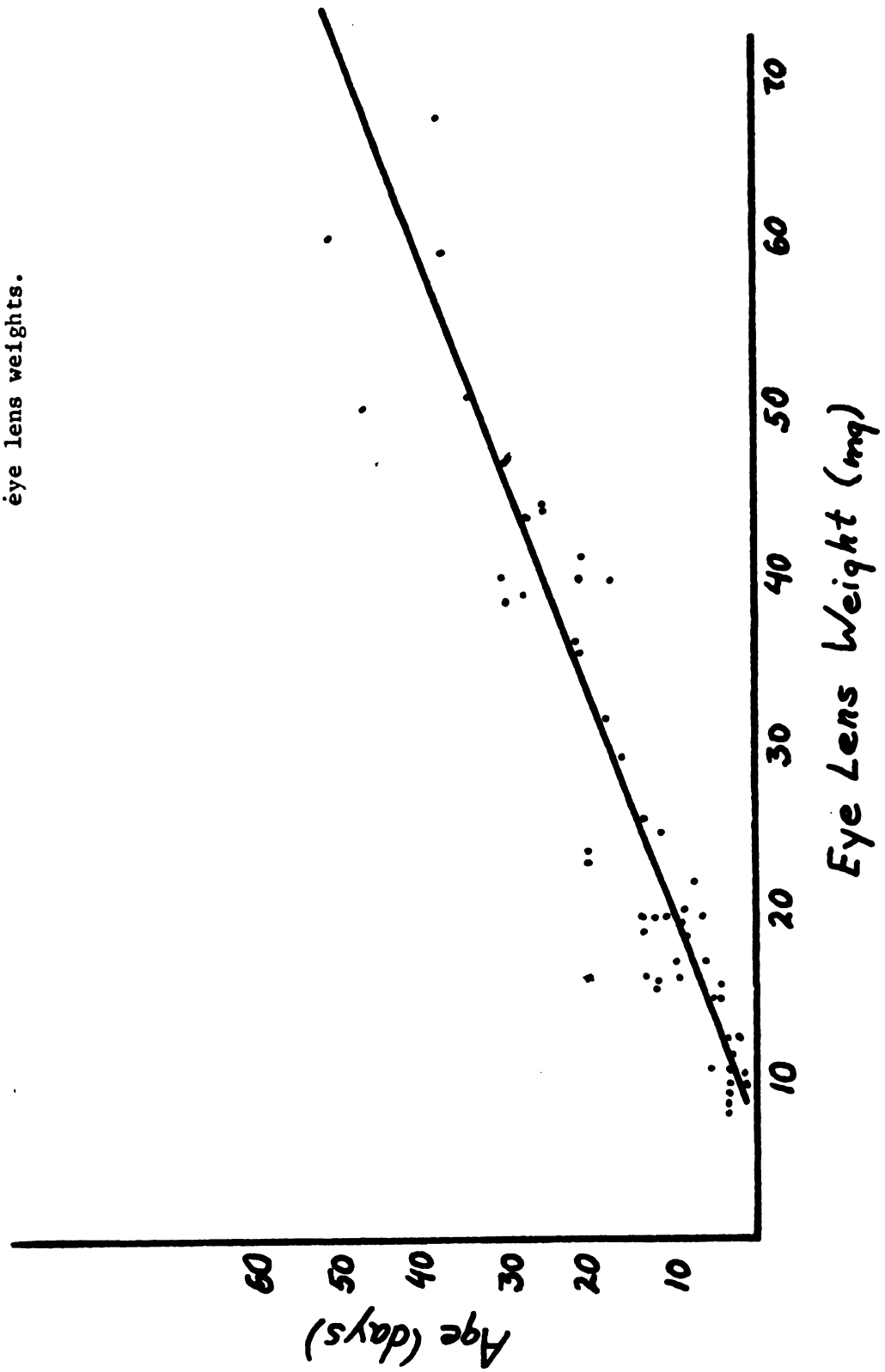

Figure 44. Early juvenile ages related to eye lens weights.

Table 23. Extremes of juvenile cottontail eye lens weights reported in this study and by Rongstad (1966b).

Cottontail age (days)	Eye lens weight (mg)	
	Present study	Rongstad (1966b)
10	15-20	15-20
20	30-35	30-35
30	45-50	42*
40	60-65	55-60
50	75-80	65-70
60	85-90	76*
70	100-105	85-90

* derived by Rongstad from a regression equation.

Sexual Development: The chance histological observation of spermatogenesis in the testis of a 14-day old cottontail (Fig. 45), led to further examination of spermatogenesis in juveniles because breeding by young males is not considered possible before 2.5 months of age (Negus, 1959).

The following generalized findings were observed in the seminiferous tubules of a sampling of juveniles testes.

3 days: No spermatozoa present, no spermatogenesis; a basophilic material within the tubules was positive when stained by Periodic acid Schiff.

12 days: An increased cellularity was evident with binucleated primary spermatogonia present; no spermatozoa.

39 days: Primary spermatogonia and increased cellularity present; no spermatozoa.

91 days: Active mitoses visible in tubules; no spermatozoa seen in the epididymis.

Conaway and Wight (1963) found that the reproductive condition of the seminiferous tubules of 3-5 month old rabbits ranged from infantile to mature. They found no signs of juvenile spermatogenesis before late July while the spermatogenesis seen in the 14-day old cottontail occurred in early May. Whether the latter animal was capable of breeding is unknown since no sections of epididymis were examined. Ecke (1955) considered the presence of sperm in the epididymis to be the best evidence of fertility.

Surgical Removal of Young and Feeding of Newborn

Five litters of Dutch Belt domestic rabbits and four litters of cottontails were successfully removed by cesarean derivation (CD), all

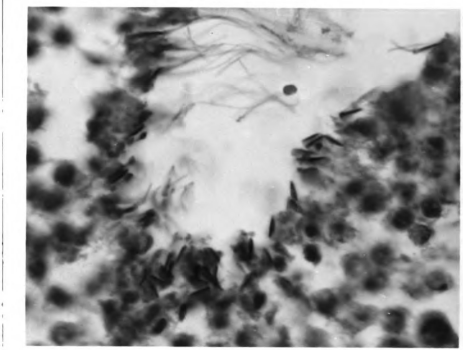

Figure 45. An unusual occurrence of spermatogenesis in the seminiferous tubules of a 14-day old cottontail. HE. X1875.

but three litters of cottontails being removed under conventional (non-sterile) conditions. These latter litters of cottontails were taken under sterile conditions within a germfree isolator (Lopushinsky and Youatt, 1968). Two of these litters were maintained germfree for at least four days, and two individuals lived 16 days germfree. The third germfree litter, removed from the doe inside the isolator, was immediately taken from the isolator and reared conventionally.

Hand Feeding: All hand feedings began on day two. Weight losses between birth and day two, for 13 domestic young and 7 young cottontails, averaged nine and six percent, respectively. When possible, feedings were continued until the stomach was observed to be full of milk.

The best method for feeding the diet (see Materials and Methods) was stomach intubation with polyethelene tubing. This technique resulted in daily gains of 6-10 grams. But, frequent esophageal perforations eliminated this method of feeding.

When fed with an eyedropper, the young initially nursed vigorously on their own, but for unknown reasons refused to nurse after the third or fourth feeding and had to be force-fed. Even prepared latex nipples were refused. The diet was also refused if their urinary bladder was full. After several feedings though, the rabbits urinated during the feeding. The largest amount of diet consumed by this method was 4 ml, whereas up to 10 ml could be intubated without apparent discomfort to the animal. Ecke (1955) found that the filled stomachs of wild nestlings comprised one-sixth

of their body weight and, if confirmed, may serve as a standard for feeding cottontail nestlings. The inefficiency of feeding by eyedropper was evident when the mean volume of diet fed per day was compared to mean daily weight gains (Table 24). Through day seven, the daily volumes of diet fed and daily weight changes are the mean data obtained from five animals; only two animals being measured thereafter. Contrary to expectations, the amount of food consumed remained rather constant with increasing age. Losses of weight occurred on days eight and twelve, while all weight gains were below normal growth rates (see Table 18).

The quantity of formula rather than its quality was suspected in the failure to rear CD cottontails. One of the reasons for suspecting quantity was the occurrence of normal physiological phenomena within normal time limits. Thus, eyes opened and furring-out occurred normally. Both of the germfree animals previously described which lived 16 days, although runts, were very active. When these died, clinical signs included the loss of fur about the head, which began on day eight, and a lacrimal secretion, which began on day twelve.

Foster Mother Trials: As an alternative to hand-feeding, the nestlings were placed on domestic does which had just kindled. Initially, the does did not readily accept the cottontails and it was decided to place young on a doe only when feeding. The greatest amount of milk was obtained by the cottontails when placed on the doe at night for a single feeding, after she had been isolated all day. The does did not remain still for the cottontails, however, and had to be

Table 24. Mean daily volumes of hand-fed diet compared to mean daily weight gains of young cottontails.

Age of nestlings (days)	Mean volume* fed/day (ml)	Mean weight change/day (gm)
2	1.8	1.4
3	3.7	1.0
4	3.0	0.8
5	4.2	4.0
6	4.2	1.6
7	4.1	3.3
8	5.5	- 0.5
9	5.8	2.9
10	5.9	0.9
11	3.9	1.1
12	5.0	- 2.0
13	3.5	1.6
14	2.8	1.3
15	5.1	2.6

* fed every 3-4 hours.

held. Once the cottontails fed, the remaining domestic nestling (see Materials and Methods) was placed with the doe to nurse and remained with her all night.

Daily cottontail weight gains by hand-feedings (every 3-4 hours) and the single foster-mother feeding were compared (Table 25). The main difference was in the initial feeding. Although the young cottontails were kept on the doe for over half an hour, the doe either prevented the let down of milk, or the young could not draw enough out.

The domestic nestling, which fed after the cottontails, always quickly obtained sufficient milk. Within one hour's time, it gained as much as 16 grams, while the total weight gained by the 3-5 cottontail nestlings, feeding just prior to the domestic, rarely exceeded 6-7 grams. For whatever the reason, cottontails could not match the domestics in obtaining sufficient milk (Table 26).

Juvenile Mortality

A total of 167 cottontail juveniles was observed in this study. Mortality associated with juveniles born in captivity was noted from the day of birth while juveniles turned in as orphans could not be so observed. Forty of the 88 young born in the pens were experimentally killed and are excluded from mortality analysis.

One litter of six, born indoors, was trampled accidentally by the doe which became excited in the cage. All of these young had nursed well. Three litters, totaling nine young, were deserted by the does. In two of these, the does had been captive for just a few

Table 25. Cottontail nestling weight gains resulting from hand-feedings and foster-mother feedings.

Nestling age (days)	Mean weight gains from previous day (in gm)	
	Hand-feedings*	Foster-mother feedings**
3	1.4	4.0
4	1.0	1.0
6	4.3	2.5
7	1.6	1.7

* for six nestlings.
** for seven nestlings.

Table 26. Mean percentage weight increase of two cottontail and one domestic nestlings nursed on the same domestic doe.

Age (days) of all nestlings	Percent weight increase*	
	Cottontails	Domestic
9	62	207
10	82	248
11	91	289

* based on the initial weight of day 1.

months which may have contributed to their desertion. The third doe, No. 65, even though captive several years, was always highly excitable. The 15% nest desertion (3 of 20 litters) observed here, equalled that reported by Sheffer (1957b) in his penned litters. Rongstad (1966a), however, only had about 1% of his penned litters abandoned. Factors causing variable rates of nest desertion are unknown. In each of two deserted litters, a nestling was partially eaten by the doe.

At least five young from two litters were aborted. One of these does was newly captured and the second was the highly excitable No. 65. In one litter of eight, there was one congenital death. Its dissected lungs sank in water, indicating that it was born dead. It had no eyelids; the eyes were covered by skin. It also was otherwise malformed and weighed only 17 grams compared to its littermates' mean weight of 26 grams. Two nestlings died from unknown causes (see below). Wohlfahrtia vigil myiasis (Appendix J) killed 10 nestlings. Two other nestlings had their larvae removed and survived. Whether these would have survived had the larvae not been removed is unknown.

From a total of 41 nestlings born and left in the pens (the six cottontails born indoors and the single congenital death are excluded) only 17 (40.5%) survived at least one month.

Eighty-two of the 167 juvenile cottontails studied (both pen-born and orphans) were experimentally killed. Of the remaining 85 juveniles, 26 (30.5%) died from unknown causes. At the time of death, approximately three weeks of age, all but two of these

26 dying juveniles were caged indoors with littermates that remained healthy. Prior to any signs of sickness, all littermates had comparable and normal growth rates and behavior. Death occurred within 24 hours once the following clinical signs were seen: the animal with fur erect, remained hunched-up rocking slowly from side to side; all were reluctant to move and when forced to, did so shakily and in apparent discomfort; a rapid loss of weight. Before death, the animals were comatose and stretched out displaying opisthotonus. The only lesions found on necropsy were gastric ulcers (Appendix L). Microbiological examinations yielded no pathogenic organisms.

Two possible explanations for these unexplained deaths are available. The first of these concerns Escherichia coli, a bacterium present normally in the intestine. Yuill and Hansen (1965) found that an overgrowth of E. coli killed the animal in less than 24 hours. A loss of gut motility was postulated for the overgrowth, but arose differently in adults and juveniles. Extreme cold caused the adult overgrowth, while juveniles died from this coliform enteritis because of captive conditions. Adults dying always had full gastrointestinal tracts. Lesions included hemorrhages of the duodenum, ilium and cecum. Tissue invasion by E. coli occurred only in juveniles.

In the present study, several adults with full gastrointestinal tracts were found frozen. One adult found dead in May had E. coli present in the liver, a condition not observed by the above authors. Some of the juveniles dying in this study had the gross lesions

described for coliform enteritis by Yuill and Hanson (1965). Although clinical signs differed between the coliform enteritis study and this study, E. coli deserves further investigation as a pathogen of cottontails.

As to the second explanation, Pederson (1963) observed springtime deaths in captive adult and juvenile cottontails due to a condition of unknown etiology he termed ulcerative enteritis. This condition involved the adrenal hormones and stress was postulated as the cause of the enteritis. Clinical signs found in dying animals included a progressive weakness, sporadic kicking, respiratory distress and severe diarrhea. Death occurred within 24 hours and was preceded by coma and opisthotonus. Lesions included inflammation of the intestine and serosal hemorrhages of the cecum. Over 70% of the dead animals had gastric ulcers. Reduced spleen size occasionally occurred. In the present study, both adults and juveniles were found with gastric ulcers and cecal hemorrhages. Small spleens were present in some adults. Whether the stress syndrome postulated by Pederson (1963) is important in captive cottontails is unknown.

CONCLUSIONS

Enzymology: This study showed the feasibility of using intracellular enzymes as a technique for determining host damage from sub-lethal coccidial infections. Enzymes of the intestinal tract of cottontails proved to be similar to those of other species in which enzyme concentrations have been used to demonstrate host damage from parasites (Jervis et al., 1966; Maronpot and Whitehair, 1967).

Of the four enzyme systems studied, alkaline phosphatase, glucose-6-phosphatase and succinic dehydrogenase were present in sufficient concentration for use in physiological measurements. Their histological identification, furthermore, was sufficiently reproducable so as to minimize possible bias from procedural techniques. The same consistent reproducability of results was not found for non-specific esterase. In the cottontail gastrointestinal tract, each enzyme had an uneven distribution; each enzyme had its maximum concentration in a different area. Thus, any area of the gastrointestinal tract may be appraised for sublethal physiological damage by determining the concentrations of a specific intracellular enzyme.

The finding that fertility was associated with an increase in the concentration of alkaline phosphatase in the testes may offer a method for determining the reproductive status of a male. This method might be of value in better defining the age at which young cottontails are capable of reproduction.

Normal concentrations of serum alkaline phosphatase were inhibited by L-phenylalanine, indicating that the main alkaline phosphatase isoenzyme may have originated in the small intestine. But, this remains to be tested in the cottontail. If proven true, then a simple blood test may be available for measuring intestinal damage in the living animal. Additional studies must then determine what factors (i.e., coccidia) are identifiable by increased, or decreased, amounts of the small intestinal isoenzyme. Since total reduction of normal serum alkaline phosphatase concentrations by the summed inhibitions of L- and D-phenylalanine was greater than 100 percent, the isoenzyme inhibitions theorized for the phenylalanine stereoisomers, as determined from studies of other species, may not pertain to the cottontail rabbit.

Cottontail Coccidiosis: No conclusions were possible concerning the pathogenicity of coccidia to cottontail rabbits. Although few reports existed of cottontail mortality due to coccidiosis, this uncommonness could merely reflect characteristic difficulties in mortality studies of natural populations. Valid postmortem diagnoses for a significant segment of a natural population have rarely, if ever, been done. The single nestling presumed to have died from coccidiosis reported here should not be considered evidence against coccidia being a pathogen of significance.

Investigative efforts should have focused on younger cottontails, but few wild coccidia-free nestlings were found. Natural coccidial infection was possible immediately after birth. Because sufficient

numbers of coccidia-free nestlings could not be found the surgical germfree removal and rearing of young was undertaken. The surgery itself proved successful, but the mechanics involved in artificially feeding the newborn were apparently responsible for failures to raise the young to weaning age, whether they were born naturally or cesarean-derived. As Glimstead (1959) has pointed out, two major aspects exist for germfree research; the first investigates the germfree animal per se; the second uses the germfree animal as a research tool. Cottontail germfree research must still center on the former area - specifically, the discovery of successful feeding techniques.

Perhaps the reliance on obtaining a germfree cottontail was premature. It may have been more practical to begin coccidial studies with adult cottontails which had received special treatment to rid them of their coccidia. Young born of these adults would hopefully be kept free of coccidia while still being nursed by the natural mother. These young could then be used to investigate cottontail pathology from inoculations of known species of coccidia. Should a correlated mortality over uninfected control animals be proven, then the more demanding germfree techniques would have to be reinstated. Germfree techniques would be necessary to insure against mortality or sublethal damage by other pathogens, perhaps even including the normal microbial flora. Phillip and Wolf (1959) demonstrated, with germfree techniques, that intestinal amoebiasis in guinea pigs was due to an interaction of several normally harmless organisms. Entamoeba

histolytica, the suspect pathogen, was non-pathogenic in pure infection, but when associated with other equally "harmless" micro-organisms, it produced death. Thus, microbial interactions can be involved in the etiology of enteric disease.

It is only a matter of time before wild species will be successfully reared under germfree conditions. Even with this artificial ecosystem, unique opportunities for ecological research exist using wild species. Just (1959), in fact, considered an ecological approach as the best regarding the development and use of germfree animals as a research tool.

Penned Cottontails: From results in the breeding pens, obviously it is not necessary to keep the sexes constantly together as was done by other workers. Adults can be separately housed in small indoor cages and temporarily released into small breeding pens. Cottontails kept indoors for over a year had no ill effects and, if caged young enough, became somewhat domesticated. The small breeding pens used in the present study were successful and the minimal size of such temporary breeding pens cannot now be estimated. The existence of a synchronized initial pregnancy period for female cottontails (Marsden and Conaway, 1963) would require only a few males to successfully breed many females at the same time. If mated immediately post-partum, maximum numbers of litters per female per year could be obtained, providing the large numbers of young needed for coccidial investigations.

SUGGESTIONS

The various laboratory procedures for evaluating coccidial infection in cottontails have yet to be established. Such an evaluation requires the availability of juveniles since this age class can be expected to suffer most from coccidia. Since they are not available from the wild, it is of primary importance that coccidia-free young be produced in the laboratory. Inability to feed sufficient diet to newborn cottontails prevented acquisition of coccidia-free juveniles for this study. Therefore, as preliminary guidelines for obtaining coccidia-free young it is suggested that either: 1) the mothers be allowed to nurse their natural-born young under coccidia-free conditions; or 2) the young be removed from the mother immediately after natural or cesarean birth. The former would be easiest but has the disadvantage of requiring the maintenance of both adults and young in a coccidia-free environment, while the latter requires finding a successful synthetic feeding procedure. Most desirable would be a method eliminating handling of the young. Initially, domestic rabbits comparable in size to cottontails should be used so that year-round research on nestling feeding methods is possible. Once such young are reared coccidia-free, the technique could be applied to the cottontail.

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APPENDICES

Appendix A

THE COCCIDIA

Taxonomy: According to Hoare (1956) these parasites belong to the Phylum Protozoa; Class Sporozoa; Order Coccidia. The term coccidia is generally used in referring to those species belonging to the four genera within the family Eimeriidae. These genera are separated by the number of sporocysts and sporozoites within the oocyst. Cottontail coccidia (subfamily Eimerinae; Genus Eimeria) have four sporocysts within the oocyst (tetraspores) each containing two sporozoites.

These protozoa are typified by gametes of different size, by the number of male gametes (microgametes) greatly exceeding that of the female gametes (macrogametes), and by the fusion of the micro- and macrogamete producing an oocyst. This structure contains the zygote and is passed with the host feces.

Eimeria species are found in a variety of hosts ranging from flatworms to man (Pellerdy, 1965). The majority of Eimeria, including those of the cottontail invade the intestinal epithelium, but coccidia are found in the mammary glands of mice, the kidneys of geese and horses and the liver and bile ducts of domestic rabbits.

Life History (based on Davies, Joyner and Kendall, 1963; Pellerdy, 1965): The development of coccidia is characterized by a regular alternation of sexual and asexual generations. Coccidial infection follows ingestion of viable sporulated oocysts contaminating food or water. Opposing theories exist concerning the mode of sporozoite release in the host digestive tube. Whatever the means of sporozoite

excystation (proteolytic enzymes dissolving the micropyle, mechanical trauma, or both), once liberated, the sporozoites are transparent, motile, and about 10 x 1.5 microns in length and width.

After penetrating the epithelial cells, which is the beginning of schizogony (the asexual phase), the parasite (now termed a trophozoite) increases in size as does the parasitized cell. The trophozoite displaces the nucleus and lies either above or below it, depending on its species. Within a few hours the trophozoite nucleus becomes segmented and is termed a first-stage schizont. Soon, the cytoplasm divides and separately surrounds each of the newly formed nuclei forming the first stage merozoites. The number of merozoites in a schizont varies with the Eimeria species. The mature first-stage schizont with fully developed merozoites may distort the parasitized cell causing it to protrude into adjacent tissues. Mature merozoites when released re-invade other cells. Some species, i.e., E. bovis of cattle, undergo only the first stage schizogony with the merozoites differentiating into sexual forms; other species form second, third or higher stage schizonts.

First-stage merozoites may initiate second-stage schizonts which increase in size and segment as did the original schizont. These second-stage schizonts may be larger than their first-stage form causing greater damage to the host tissues, or they may be smaller with fewer but larger merozoites. A position in the tissue different from that occupied by the first stage is possible. Thus, E. tenella second-stage schizonts may be located in the sub-epithelial,

sub-mucosal, or muscular layers whereas the first stages occupied the epithelial layer.

Host cells adjacent to whatever schizont stage initiated gametogenesis usually are suitable for sexual reproduction. Sometimes a migration to a more favorable location is required, i.e., E. necatrix in the chicken migrates from the small intestine to the cecum.

Microgametocytes and macrogametocytes are formed from last-stage merozoites. The former enlarge and undergo multiple divisions producing numerous microgametes. The microgametes have two flagella at one end, are fusiform, motile and about 5 microns long. Macrogametes are generally larger than microgametes, a single last-stage merozoite producing a single macrogamete. The macrogamete has a central nucleus with mucoprotein granules in the cytoplasm. These granules are largest at the periphery and disappear once fertilization occurs. They are assumed to form the cyst wall around the zygote in the developing oocyst.

When the cyst wall is complete, the oocyst is extruded from the host's gut tissue and passes out into the feces. When first passed in feces, oocysts are non-infective. After a prescribed length of time and under proper conditions of heat and moisture, they sporulate and become infective. Identification of the Eimeria spp. relies on the morphological changes of sporulation. Two of the more important structures are shown in Figures 46 and 47. The first demonstrates an oocyst residual body with the latter showing sporocyst residual bodies. The period of time from first infection to first oocyst appearance in

feces is called the prepatent period. The duration of this period is a species characteristic and is useful in species identification. Eimeria minima of the cottontail rabbit has a prepatent period of six days while E. arloingi's in sheep and goats is 22 days in length. The patent period refers to the length of time from the first appearance of oocysts in the feces to when oocysts are no longer passed.

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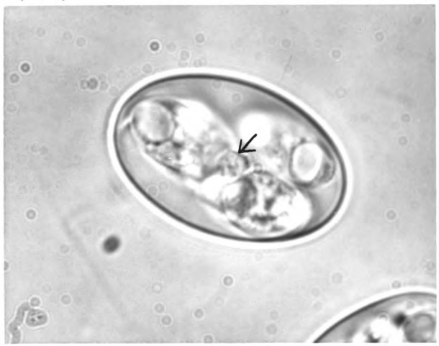

Figure 46. A sporulated cottontail rabbit Eimeria oocyst demonstrating an oocyst residual body (arrow). Unstained sugar solution. X1875.

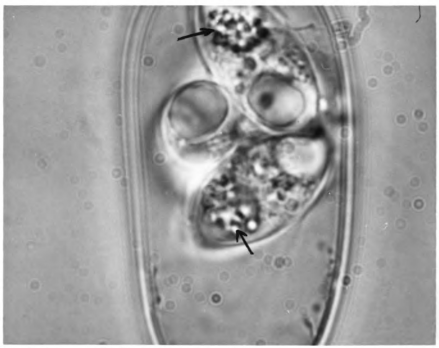

Figure 47. A sporulated cottontail rabbit Eimeria oocyst demonstrating sporocyst residual bodies (arrows). Unstained sugar solution. X1875.

Appendix B

ENZYMOLOGY (based on Dixon and Webb, 1964)

Enzymology is a comparatively recent science; it's beginnings can be traced back to the early nineteenth century, but only in the past forty years have the outstanding developments occurred. Payen and Persoy, in 1883, first clearly recognized an enzyme when they found an alcohol precipitate of malt extract which contained a thermolabile substance that converted starch into sugar. They named this substance "diastase" (now called amylase) and this word came to be used as a general term for enzymes. The term "ferment" was also used for enzymes. The controversy between Leibig, who held that fermentation and similar processes were due to the action of chemical substances, and Pasteur, who was of the opinion that fermentation was inseparable from living cells, produced the term "unorganized ferments" and "organized ferments" (today called extracted enzymes and micro-organisms, respectively). In order to avoid these confusing terms, Willy Kuhne, in 1878, introduced the term "enzyme" to replace the older term "unorganized (intracellular) ferment". Enzyme literally means "in yeast" and Kuhne's intention was to denote something present <u>in</u> yeast in contrast to the yeast itself. Duclaux, in 1883, proposed enzymes be named by adding the suffix -ase (from diastase) to a root indicating the nature of the substance on which the enzyme acts, i.e., phosphatase. This system is still in use; a few digestive enzymes ended in -in, i.e., pepsin, and have persisted. Currently the large

number of enzymes make it necessary to also name the nature of the reaction, i.e., lactic dehydrogenase.

Obligatory association of enzymatic function with living cells was retained until 1897, when Edward Buchner extracted zymase and demonstrated this cell-free liquid capable of fermenting sugar. Sumner, in 1926, first crystallized an enzyme (urease) and showed it to be a globulin. Subsequent studies have shown all enzymes to be proteins* having a prosthetic (non-protein) group in which the enzyme activity resides and which may or may not be an integral part of the protein molecule. Early attempts at enzyme purification were carried out by Wilstatter and co-workers in 1922-1928. To date, over 600 enzymes have been purified with over 100 available in crystalline form.

Most early studies centered on the industrial significance of the enzymes of digestion and fermentation. Despite the fact that they form the majority of all enzymes, it was not until 1937 that the importance of intracellular enzymes was recognized.

The medical significance of enzyme alterations is of relatively recent origin. In 1908, Wohlegemuth first demonstrated the diagnostic application of such alterations. He observed that ligation of the pancreatic duct produced an increase in amylase activity in both serum and urine. Stocks, in 1915, complemented this finding by showing the elevated amylase activity levels to be present in the serum of individuals with acute pancreatic disease (Bodansky, 1961).

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* Hess (1963) lists one exception to this: pig kidney alkaline phosphatase contains glucose, a pentose and a substituted pyrimidine as the nitrogen containing compound.

Chronologically, the clinical application of enzymology has had two distinct phases: 1) measurement of certain highly localized enzymes which are contained in relatively few organs, i.e., the great amounts of alkaline phosphatase in bone, liver and small intestine; and 2) serum activity determinations of a more ubiquitous series of enzymes such as the transaminases.

Those enzymes concentrated in certain organs offer a useful diagnostic tool in certain diseases. Damage to these organs allows their enzyme content to escape into the serum. Elevated serum activities of these enzymes are thus associated with specific organ damage. The damage may be due to tissue necrosis or from modification in cellular permeability which results in the outpouring of the intracellular enzymes (Innerfield, 1960). Recent progress utilizing assays of the widespread glycolytic enzymes have resulted in major advances for diagnosing myocardial infarction, hepatitis, muscular dystrophy and various cancers.

Three major approaches exist to the study of the enzyme-tissue relationship: 1) biochemical measurements relate and quantitate the activity of a single enzyme system to a specific quantity of tissue or body fluid; 2) histochemical analysis records the presence or absence of an enzyme and its location within a tissue and employs a stain specific for the enzyme under consideration; 3) cytochemical examination utilizes a suitable enzyme stain with the results observed through an electron microscope relating enzyme location to ultrastructure.

This study uses the second technique. Histochemistry is the study of the chemical and physical properties and reactions of animal and plant tissue or their structural components (Thompson and Hunt, 1966). General considerations include: 1) a suitable substrate for the enzyme to act upon which can be coupled to a colored compound (or one which fluoresces); and 2) finding suitable in vitro conditions which allow the reaction to take place. Tissue fixation is an exceedingly important phase of enzyme histochemistry. A few enzymes are tolerant of a wide range of chemical agents including fixatives, i.e., alkaline phosphatase, whereas others, i.e. glucose-6-phosphatase, are completely inactivated by even brief exposure to most tissue fixing and processing fluids. For this study, the preferred method for tissue preparation was the immediate freezing of fresh tissue with subsequent sectioning done at freezing temperatures. The problem of heat and chemical lability was thus circumvented.

Unlike many other chemical reactions, enzyme histochemistry uses not only positive control sections, but also negative controls. The former sections are essential to insure that the reaction is working and uses tissues known to contain high activities of the particular enzyme studied. Negative controls are incubated in medium without the substrate required by the specific enzyme studied. Thus, any observed staining reaction is known not to be the result of enzyme activity since the specific substrate was missing. The following lists positive and negative controls for the enzymes studied here:

Enzyme	Positive control	Negative control
Alkaline phosphatase	small intestine, kidney	substrate solution minus specific substrate.
Glucose-6-phosphatase	liver	eliminate glucose-6-phosphatase from media or use formalin fixed tissue.
Esterase	liver, kidney	substrate solution minus specific substrate.
Succinic-dehydrogenase	myocardium	eliminate sodium succinate from media.

Enzymes Selected for Study

Alkaline phosphatase (Phosphomonoesterase I; non-specific alkaline phosphatase): The phosphatases are hydrolic enzymes catalyzing the hydrolysis of phosphoric esters:

Ester plus water $\longrightarrow$ alcohol and phosphoric acid.

Alkaline phosphatase is found in all cells and hydrolyzes most orthophosphomonesters at an alkaline pH. Organically bound phosphoric acid esters are transformed into inorganic phosphates. Functionally, the enzyme is concerned with maintaining the concentration of intracellular inorganic phosphate required in bone formation and in dephosphorylation, necessary for absorption and transport. Metabolic control is exerted by the rate of dephosphorylation (Thompson and Hunt, 1966). The kidney cortex is rich in alkaline phosphatase and it has been suggested (Lundsgaard, 1933; in Innerfield, 1960) that it aids in glucose reabsorption within the convoluted tubules. The function in the small intestine, the richest source of the enzyme, is one of glucose and fatty acid absorption.

Glucose-6-phosphatase: This hydrolytic enzyme specifically dephosphoralates glucose-6-phosphate to glucose in glycogenolysis. Most of the body fluid glucose is derived from either intestinal absorption or hydrolysis of glucose-6-phosphate which occurs mainly in the liver. This enzyme is one of the most important in the intermediate metabolism of carbohydrates (Wachstein and Meisel, 1956).

Non-specific Esterase: Hydrolyzation of carbonylic acid esters is the primary reaction performed by these enzymes:

Ester and water $\longrightarrow$ acid and alcohol.

Three somewhat overlapping divisions exist: 1) true esterases hydrolyzing simple esters of monohydric alcohols; 2) lipases hydrolyzing fats and oils; 3) cholinesterases hydrolyzing esters of choline. No attempt was made to differentiate among the divisions in this study.

Lewis and Hunter (1966) reported an increase in intestinal esterase activity with increased fat ingestion. A role in the resynthesis and exit of cellular material is suggested by Friedman, et al. (1966).

Succinic dehydrogenase: From a histochemical point of view, this enzyme is one of the best indicators of Kreb's cycle activity. Succinic dehydrogenase is involved with oxido-reduction reactions. Oxidation of a substance may occur by: 1) addition of oxygen; 2) the loss of hydrogen ions; and 3) the loss of electrons. The dehydrogenases act via the second method. The removed hydrogen ion combines with oxygen to form water. This transfer is not direct and the hydrogen must pass

through a system of intermediate carriers, viz., (DPN), (TPN) and the flavo-proteins. Of all the dehydrogenases, succinic dehydrogenase alone bypasses these intermediate carriers and transfers the hydrogen ion removed from succinate directly to the cytochrome system.

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Appendix C

ISOENZYMES

Isoenzyme refers to the multiple molecular varieties of an enzyme which catalyze the same reaction and occur in the same species, but differ in certain physico-chemical properties (Wilkinson, 1965). Markert and Moller (1959) first proposed the term 'isozyme' to describe the different proteins with similar enzymatic activity. Although isozyme is acceptable, isoenzyme is the preferred term.

Isoenzymes may differ from each other in ways ranging from small variations in secondary structures to broad differences in amino acid sequences and molecular weight (Goodfriend and Kaplan, 1965). At one extreme are isoenzymes with marked structural differences but a common substrate, i.e., the esterases, while at the other end are molecules identical in all respects but their degree of denaturation.

Isoenzymes probably originated when a gene which produced one enzyme, mutated and produced two or more genes resulting in two or more enzymes (Goodfriend and Kaplan, 1965). Subsequent evolution and environmental influences produced isoenzymes modified by varying degrees in different organs. It is the organ-specific isoenzyme localization which renders isoenzymes useful for clinical application.

The physiologic significance of isoenzymes can be illustrated using the isoenzymes of lactic dehydrogenase (LDH). Lactic dehydrogenase is essential for converting pyruvate to lactate anaerobically. Heart and skeletal muscle are rich in LDH but each contains a different subunit, or isoenzyme of LDH; H subunits and M subunits respectively.

M subunits function at high concentrations of pyruvate when it cannot be oxidized, as frequently occurs in skeletal muscle. On the other hand, H subunits are inhibited by high pyruvate concentrations, retarding the pyruvate-to-lactate reaction. This promotes the shunting of pyruvate into the oxidative pathways of the Krebs cycle and favors a more complete utilization of the available energy in glucose. Perhaps isoenzymes can be considered environmentally adapted biochemicals.

Only the alkaline phosphatase (AP) isoenzymes were considered in this thesis. The study of phosphatase relies on the determination of one of the products of hydrolysed substrate liberated under standardized conditions. The activity of the tissue extracts or blood is expressed in relative units corresponding to amounts of phosphoric acid liberated. Serum AP activities will be expressed in International Units (IU) as suggested by the International Union of Biochemists (1961). An IU is defined as that amount of enzyme which will catalyze the transformation of one micromole of substrate per minute. Serum activities should also be recorded as IU/liter of serum (Mather, 1965). Alkaline phosphatase is relatively non-specific in its substrate requirements and various substrates have been used in studying its isoenzymes, i.e., beta-glycerophosphate, p-nitrophenol phosphate, phenyl phosphate and phenolphthalein phosphate. The last mentioned substrate was used for the serum analyses in this study.

Serum AP activities are elevated in two chief groups of diseases--those involving the integrity of the liver and those affecting the

bone. More recently, Haije and de Jong (1965; in Wilkinson, 1965) have found that intestinal damage elevated AP activity in human serum.

The use of enzyme inhibitors as a means for isolating AP isoenzymes has received increased attention (Fishman et al., 1962, 1963; Hugon and Borgers, 1966). These workers observed organ-specific inactivation of rodent AP isoenzymes. The amino acid phenylalanine inhibited the intestinal AP while not affecting the isoenzymes of bone, spleen, lung, liver and bile. D-phenylalanine, however, did not affect the intestinal AP, but did inhibit the other isoenzymes. Through the use of such isoenzyme inhibitors and sparers, it may be possible to monitor for intestinal damage in the living animal. This could possibly result in a clinical test for field use which would not require the killing of the animal.

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Appendix D

GERMFREE TECHNIQUES

Terminology: As with any expanding scientific field, terminology can be overabundant and often confusing. When applied to higher animals, germfree indicates an absence of all other forms of life. Germfree conditions are measured in terms of failure to demonstrate viable forms of life. Because newer methods of diagnosing contamination are continually disclosing heretofore undiscovered organisms, germfree is a relative term. A description nomenclature has evolved and is used rather indiscriminately in describing an animal's health. Such terms include: disease-free, axenic (without strangers), pathogen free, Cesarean-derived, specific pathogen free (SPF) and autobiont. In 1942, Reyniers et al. proposed gnotobiote meaning either free from micro-organisms, or in association with other known forms of life. With such a variety of terms, it is no wonder clarification is needed at times.

Although these many terms will continue in use, a clearer, more concise word is required regarding the quality of a research animal, especially when defining experimental conditions. Patterson (see Hill, 1963) defined an animal's quality as "a combination of a variety of factors dependent upon parentage, environment, nutrition and exposure to pathogens". Hill (1963) considers the term "defined animal" to best describe an animal's physiological status and includes the characteristics of the apparatus in which the animal was reared and maintained, information on the various ambient and fixed conditions to

which it has been exposed, i.e., humidity, temperature, what techniques were used for diagnosing disease and contaminants, and information on the diet as well as its nutritional value. A genetic history would also be desirable including the mating system used and how long each system was used. Although many of the above requirements cannot be met when working with wild species, an attempt to adhere as closely as possible to this procedure should be made.

When describing animals other than gnotobiotes, Gordon (1959) expressed the idea that the term normal should not be used because it refers to a favorable adaptation of an organism to its environment, an adaptation displayed by many gnotobiotes. Instead, the term conventional refers to the polycontaminate animal. At the other extreme, germfree should mean just that; free of all demonstrable forms of life; gnotobiote referring to a known-contaminant infection. Thus, cottontails cesarean-derived and maintained under aseptic conditions should be called germfree. When these are experimentally infected with coccidia they should then be referred to as gnotobiotic.

History (based on Luckey, 1963): Providing research animals free of the stress of pathogens has been the goal of animal specialists for many years. Various techniques have been tried; intensive culling, selective breeding for specific disease resistance and development of new diagnostic tests and therapeutic agents designed to reduce or eliminate certain pathogens. But none of these proved entirely successful. It remained for the system of cesarean-derived young

maintained under sterile conditions to pave the way towards obtaining high quality research animals.

With the availability of such animals, studies of host-parasite interrelationships, vitamin metabolism, immunology, radiation effects, etc. are receiving increased emphasis. In disease studies, these animals offer the best opportunity to observe true physiological interactions. Any pathological deviation is more easily attributable to a specific agent in an animal free of other contaminations. The animal breeder has also benefited because "seed" stock of the highest quality is available for commercial herd production.

Exactly when the concept of germfree life originated cannot be stated with certainty. But such a concept must have been considered from the time microbes became incriminated in diseases of man and animal. Such a "pure culture" concept must also date to the first realization that study of specific entities requires their isolation from their natural complex before the demands of the experimental method are met.

The first experiment on germfree life was by Duclaux in 1885. But this work could have gone unnoticed had not Pasteur, in the introduction, stated his belief that microbes were necessary to life. This theory received immediate attention from opposing schools of thought.

Experimental evidence supporting Pasteur was offered by Schottelius. Although he contributed much to the rearing methods for germfree chickens, inadequate diets prevented his obtaining successful

survival rates. Opposed to an obligatory role of microbes for life, was Nencki. He was impressed by the ability of various flora to produce toxic substances and on theoretical grounds (1886) first attacked Pasteur's theory. About 10 years later, Nuttall and Thierfelder, attempting to corroborate Nencki, used mammals in germfree research for the first time. Once again, inadequate diet prevented valid conclusions. They did observe for the first time a mammalian characteristic still of concern today; the greatly enlarged cecum filled with a copious amount of fluid.

Metchnikoff championed the theory that body flora can actively fight against the host. One of his students, Cohendy, along with Wollman, in 1910, successfully proved this using germfree chickens of good quality. Credit for realizing the value of the germfree animal as a research tool belongs also to Cohendy.

Shortly prior to World War I, Küster raised two germfree goats for more than two weeks and settled the issue in favor of those supporting germfree life. His basic environmental design is used today.

The first investigator to undertake extensive germfree research was Glimstedt in Sweden. He demonstrated an inhibited development and lower content of lymphoid tissue in germfree guinea pigs. This was especially true for organs normally in close association with the body's flora, i.e., small intestine. Balzan in 1937 studied nutrition in chickens. In 1948, the Swedish worker Gustaffsen raised germfree rats. Kyoichi and Miyakawa in Japan have also added to the technology

of germfree research. In studying wound healing and inflammatory reactions, Miyakawa has contributed much to their understanding.

The University of Notre Dame initiated in 1928 the first long-range program investigating the problems of germfree life. It was here that Reynier, in 1942, developed the stainless steel isolator within which much of the early work was conducted. But it remained for Trexler and Reynolds (1957) to open the field for commercial application with their development of the flexible film isolator. Successful rearing of various larger vertebrates has only occurred within the past few years. These include the dog (Griesemer, 1963); pig (Landy et al., 1961; Waxler, 1961) and sheep (Smith, 1961).

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Isolator materials required for cesarean removal of germfree cottontails.

Plastic mouse box, metric ruler, ear punch, gram scale (one of each).

Scalpels, scissors, thumb forceps, eye droppers and 6-cc syringes (two of each).

Kelly hemostatic forceps (4-6).

100-cc Erhylemeyer flasks with water (4).

Autoclavable latex nipples* (Lotol-6982U; Naugatuck Chemical Corp., Naugatuck, Connecticut) (4).

Thioglycollate vials (20).

Paper towels (100).

As many gauze pads, cotton swabs, and blood agar plates as required.

* As prepared by Pleasants (pers. comm.), Lobund Institute, Notre Dame University, South Band, Indiana (Appendix E).

Appendix E

PREPARATION OF AUTOCLAVABLE LATEX NIPPLES*

For younger cottontails, a 1/8" glass rod drawn out to a point or a wooden applicator stick sharpened at one end sufficed to form the nipple "core".

1. Stir the Lotol.
2. Dip the core into vinegar.
3. Dip into Lotol until desired thickness is reached.
4. Dip into vinegar and invert overnight.
5. Place in distilled water for 1 hour.
6. Dry in oven @ 78^{o} (or overnight at room temperature).
7. Boil in distilled water for 7 minutes.
8. Remove nipple slowly from model and dry for 3 hours.

* Silicone infant rodent nipples are also available from R. E. Darling Co., P.O. Box 666, Gaithersburg, Maryland.

Appendix F

COTTONTAIL HEMATOLOGY

Hematological data for wild mammals, especially for healthy individuals, are scarce. Only two reports were found on the blood values of the Mearnsii cottontail (Geise, 1957; Youatt et al., 1961). Such data may be used to measure environmental stress. Sealander (1961) demonstrated significantly lower hemoglobin concentrations and packed cell volumes in deer mice parasitized by bot flies. Since cottontails are parasitized by various flies, i.e., bots and flesh flies, a knowledge of the host's blood parameters may prove valuable.

Materials and Methods: Whole blood was collected by cardiac puncture or withdrawn from a peripheral ear vein. Only oxygenated blood was used for the cardiac samples. A one inch 22-gauge hypodermic needle was attached to a 6-cc syringe for the cardiac punctures and to a 2-cc syringe for the ear vein bleedings. Two of the 5-6 cc of the withdrawn cardiac blood were quickly added to an EDTA*-coated blood vial and used for the total hemogram determinations. Cottontail blood clotted rapidly and required flushing the small syringe with anticoagulent. Care was taken to prevent blood dilution by the anticoagulent. Juveniles too small to bleed by cardiac puncture were killed by a blow at the base of the skull and the still-beating heart exposed. If blood still could not be withdrawn, the heart was cut and as much blood as possible caught in an EDTA-coated vial. Cardiac or ear vein samples

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* Dipotassium Ethylenediamine tetraacetate (tablets): Cambridge Chemical Products, 5850 Chase Road, Dearborn, Michigan.

usually could not be taken from living animals which weighed less than 100 grams. The smallest living animal successfully bled by cardiac puncture was eight days old and weighed 85 grams.

The following were run in duplicate for the ear vein and cardiac blood samples and constituted the hemogram. Total red (RBC) and white (WBC) blood cell counts followed standard procedures (Benjamin, 1961) using a Spencer Brightline Hemocytometer. Packed cell volumes or hematocrits (Hct) were determined in microhematocrit capillary tubes spun in an International hematocrit centrifuge. Hemoglobin concentration (Hb) was determined by the cyanmethemoglobin method (Banjamin, 1961) using a Bausch and Lomb Spectronic-20.

Blood smears for differential white cell counts (relative percentages of heterophils, eosinophils, lymphocytes, monocytes and basophils) were stained with a buffered Wright's stain (Camco Quick Stain: Cambridge Chemical Products, Scientific Products). Total time for staining required three minutes with 15 seconds in the stain and 2½ minutes of buffering in distilled water. The slides were washed in tap water and then air dried. A total of 200 white blood cells were counted per blood smear.

Results and Discussion: Cottontails when handled demonstrated a wide range of excitability. Heart beats per minute were used as an index of physiological excitability (Table 27). Most noticeable were the wide extremes in counts for the same animal and between animals. The two most visibly excitable animals, Nos. 04 and 65, sometimes had such rapid and erratic heart beats that they were non-countable. It seems

Table 27. Heart beats per minute for adult female cottontail rabbits.

Rabbit number	Number of counts	Mean	Range
04	8*	79	(72-88)
64	11	65	(59-76)
65	6*	85	(61-117)
66	12	80	(62-101)

* Additional counts not included because of high and erratic heart beats.

that stress induced by handling varies for a cottontail and between cottontails.

Hemograms

Adults: The hemogram subtotals for seven males and six females (Table 28) were considered similar and combined into an adult category. Red cell counts and hemoglobin concentrations were most constant and had the narrowest range of values. Ear vein hematocrits had generally higher values than cardiac hematocrits. Youatt et al. (1961) reported similar hemogram values for adult cottontails, but Geise (1957) found lower mean red and white blood cell counts.

Subadults: Hemogram subtotals for five males and three females were combined into a subadult category (Table 29). The red cell counts and hemoglobin values were again the most constant parameters.

Juveniles: Thirty-four juveniles had hemograms recorded (Table 30). The narrowest range of values occurred in red blood cell counts and hemoglobin concentrations. Ear vein hematocrits were higher than cardiac hematocrits. Hemoglobin values were most often missing because of spectrophotometer problems.

Both red and white blood cell counts increased with age (Table 31). Hemoglobin values appeared to be higher in non-adults, but hematocrit values appeared to be similar for all ages. The increase in red and white blood cells with cottontail age was also reported by Youatt et al. (1961), as was the similarity of adult and non-adult

Table 28. Hemograms for adult cottontail rabbits.

Number	Weight	Month of sample	RBC 10^6/cmm	WBC 10^3/cmm	Hb gm/100 ml	Hct (%) Cardiac blood	Hct (%) Ear vein blood
Males							
01	1420	July	5.6	8.7	11.0	37.5	--
64	1360	August	6.2	10.9	10.6	43.0	47.0
	1380	November	5.5	8.7	10.1	47.0	35.0
02	1163	August	7.0	12.1	11.5	47.5	45.0
06	1050	September	5.8	5.5	10.8	42.0	49.5
48	1352	July	5.7	7.1	12.2	42.0	37.0
49	1307	November	6.0	5.6	14.0	41.5	44.0
50	1160	November	9.4	7.5	14.8	60.0	47.0
Subtotal Mean			6.4	8.3	11.9	45.0	43.5 *
Females							
65	1260	July	7.0	8.0	10.6	40.5	--
	1150	August	6.2	8.4	11.3	39.0	41.5
	1447	September	6.1	9.3	14.0	41.0	44.0
	1220	November	6.2	5.5	9.8	34.0	42.5
66	1590	July	5.8	17.1	9.5	39.0	--
	1620	August	5.9	8.6	--	34.0	38.5
	1580	November	6.3	6.3	9.1	32.5	38.0
16	1405	September	6.2	6.5	12.9	40.0	40.5
04	1460	July	6.3	9.4	10.0	46.0	--
	1476	August	7.8	7.3	11.7	48.5	55.0
	1380	November	7.5	14.5	9.5	40.5	47.5
05	1580	July	4.9	7.8	10.0	31.5	42.0
	1476	August	5.7	7.2	11.3	39.0	43.0
	1490	September	5.1	7.2	10.6	47.5	41.5
09	1010	September	5.8	4.8	11.1	40.5	45.0
Subtotal Mean			6.6	8.5	10.8	39.5	44.1 *
Adult range:			(4.9-9.4)	(4.8-17.1)	(9.1-14.8)	(31.5-48.5)	(35.0-55.0)
Adult mean:			6.5	8.4	11.4	42.0	44.0 *

* Hematocrit mean values based on additional samples not included above.

Table 29. Hemograms for subadult cottontail rabbits.

Number	Weight	Month of sample	RBC 10^6/cmm	WBC 10^3/cmm	Hb gm/100 ml	Hct (%) Cardiac blood	Hct (%) Ear vein blood
Males							
658-BT	532	July	5.5	7.8	10.6	42.0	--
07	540	July	6.0	6.8	10.7	44.0	45.0
08	850	--	5.4	4.3	9.9	50.0	33.5
06	936	July	6.7	5.3	14.1	56.5	--
	995	August	6.5	6.9	12.8	46.0	50.0
12	765	July	5.5	2.8	12.2	36.0	41.0
Subtotal Mean:			5.9	5.7	11.7	45.5	42.5
Females							
02	930	July	5.7	8.8	9.7	46.1	46.5
10	470	July	6.5	6.0	10.3	43.5	41.5
658-UL	589	July	4.4	14.7	10.3	32.5	--
Subtotal Mean:			5.6	9.8	10.1	40.5	44.0
Subadult range:			(4.4-6.7)	(2.8-14.7)	(9.7-14.1)	(32.5-56.5)	(33.5-50.0)
Subadult mean:			5.8	7.0	12.4	44.0	42.0

Table 30. Hemograms for juvenile cottontail rabbits.

Rabbit number	Weight (grams)	Age (days)	RBC 10^6 /cmm	WBC 10^3 /cmm	Hb gm/100 ml	Hct (%) Cardiac blood	Hct (%) Ear vein blood
6512-UL	52	4	4.2	2.8	10.8	33.0	--
6610-0	85	8	3.8	4.1	12.6	41.5	--
672-LR	50	8	--	--	--	--	36.0
671-0	--	9	--	--	--	44.0	--
6611-LL	155	10	4.8	2.8	--	41.0	50.0
668-UR	115	13	4.0	6.2	11.4	41.0	--
6610-UR	133	16	4.3	2.0	13.1	40.5	51.0
6614-0	110	17	4.4	3.2	--	52.0	--
6614-UL	95	17	4.3	2.7	--	56.0	--
6613-UL	151	21	5.1	3.4	--	39.5	--
6613-BT	131	21	4.2	7.9	--	44.0	--
665-0	167	23	--	--	--	33.0	45.0
6518-LR	172	24	4.1	5.1	11.0	36.5	--
6518-LL	178	24	6.0	5.2	14.0	52.5	--
6610-LL	202	26	5.2	4.2	12.7	53.5	51.5
6610-UL	220	27	5.0	4.4	11.2	47.0	47.5
658-LR	--	28	4.7	13.7	--	42.0	--
664-LR	177	28	--	--	--	--	36.0
6613-UR	220	30	4.4	10.4	--	34.5	--
669-UR	332	30	5.9	7.2	11.9	39.0	43.0
66-A	240	33	5.5	4.3	--	40.5	46.5
664-UL	201	33	5.0	7.1	11.3	44.5	--
6610-LR	309	35	4.9	5.7	11.8	42.0	50.0
6613-LL	369	38	4.5	5.1	--	40.0	44.5
6613-LR	319	38	4.1	6.5	--	44.0	--
6517-LL	368	45	4.2	4.0	11.1	38.5	--
6514-0	233	47	6.5	11.5	14.0	47.0	--
664-0	347	49	5.0	4.8	10.8	40.0	--
6516-0	378	51	5.0	9.5	12.7	42.5	--
6613-LR	437	52	5.2	11.6	14.2	44.5	45.5
6514-UR	203	57	5.5	4.6	13.5	50.5	--
6517-LR	145	58	4.8	5.8	12.9	43.0	--
656-BL	436	74	5.4	5.5	13.6	47.0	--
Juvenile range:			(3.8-6.5)	(2.0-13.7)	(9.0-14.2)	(30.5-56.0)	(36.0-51.5)
Juvenile mean:			4.8	5.8	12.2	42.5	45.5

Table 31. Mean hemogram values for adult, subadult and juvenile cottontails.

	RBC 10^6/cmm	WBC 10^3/cmm	Hb gm/100 ml	Hct (%) Cardiac blood	Hct (%) Ear vein blood
Adults	6.5	8.4	11.4	42.0	44.0
Subadults	5.8	7.0	12.4	44.0	42.0
Juveniles	4.8	5.8	12.2	42.5	45.5

hematocrits. In domestic rabbits, white blood cells also increased with rabbit age (Cheng, 1930).

Although hemograms are vital physiological parameters, care may be necessary in interpreting those of cottontails. Schermer (1967) considered the domestic rabbit to be unreliable in terms of constancy of blood factors. Among the factors implicated as causes of fluctuation in blood values were seasonal influences and feeding patterns. It was for the former reason that the dates of the blood samples were included in the previous cottontail hemograms. To what extent outside factors should be considered in the interpretation of cottontail hematology has yet to be determined.

Blood Cell Types

Red Blood Cells: A characteristic feature of the blood of domestic rabbits is the crenated erythrocyte (Schermer, 1967), which also occurs in cottontails (Fig. 48). Slight amounts of polychromasia occurred in cottontail blood, and were seen as a light blue-to-pink color in various red blood cells. Polychromasia in domestic rabbit blood was considered to be due to the presence of reticulocytes (Schermer, 1967).

Heterophils (Pseudoeosinophils): These cells are comparable to the neutrophil in other species and are unique in cottontail blood. Neutrophils of other species rarely show granules, but the cottontail heterophil contained round, red granules in a pinkish cytoplasm (Fig. 49). The granules were at times rod-shaped, but were never very numerous. The nucleus was lobulated and stained a light purple to blue.

Eosinophils: This cell was larger than the heterophil. Its rod-shaped granules were larger, thicker, stained a bronze-like hue and were densely packed into the cell (Fig. 50). The nucleus was blue and segmented, often in the shape of a horseshoe. No problem existed differentiating an eosinophil and heterophil once the former was observed.

Basophils: Although the basophil was similar in size to the heterophil, it's granules stained intensely blue (Fig. 48). The nucleus was relatively large, stained pale blue and, at times, was hidden by the granules.

Lymphocytes: Both a small form (Fig. 51) and a large form were seen. The former, not much larger than a red blood cell was circular; the nucleus staining dark blue. Occasionally, a small rim of pale blue cytoplasm was present. The large form, the size of a heterophil, stained the same as the smaller form with occasional basophilic granules present in the cytoplasm (Fig. 52).

Monocytes: When present in the classical form, identification was no problem. The monocyte was the largest of the leukocytes and contained a large nucleus with visible strands of chromatin (Fig. 51). A large amount of greyish-blue cytoplasm was present as were vacuoles. Basophilic granules were occasionally present in the cytoplasm.

More often than not, transitional forms were found which could not be easily identified as either a lymphocyte or a monocyte (Fig. 53). Schermer (1967) also mentioned these transitional cells for

domestic rabbits. When classifying transitional cells, the main characteristics I looked for were the amount of cytoplasm and whether or not vacuoles were present. Large amounts of vacuolated cytoplasm classified the cell as a monocyte.

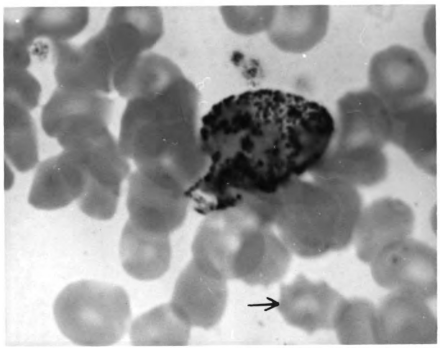

Figure 48. Cottontail blood smear showing a basophil and a crenated red blood cell (arrow). Buffered Wrights. X1875.

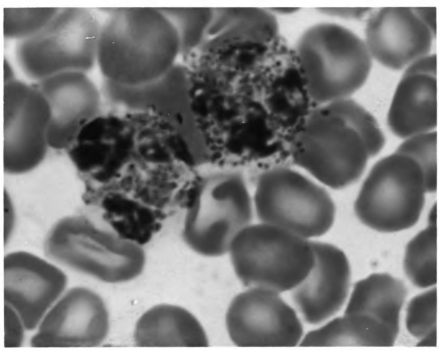

Figure 49. Blood smear showing two heterophils. The round eosinophilic granules are evident. Buffered Wrights. X1875.

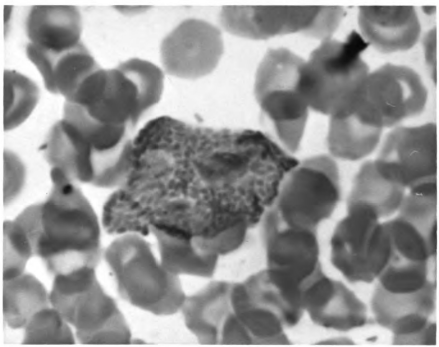

Figure 50. Blood smear showing an eosinophil containing numerous large rod-shaped granules. Buffered Wrights. X1875.

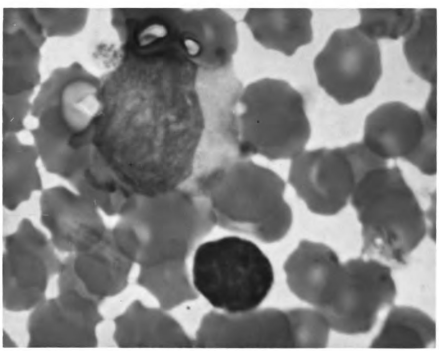

Figure 51. Blood smear showing a monocyte at the upper left with a small lymphocyte below it. Buffered Wrights. X1875.

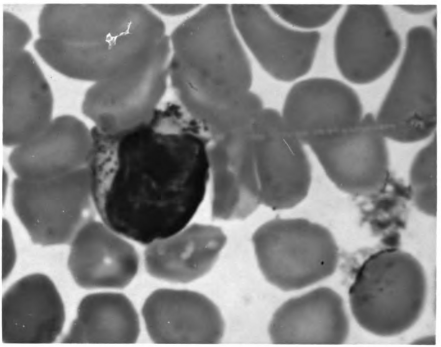

Figure 52. Blood smear showing a large lymphocyte with basophilic granules in the cytoplasm. Buffered Wrights. X1875.

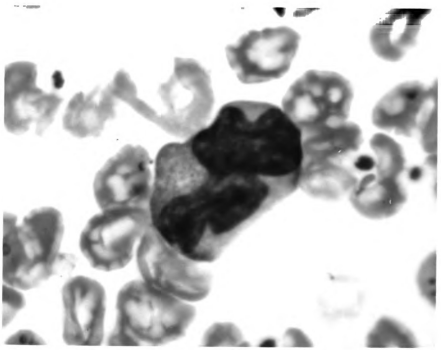

Figure 53. Blood smear showing a transitional leukocyte classified as a monocyte. Buffered Wrights. X1875.

Adult Differential Leukocyte Counts

Males:

Cardiac Blood: Differential leukocyte counts for male cottontails (Table 32a) showed some rabbits having wide variations in heterophil and lymphocyte numbers. Rabbit No. 38 (August) had nearly all lymphocytes in the smear, while Nos. 47 and 48 had heterophils in the majority. Eosinophils and basophils had the narrowest range of values while heterophils and lymphocytes had the widest range.

Ear Vein Blood: All cell types had percentages similar to those of cardiac blood, except for eosinophils which had higher mean values (Table 32b). All cell types had narrower ranges than found in cardiac blood.

Females:

Cardiac Blood: Some females had extremely high lymphocyte values, while others had high heterophil percentages (Table 33a). Heterophils and lymphocytes had the widest range of values.

Ear Vein Blood: Narrower ranges for all cell types, as compared to cardiac blood, occurred with higher heterophil percentages present (Table 33b).

In Summary: For both sexes, ear vein blood had higher heterophil values than cardiac blood with similar values for the other four cell types. Ear vein differentials always had narrower ranges for the five types of leukocytes. It was not determined why certain animals

Table 32. Differential leukocyte counts for adult male cottontails.

Rabbit number	Month of sample	Leukocyte Differential Counts (%)				
		Heterophils	Eosinophils	Lymphocytes	Monocytes	Basophils
(a) Cardiac Blood						
13	August	23	4	73	0	0
	September	22	7	50	21	0
	November	46	5	37	11	1
24	August	4	1	59	36	0
	November	44	4	41	9	2
36	July	25	12	46	16	1
38	August	2	2	96	0	0
	November	37	5	50	8	0
46	July	25	2	42	27	4
47	August	59	3	23	13	2
48	July	56	3	20	21	0
49	August	33	5	51	11	0
	November	31	14	40	13	2
50	August	41	3	49	7	0
	September	16	6	47	30	1
	November	35	3	54	7	1
51	July	28	3	55	13	1
Range:		(2-59)	(1-14)	(20-96)	(0-36)	(0-4)
Mean:		31	5	49	14	1
(b) Ear Vein Blood						
13	August	18	4	77	0	1
	November	43	5	43	8	1
24	November	50	3	37	8	2
49	July	44	3	30	23	0
	November	37	10	31	20	2
50	November	42	5	46	7	0
Range:		(18-50)	(3-10)	(30-77)	(0-23)	(0-2)
Mean:		39	5	44	11	1

Table 33. Differential leukocyte counts for adult female cottontails.

Rabbit number	Month of sample	Differential Leukocyte Counts (%) Heterophils	Eosinophils	Lymphocytes	Monocytes	Basophils
(a) Cardiac Blood						
04	February	26	14	35	24	1
05	September	20	4	58	17	1
09	September	29	16	40	12	3
20	August	8	18	68	4	2
	November	28	5	54	13	0
23	August	21	9	62	6	2
	November	26	3	62	9	0
27	August	14	16	65	4	1
	November	39	1	36	23	1
30	August	21	2	76	1	0
	November	44	3	44	8	1
35	August	9	4	86	0	1
	November	24	2	55	19	0
39	August	40	8	50	0	2
	November	22	10	57	9	2
40	August	1	0	98	0	1
	November	39	9	45	6	1
43	August	8	10	80	1	1
	November	33	13	45	8	1
46	August	12	5	83	0	0
	November	28	1	51	19	1
48	August	0	1	98	0	1
	November	22	6	48	23	1
49	August	0	1	96	0	3
	November	13	7	61	14	5
64	November	37	3	40	20	0
65	September	33	12	34	20	1
Range:		(0-44)	(0-18)	(34-98)	(0-24)	(0-5)
Mean:		22	7	60	10	1

Table 33 (Cont.) Differential leukocyte counts of adult female cottontails.

Rabbit number	Month of sample	Differential Leukocyte Counts (%) Heterophils	Eosinophils	Lymphocytes	Monocytes	Basophils
(b) Ear Vein Blood						
04	February	50	0	27	23	0
09	September	35	2	56	6	1
10	May	31	2	49	16	2
20	November	29	8	50	11	2
23	November	28	2	65	4	1
29	November	43	2	35	19	1
30	September	43	4	48	4	1
	November	51	5	39	4	1
35	November	24	3	68	5	0
39	August	8	8	83	0	1
	November	26	3	63	7	1
40	November	31	6	55	6	2
43	November	32	10	53	4	1
46	August	30	6	62	0	2
	November	32	1	47	20	0
48	August	28	2	69	0	1
	November	20	4	68	7	1
65	February	35	4	47	13	1
Range:		(8-51)	(0-8)	(27-83)	(0-23)	(0-2)
Mean:		32	4	55	8	1

had unusually high heterophil or lymphocyte values. Males always had higher mean heterophil values regardless of whether blood was sampled from the ear or heart. The mean values for the other four cell types were similar in both sexes.

In order to obtain adult differential leukocyte values, the male and female subtotals were combined (Table 34). The only report found for adult cottontail differentials was that of Youatt et al. (1961). They found, for cardiac blood, values of 28% (range: 13-52) for heterophils, 3% (0-8) for eosinophils, 65% (42-78) for lymphocytes, 3% (1-6) for monocytes and 1% (0-2) for basophils. Their findings agreed with those in Table 34a. Their narrower range of values was probably due to a small sample of just three rabbits. The greatest differences between their results and mine were in the lymphocyte and monocyte values. This would be expected due to the difficulty of classifying the transitional forms between the two cell types. But, if the lymphocyte and monocyte values are combined into a single category, almost the same value occurs in both reports.

Adult Differential Leukocyte Counts from Cardiac and Ear Vein Samples Taken at the Same Time

Certain cottontails had cardiac and ear vein blood samples taken within five minutes of each other (Table 35). Cardiac samples were taken first in males with the ear vein blood sampled first in females to eliminate sample sequence as an influence on blood counts. One male, No. 49, and five of the fourteen female comparisons had widely

Table 34. Adult cottontail differential leukocyte counts from cardiac and ear vein blood.

	Differential Leukocyte Counts (%)				
	Heterophils	Eosinophils	Lymphocytes	Monocytes	Basophils
(a) Cardiac Blood					
Mean (range):	26 (0-59)	6 (0-18)	56 (20-98)	11 (0-36)	1 (0-5)
(b) Ear Vein Blood					
Mean (range):	34 (8-51)	4 (0-10)	52 (27-83)	9 (0-23)	0 (0-2)

Table 35. Cardiac and ear vein differential leukocyte values derived from samples taken at the same time from adult cottontails.

Rabbit number	Month	Sample source*	Differential Leukocyte Counts (%) Heterophils	Eosinophils	Lymphocytes	Monocytes	Basophils
Males							
13	August	C	23	4	73	0	0
		E	18	4	77	0	1
	November	C	46	5	37	11	1
		E	43	5	43	8	1
24	November	C	44	4	41	9	2
		E	50	3	37	8	2
49	July	C	33	5	51	11	0
		E	44	3	30	23	0
50	November	C	35	3	54	7	1
		E	42	5	46	7	0
Females							
04	February	C	26	14	35	24	1
		E	50	0	27	23	0
09	September	C	29	16	40	12	3
		E	35	2	56	6	1
20	November	C	28	5	54	13	2
		E	29	8	50	11	1
23	November	C	26	3	62	9	0
		E	28	2	65	4	1
30	November	C	44	3	44	8	1
		E	51	5	39	4	1
35	November	C	24	2	55	19	0
		E	24	3	68	5	0
39	August	C	40	8	50	0	2
		E	8	8	83	0	1
	November	C	22	10	57	9	2
		E	26	3	63	7	1
40	November	C	39	9	45	6	1
		E	31	6	55	6	2
43	November	C	33	14	45	8	1
		E	32	10	53	4	1
46	August	C	12	5	83	7	1
		E	30	6	62	0	0
	November	C	28	1	51	19	1
		E	32	1	47	20	0
48	August	C	0	1	98	0	1
		E	28	2	69	0	1
	November	C	22	6	48	23	1
		E	20	4	68	7	1

* C - cardiac blood.
E - ear vein blood.

differing values. Excessive differences between cardiac and ear vein samples existed for the heterophil values for Nos. 04, 09 and 39, and the monocyte values for Nos. 09, 35, 46 and 48. The reason for these differences is unknown.

Seasonal Differences in Adult Differential Leukocyte Counts

Mention must be made of the unexplained differences for monthly differential values of the same animal. Thus, for the August and November cardiac values of males Nos. 13, 24 and 38 (Table 32a), and females Nos. 20, 27, 30, 35, 40, 43, 46, 48 and 49 (Table 33a), increased heterophil and decreased lymphocyte values occurred in the November sample. The ear vein samples of August and November for male No. 13 (Table 32b) and female No. 39 (Table 33b) also demonstrated the heterophil and lymphocyte differences.

These changes appeared real and not due to faulty technique or sample sequence. The August and November cardiac samples for males Nos. 49 and 50 (Table 32a), females Nos. 23 and 39 (Table 33a), and the ear vein samples for females Nos. 46 and 48 (Table 33b) did not demonstrate the increased heterophil values. Seasonal differences may thus exist for cottontail differential leukocyte values.

Juvenile Differential Leukocyte Counts

Values were obtained only from cardiac blood (Table 36). When compared to the adult values (Table 34a), juvenile monocyte values were twice as high. But, when lymphocyte and monocyte values were summed, values of 72 and 67 percent were obtained for juveniles and

Table 36. Differential leukocyte values obtained from cardiac blood of juvenile cottontails.

Rabbit number	Age (days)	Differential Leukocyte Counts (%) Heterophils	Eosinophils	Lymphocytes	Monocytes	Basophils
Males						
613-UL	21	33	0	33	34	0
613-UR	30	6	2	44	47	1
Females						
614-0	17	39	1	40	20	0
614-UL	17	42	1	33	23	1
613-LL	28	23	5	54	16	2
Sex Unknown						
613-BT	21	40	0	37	22	1
613-LR	51	22	2	25	51	0
671-UL	8	0	0	80	20	0
Range:		(0-42)	(0-5)	(25-80)	(16-51)	(0-2)
Mean:		25	> 1	43	29	< 1

adults, respectively. Thus, mean differential leukocyte values appeared to be similar for adult and juvenile cottontails provided the lymphocytes and monocytes are combined.

Juvenile differential values from cardiac blood were reported by Youatt et al. (1961). Their values of 42% (24-67) for heterophils, 1.5% (0-9) for eosinophils, 52% (27-73) for lymphocytes, 4% (0-8) for monocytes and 1% (0-4) for basophils, differed from those reported in Table 36.

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Appendix G

COTTONTAIL SERUM AND PLASMA PROTEINS

Changes in the electrophoretic distribution of the blood proteins have been noted in wild animals suffering from a variety of parasitic and infectious diseases (Payne et al., 1967). This technique could also find application for the screening of parasitic damage in living cottontails.

With the possibility of Eimeria steidae infection in cottontails, such protein values could prove of value. Dunlop et al. (1959) examined the serum protein fractions of domestic rabbits infected with E. steidae, and found a significant increase in the beta and gamma globulin fractions of infected animals.

Materials and Methods

Cellulose acetate membrane, accommodating eight samples simultaneously, were used with the microzone electrophoretic cell (Model R-101; Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, California). The 0.25 microliter samples were taken from the mid-portion of the plasma section of a micro-hematocrit capillary tube and from a drop of serum. The membranes were read on a Model RB Analytrol with Model R-102 Microzone Scanning attachment (Beckman Instruments, Inc.). Protein fraction nomenclature followed the classical scheme of naming the fastest migrating fraction as albumin with the sequentially slower globulins called alpha-1, -2, beta-1, -2, and gamma, respectively.

Results and Discussion

Similar protein patterns were observed in both serum and plasma samples. Comparable peaks for the albumin, alpha-1, -2, beta-1, -2 and gamma globulin fractions were present in both types of samples except for an unidentified globulin in serum that occurred between the beta-2 and gamma globulin fractions. Because no identifying tests were done, this serum fraction was merely called an unidentified fraction. The possibility that this fraction represented residual fibrinogen from incomplete clot formation was not investigated, but it was located in the same position as was plasma fibrinogen.

The electrophoretic protein values for cardiac serum, cardiac plasma, and ear vein plasma of adult males and females are listed in Table 37. Regardless of the sample type, males always had lower alpha-1, -2 and fibrinogen fractions, but higher gamma globulin values. These male and female values were combined into an adult category. When the cardiac serum protein values for juveniles (Table 38) were compared to those of adults, the greatest difference was in the alpha-1 globulin fraction, juveniles having a lower value.

Schermer (1967) summarized serum protein percentages for domestic rabbits and when compared to those of adult cottontails, similar values for all fractions were present in both species except for higher beta-globulin values in cottontails.

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Table 37. Adult cottontail serum and plasma protein fractions.

Protein fraction	Protein percentages in: Cardiac serum Males n = 4	Females n = 13	Adult mean	Cardiac plasma Males n = 3	Females n = 8	Adult mean	Ear vein plasma Males n = 4	Females n = 10	Adult mean
Albumin	58.6	61.6	60.1	59.8	59.1	59.5	58.3	56.4	57.4
Alpha-1	4.3	5.7	5.0	3.9	5.5	4.7	4.1	6.1	5.1
Alpha-2	6.6	7.8	7.2	5.1	6.7	5.9	5.4	6.9	6.2
Beta-1	7.1	6.3	6.7	7.5	7.0	7.3	7.3	7.8	7.6
Beta-2	7.4	7.4	7.4	6.8	7.4	7.1	7.9	7.6	7.8
Unidentified Globulin	9.0	6.1	7.6	---	---	---	---	---	---
Gamma	6.9	5.3	6.2	7.4	4.2	5.8	6.5	3.8	5.2
Fibrinogen	---	---	---	9.4	10.2	9.8	10.7	11.3	11.0

* n - sample size.

Table 38. Juvenile cottontail serum and plasma protein fractions.

Protein fraction	Protein percentages in: Cardiac serum Males n = 2	Females n = 1	Juvenile mean	Cardiac plasma Females n = 1	Ear vein plasma Females n = 1
Albumin	61.7	58.1	59.9	58.6	60.1
Alpha-1	2.3	3.3	2.8	4.1	4.3
Alpha-2	9.0	5.7	7.4	7.0	7.5
Beta-1	7.8	8.0	7.9	9.8	8.0
Beta-2	8.0	8.2	8.1	8.9	8.7
Unidentified Globulin	6.6	9.7	8.2	---	---
Gamma	4.9	6.9	5.9	3.6	3.0
Fibrinogen	---	---	---	8.0	8.4

* n - sample size.

Appendix H

SEASONAL WEIGHT CHANGES FOR ADULT COTTONTAILS

Seasonal Fluctuations in Weight: Wild cottontails have had their yearly weight fluctuations recorded. Elder and Sowls (1942) showed a significant loss of weight to occur during the winter-spring period for adult males, with a parallel decrease for adult females. Haugen (1942) demonstrated the lowest weights for adult females to occur during the winter, while males had their lowest weights during the winter and breeding season. Lord (1963) demonstrated the breeding season to be the period of lowest weight for adults.

The penned adult females used in the present study also demonstrated a seasonal fluctuation in weight (Table 39). The year was divided into three periods of four months each with November through February considered the period of greatest hardship because of adverse weather. Within each period, the mean weight of each animal was based on at least five weighings. All females had their lowest weights in the November-February period which agreed with all the above reports except that of Lord (1963).

From the few comparisons available between adult males and females, the latter averaged heavier in weight, a finding reported in other studies (Elder and Sowls, 1942; Haugen, 1942).

Of interest was the fact that the penned rabbits had weight fluctuations similar to those reported for wild unconfined cottontails, even though the former were provided with quality food all year, a condition assumed not to exist for wild animals. Why this should be

Table 39. Mean seasonal weights for individual penned adult female cottontails.

Rabbit number	November-February	March-June	July-October
04	1450	1484	1487
05	1366	1404	1412
09	1130	1195	1165
65	1338	1432	1348
66	1618	1677	1641

is not known, unless the assumption of a dissimilar quality of food for wild and penned cottontails in winter is false.

Weight Changes After Capture: Eight of nine adult cottontails weighed within a week to 10 days after capture, averaged a 7% weight loss (range: 3-14%). A single female, No. 66, gained weight. This latter animal was the calmest of some 40 adults handled during the study, and consistently had high weights, including the highest recorded for this study - 1844 grams. Within three weeks, most animals regained their lost weight and, over the years, slowly increased in weight. One female, however, did not regain its lost weight and remained, two years later, some 10-20% below its capture weight.

Of six subadult cottontails captured, five averaged an 8% weight gain during the first week. A single animal lost weight and died five months later of unknown causes.

Female Weight Changes Associated With Breeding: During the two day period with the male, female cottontails lost weight 75% of the time (Table 40). Weight losses ranged from 0.4 to 8.4 percent body weight while weight gains ranged from 0.1 to 12.5 percent. Pen observations showed that the buck would give the female constant attention, the pair always being on the move from one end of the pen to the other. This behavior, termed <u>following</u> (Marsden and Conaway, 1963), has the male constantly following the female, sniffing and attempting to mount. Female loss of weight was assumed to be the result of this constant attention from the male. Each of the three males listed in

Table 40. Weight changes in female cottontail breeders after two days with a specific male.

Females	Males: No. 36 — Percent female weight change*: Month of pairing		Males: No. 13 — Percent female weight change*: Month of pairing		Males: No. 12 — Percent female weight change*: Month of pairing	
04	- 4.1	March	- 4.1	May		
	- 0.4	April	- 1.2	May		
	+ 0.1	June				
	- 4.0	July				
	- 1.6	July				
	+ 0.5	August				
05	- 2.3	February	- 2.4	March	- 7.4	June
	- 1.2	March	- 1.8	May	- 2.5	June
	- 1.3	April	+ 5.0	June		
	- 4.4	July	- 3.2	July		
	+12.5	August				
	+ 0.9	September				
09	- 7.7	April	+ 0.3	March		
	- 1.0	April	+ 1.5	March		
	- 2.5	June	- 2.2	May		
	- 2.1	August	- 7.0	July		
			- 3.4	August		
15	- 4.1	August	- 7.9	June		
			- 8.4	July		
16	- 4.7	May	+ 2.1	July		
			- 1.8	August		
65	- 4.2	February	- 5.5	March	- 0.9	May
	+ 0.7	May	- 2.0	April	+ 2.6	June
	- 7.6	June			+ 2.2	July
	- 0.8	July			- 1.2	July
	- 2.8	August				
	- 4.1	August				
66	- 1.0	March				
	+ 2.2	May				
	+ 3.4	June				
	+ 3.3	July				
	- 4.2	July				
	+ 3.8	September				

* - () = percent loss of weight.
+ () = percent weight gain.

Table 40 seemed equal in his "attention" to the female. The percent of negative female weight changes due to these males, Nos. 36, 13 and 12, was 70, 76 and 67 percent, respectively. Female receptivity to a male seemed to vary. Female No. 66, when paired with No. 36, gained weight 67% of the time. But, other females lost weight 80% of the time when paired with this male.

Females placed with males during the early months of breeding season lost weight more often than during the latter months (Table 41). Whether this is due to changes in the male or female attitudes and activities, or both, is not known.

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Table 41. Monthly ratios of negative female weight losses from breeding.

Month	Negative weight changes/ total number of pairings	Percentage negative weight changes
February	2/2	100
March	5/7	71
April	5/5	100
May	6/8	75
June	5/9	56
July	9/12	75
August	6/8	75
September	0/2	0

Appendix I

COTTONTAIL FOOD AND WATER CONSUMPTION AND DEFECATION RATES

Eight adult cottontails, caged indoors, had average daily food (F) and water (W) intakes determined (Table 42). Commercial rabbit ration and water were given freely. Their listed weights are means for the sampling period. Thus, No. 10 averaged 1120 grams in weight during the 31 days of sampling. In general, an animal's weight fluctuated less than 5% during the sampling period. Although adult weights differed widely, the 95% F and W confidence limits overlapped for all rabbits.

When these F and W values were compared to body weight (Table 43), approximately 7% and 16% of body weight were eaten and drunk, respectively, each day. Daily water consumption (in mls) was 2-2½ times that of food consumption (in grams). Rongstad (1966) reported adult cottontails eating one-two pounds of pelleted ration per week which compared to the approximately 700 grams eaten per week in this study.

Two subadults and four adults had daily fecal weights (f) recorded (Table 44). Fecal weights are means of three consecutive fecal samplings and cottontail body weights are mean weights during the three days of fecal sampling. Regardless of body weight, subadult and adult cottontails had a daily fecal production averaging 5 percent of body weight.

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Table 42. Daily food and water consumption of caged adult cottontails fed commercial pellets.

Rabbit number	Sex	Mean body weight (grams)	Number of diet determinations	Mean wt. food eaten (grams)	Mean volume water drunk (ml)
10	F	1120	31	95 (±22)*	190 (±89)*
09	F	1130	31	107 (±20)	217 (±63)
02	F	1150	16	88 (±25)	289 (±179)
65	F	1400	56	107 (±35)	228 (±81)
04	F	1475	56	85 (±51)	195 (±111)
05	F	1480	9	77 (±63)	183 (±166)
64	M	1490	51	99 (±12)	247 (±82)
66	F	1600	55	91 (±49)	211 (±95)

* mean ± two standard deviations (95% confidence limits).

Table 43. Ratios of mean daily food (F) and water (W) consumption to body weight for adult cottontails.

Rabbit number	Percentage body weight eaten	Percentage body weight drunk	W/F
10	8.4	16.9	2.00
09	9.4	19.2	2.02
02	7.6	25.1	3.28
65	7.6	16.2	2.13
04	5.7	13.2	2.29
05	5.2	12.3	2.37
64	6.6	16.5	2.49
66	5.6	13.1	2.31
Mean percentage:	7.1	16.4	2.36

Table 44. Mean daily defecation related to body weight for adult and subadult cottontails.

Rabbit number	Mean body weight (gm)	Daily defecation (f) (gm)	f/body weight (%)
07	850	43	5.0
06	880	45	5.1
02	1120	60	5.3
04	1500	64	4.2
05	1500	64	4.2
66	1800	68	3.7

Appendix J

COTTONTAIL NESTLING MYIASIS FROM WOHLFAHRTIA VIGIL (the grey flesh fly)

Description and Life History of the Fly: The range of the adult fly (family: Sarcophagidae) includes northern United States and southern Canada. Females are larviparous, depositing white larvae on exposed body surfaces of living vertebrates (Faust, 1966). When laid on an undamaged body surface, the larvae migrates a distance before penetrating the skin. Younger host age classes, including human babies, are most susceptible because of their thin skin. A small raised abscess in the subcutaneous tissue is seen at the site of penetration and has an air hole to the exterior.

Mature larvae measure 17-18 mm in length, with their posterior spiracles used for species identification. The larvae complete their development in 7-9 days. After this time, a puparium is formed (9-10 mm) from which an adult emerges in 10-12 days. Adult flies, 11 mm in length, live 30-40 days, with females beginning larvae deposition 11-17 days after emergence from the puparium. Although adults have well developed mouth parts, they are presumed to feed on nectar (Faust, 1966). The first report of cottontail myiasis from W. vigil was that of Johannsen (1926).

Results and Discussion

Incidence of Infestation: In each of three successive years, at least one cottontail litter born in the pens was parasitized by W. vigil with resultant mortality (Table 45). Routine bacteriological

Table 45. Wohlfahrtia vigil infestations of nestling cottontails born in pens.

Year	Number infested	Total born in pens	Percent infested	Date of earliest parasitism
1965	7	22	32	July 1
1966	2	33	7	June 13
1967	3	12	25	June 24

examinations of dead nestlings found no pathogenic organisms. In 1965, three different litters were infested. The high percentage infestation may be due to two of these litters being born in adjacent pens on the same day, the same fly parasitizing both litters. A small total number of young born in the pens in 1967 could account for that year's high percentage infestation. All three animals infested were from the same litter. The 1966 group, with the lowest percentage infection, had nearly half the young born prior to June, generally considered to be the initial month of the fly's activity.

For ease in comparison, the numbers of young and the number of litters parasitized by W. vigil reported by others are listed with those of this study (Table 46). The Wisconsin and Michigan reports are for two and three years, respectively. Besides the small sample size, this study's higher degrees of parasitism could have been due to the closeness of the pens, none of the parasitized litters being over 50 feet apart. Rongstad (1966) suggested that a better indication of such fly parasitism would be to include only those litters born after the initial myiasis. His results would then be 14/41 (34%) and 10/40 (25%). The only other data so alterable is this report's middle value for parasitized litters which would change from 1/5 to 1/4.

Both the earliest (late May) and latest dates (early September) of W. vigil myiasis were reported by Rongstad (1966). But, a letter received at this laboratory (Rose Lake Pathology Laboratory, 1967), inquired about the presence of "white worms" feeding on living

Table 46. Listing of the number of litters and numbers of young parasitized by Wohlfahrtia vigil from all known reports for cottontail nestlings. Percentages in parentheses.

	Beule (1940) Pennsylvania	Yuill and Eschle (1963) Wisconsin	Rongstad (1966) Wisconsin	Present study Michigan
No. litters	3/26 (12)	4/18 (22)*	14/83 (17)	3/4 (75)
parasitized		6/46 (13)*	10/101 (10)	1/5 (20)
				1/4 (25)
No. young	Not given	13/81 (16)	Not given	7/22 (32)
parasitized		17/173 (10)		2/33 (7)
				3/12 (25)

* does not include suspect litters.

cottontail nestlings. Based on the writer's description of the larvae and the cottontail lesions, W. vigil myiasis was diagnosed. This occurred in early October, and assuming our diagnosis to be correct, is now the latest report for such cottontail myiasis.

The youngest animal found to be parasitized had larvae present on day 2, while the oldest was 14 days old. Most nestlings were infested during their first week. Rongstad (1966) also reported parasitisms occurring from birth to day 14. Significant size differences between parasitized and non-parasitized littermates were visible within 2-3 days after infestation. In Figure 54, the lower nestling is the uninfested sibling of the smaller parasitized animal above it. This litter had six of the seven nestlings infested. Three died within three days from large infestations, and two were killed due to their comatose state and imminent death. Seven larvae were removed from the sixth, pictured in Figure 54, and with the uninfested nestling, was returned to the nest. Three days later (age: 5 days), this infested animal was half the weight of the other. When it died (age: 11 days) it weighed 57 grams compared to the 100 gram weight of its siblings. This weight difference was noted in two other litters where the uninfested animals within three days weighed about twice as much as their parasitized siblings. The darker color, characteristic of nestlings doing poorly, from whatever the reason, is also seen in the infested nestling (Fig. 54).

The largest number of larvae removed from a living animal was 35. Yuill and Eschle (1963) reported 40 larvae in one animal. Removal of

Figure 54. Cottontail siblings. The upper nestling parasitized by W. vigil; the lower animal non-parasitized.

larvae too deep to reach with forceps, relied on closing the exit hole with water. The lack of air caused the larvae to crawl out and be grasped with forceps. The minimal larval load necessary for death is unknown for cottontails, but James and Kraft (1964) stated that as few as four maggots can kill fox pups. In the present study, a rapid loss of weight was observed in cottontails with as few as five larvae even though nursing continued after infestation. Nestlings dead from the myiasis had fresh or day-old milk curds in the stomach. Animals with 10 or more mature larvae, if they were to die, did so within 3-5 days. Yuill and Eschle (1963) also reported death to occur within three days.

Death may be related to larval location, the head and abdominal locations being more detrimental than those of the back or extremities. Evidence for this was seen from attempts to 'clean' infested nestlings. One nestling, weighing 135 grams, had 25 larvae removed from its back and legs, with other smaller larvae also present. Three days later, it weighed 187 grams and was recovered sufficiently to place in the pens. Two weeks later it weighed 262 grams. Another nestling, weighing less than 100 grams, had 10-12 small larvae removed from its head, but died two days later, suggesting that location of the larvae may affect the prognosis for host recovery. It is possible, however, that the weight of the nestling at the time of initial infestation is of equal significance to larval location in the host influencing host recovery.

Vaseline was placed over the exit holes of 'cleaned' nestlings to help prevent against secondary bacterial infections.

Gross and Microscopic Pathological Findings: Most parasitisms were found because of the characteristic matting of fur from the tissue exudates that accompanied larval penetrations. Hairs surrounding the entrance hole were matted along their entire length and glossy in appearance. This matting usually occurred in areas of longer hairs, i.e., the back and flanks. When larvae penetrated between the toes, a serosanguinous exudate was frequently present, with the shorter hairs allowing a view of the entrance hole and larvae. Findings of different sized larvae within the same animal suggested multiple infestations separated in time.

Depending on the numbers and duration, tissue damage ranged from the raised swelling of initial penetration, to one where large areas of the skin were entirely separated from the underlying muscle (Fig. 55). Some muscle fibers displayed degenerative changes with hemorrhage separating them. Infiltration by eosinophils occurred between many of the muscle fibers (Fig. 56). Adjacent to the path of the larvae were necrotic areas with cellular debris, hemorrhage, eosinophils and macrophages. Proliferating fibroblasts also occurred under these necrotic areas.

No specific mortalities are given by Rongstad (1966), but Beule (1940) reported loss of two complete litters from the parasitisms. Yuill and Eschle (1963) also recorded the loss of at least three

litters with other individuals also dying. Ten of the twelve cottontails found parasitized in this study died. The remaining two animals were saved by larval removal. Fly-induced mortalities for penned cottontails are circumstantial evidence for W. vigil being an important pathogen. But, according to the letter referred to previously, 15 of 19 young from five different wild litters were killed within 4-5 days indicating a local importance for Wohlfahrtia myiasis. Yuill and Eschle (1963), furthermore, correlated the incidence of Wohlfahrtia myiasis in ranch mink and cottontail populations. An increased mink parasitism was associated with decreased cottontail populations and suggested that in Wisconsin, cottontails may serve as the primary natural host for the flesh-fly.

Larval Pupation: Rearing of the larvae was accomplished by placing 2-3 larvae with a hind leg of a dead nestling. These were kept in a covered jar with air holes provided and partially filled with a dry, sandy soil. In two weeks, adult flies emerged.

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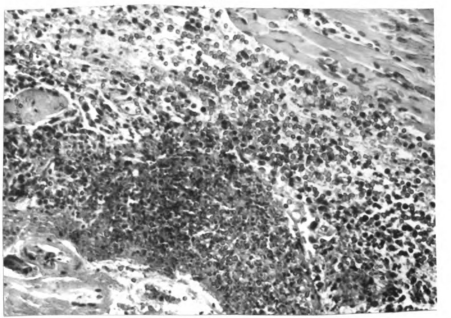

Figure 55. Necrotic area adjacent to larval migrations of W. vigil. The muscle fibers are infiltrated with eosinophils, macrophages and hemorrhage. HE. X188.

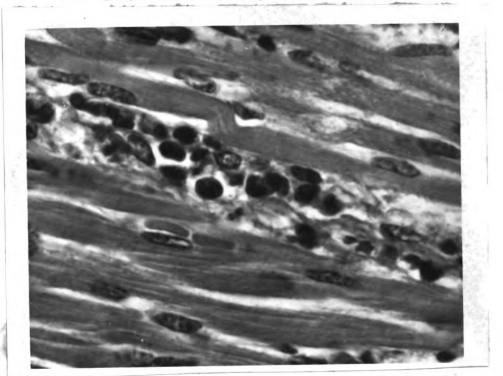

Figure 56. Eosinophils infiltrating between muscle fibers in an area of necrosis associated with W. vigil myiasis. HE. X1875.

Appendix K

HEPATIC LESIONS PRESUMED TO BE ASSOCIATED WITH TAENIA PISIFORMIS INFECTIONS

Few reports of cottontail endoparasitism exist which do not refer to these tapeworm forms. Their presence has been reported in at least 17 states, from California (Herman and Jankiewicz, 1943) to New York (Smith and Cheatum, 1944), and Minnesota (Erickson, 1947) to Alabama (Moore and Moore, 1957).

Description and Life Cycle: Stringer (1966) reported two forms: spherical and elongate with transitional stages between the two extremes. The round form may measure two centimeters across, while the elongate form reaches eight centimeters. A membranous sac, presumed produced by the host as a protective mechanism frequently encloses both forms. As many as nine cysticerci enclosed within a sac were found in the present study. The rostellum contains 38 alternating small and large hooks.

Adult T. pisiformis are found in carnivora, the parasite eggs passing out with the host feces. The intermediate hosts, rabbits, become infected from eating vegetation containing the worm eggs. The primary carnivora hosts are infected by eating the rabbit. Regardless of host, onchospheres hatch from the eggs in either the stomach or small intestine and usually penetrate into blood vessels to reach a final destination. But the circulatory system may be bypassed with the onchospheres penetrating directly through the stomach (Stringer, 1966) or small intestine (McGinnes, 1958). About

20 days later, larvae are present under the liver capsule, breaking out into the abdominal cavity some ten days later. But, larvae have been found in the liver less than 24 hours after the ingestion of eggs (Leonard, 1940).

Results and Discussion

Gross Observations: Cysticerci were found either lying free or attached in the abdominal cavity. The two youngest animals containing cysticerci were 38 and 47 days old. Some correlation between the number of cysticerci and their location was suggested by Hensler (1959). Light infections (1-20 cysticerci) were considered confined to the abdominal cavity. In heavier infections cysticerci may be present in the thoracic cavity, the kidney and even the urinary bladder. In the present study, two animals had cysticerci attached to the pleural surface of the lungs as well as within the lung tissue. These animals had over 25 cysticerci present which agreed with Hensler's suggestion. The greatest number of cysticerci ever found in a cottontail was 250 (Morgan and Waller, 1940).

Gross hepatic lesions were not always present. Eight cottontails were found to harbor cysticerci, but had no gross liver lesions. This may have been due, however, to the larvae having migrated to another organ before escaping into the abdominal cavity. When hepatic lesions were present, they varied in number and, generally, were in the form of linear scars. Circular foci of necrosis were also seen. The lesions were grey in color and sunken below the level of the surrounding tissue. Herman and Jankiewicz (1943) described similar

hepatic lesions and differentiated between such hepatic lesions due to T. pisiformis, and the yellowish raised lesions caused by E. steidae.

Histopathology: Of 12 animals presumed to have hepatic lesions from T. pisiformis, only three had cysticerci and gross and microscopic hepatic lesions. Only these three animals are used below for describing microscopic lesions in the liver. No cysticerci were found in the other nine animals exhibiting characteristic hepatic lesions. All but one of the 12 animals were in apparent good health when killed, the one being an adult, No. 65, that was found dead.

No. 6613-LL (38 days of age): Numerous necrotic areas surrounded by an acidophilic fibrillar material were present (Fig. 57). Large numbers of eosinophils occasionally occurred in dense masses outside the fibrillar material. Hemorrhage, numerous Langhan's giant cells and basophilic amorphic particles were also scattered about. Blood vessels contained numerous eosinophils as well as some lymphocytes.

No. 6514-0 (47 days of age): Necrotic foci were observed and contained what appeared to be fibrin. Eosinophils and lymphocytes occurred both in blood vessels and peripheral to the necrotic foci.

No. 65 (Adult): This animal was emaciated when found dead. Four capsules (2-4 inches in diameter) in the abdominal cavity contained numerous tapeworm scoleces. Many cysticerci were also attached throughout. The liver was scarred and a splenomegaly was present.

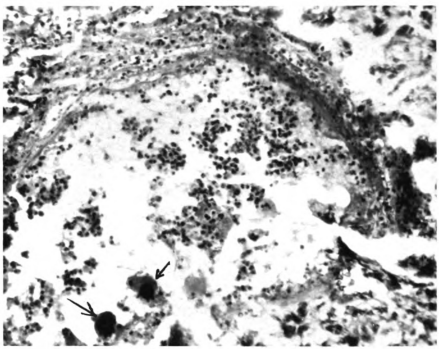

Figure 57. Hepatic necrosis with a surrounding amorphous material encompassing scattered eosinophils. The dark basophilic objects (arrows) were unidentified. HE. X188.

Histologically, normal spleen and hepatic tissue were replaced by collagen and amyloid, indicative of a chronic condition. A similar amyloidosis of the liver from T. pisiformis has been reported in snowshoe hares (Bell and Chalgren, 1943). More recent necroses occurred with and without encapsulation by fibrous connective tissue. In one of the few times noted, lymphocytes seemed to be more numerous than eosinophils. Routine microbiological examinations of the liver were negative.

The only other histopathological descriptions of T. pisiformis in the liver of cottontail rabbits were those of Morgan and Waller (1940). These authors found giant cells, fibrin and leukocytic infiltrations. These findings agreed with those in this study except their identification of the infiltrating leukocyte as a heterophil. No doubt existed in the present report, however, that the leukocytes were eosinophils. Instances were rare where eosinophils were not the prominent leukocyte present regardless of tissue type, including the blood. As described previously in the section on cottontail blood cells, confusion can exist in differentiating these two cell types especially within tissue sections. Although the above lesions were assumed to be due to T. pisiformis, this assumption must remain tentative until further investigation.

The following two animals had liver damage which could not be accounted for. Whether these represented earlier stages of T. pisiformia pathology was unknown, but are reported since the chronic lesions are in young animals.

No. 6610-0 (8 days of age): No cysticerci were present, but scattered necrotic foci were microscopically observed to contain cellular debris and large numbers of eosinophils.

No. 6518-LR (24 days of age): No gross lesions were observed. Chance histological examination of the liver revealed necrotic foci containing cellular debris and fibrin. Masses of lymphocytes, eosinophils and heterophils were also present (Fig. 58).

Pathogenicity: The majority of reports give little pathologic significance to this parasite, but Whitlock (1940) attributed a debilitating effect from the hepatic lesions. Bell and Chalgren (1943), furthermore, blamed these larvae for the death of a juvenile cottontail because of extensive hepatic damage.

The potential for damage by T. pisiformis larvae was evident from the case of lung invasion mentioned previously. This adult animal had a clear fluid within the thoracic cavity. Whitlock (1940) reported a thick fluid in the abdominal cavity of cottontails infected with T. pisiformis. Raised nodules (2 mm in diameter) were scattered on the lung, with one larger cyst measuring 2.5 cm in diameter. Histologically, the cyst contained a tapeworm. Although a Taenia, it could not be determined whether it was T. pisiformis. Congestion and hemorrhage surrounded the worm with many lymphocytes and alveolar macrophages present. Lesser numbers of eosinophils and heterophils also occurred.

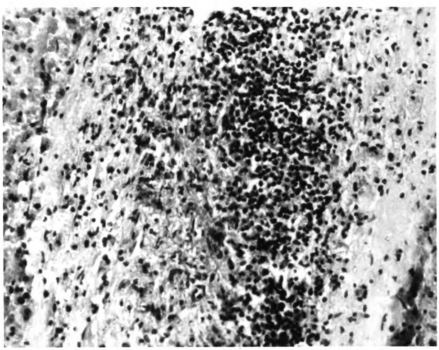

Figure 58. An unexplained area of hepatic necrosis in a 24-day old cottontail. Numerous lymphocytes, eosinophils and heterophils are seen. HE. X188.

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Appendix L

GASTRIC ULCERS

Five of 28 juvenile stomachs examined had reddish-brown areas on the epithelial surface diagnosed as free blood. The oldest rabbit found with such lesions was 85 days old, while the other animals ranged in age from 14-28 days. Several adults were also found with similar gross lesions (see Juvenile Mortality, p. 115). Microscopically, these areas of blood were found to be due to ulcers and contained necrotic debris, scattered red blood cells and an eosinophilic, amorphous material that replaced much of the normal cellular structure (Fig. 59). In one instance, an ulcer reached the muscularis mucosa. Most ulcers displayed an edema of the surrounding area with lymphocytes and eosinophils present at times. In one stomach section, a nematode was observed in the area of an ulcer.

Other workers have reported gastric ulcers and hemorrhages which they considered to be due to the stomach worm Obeliscoides cuniculi (Alicata, 1932; Chandler, 1924; Hensler, 1959; Stringer, 1966). Cottontail deaths have also been attributed to this nematode (MacLulich, 1936). This study's finding of a nematode in the area of an ulcer, suggested that the stomach worm may have been responsible for the observed lesions. But, until pure infections of this worm are given to valid experimental animals, i.e., germfree cottontails, the causal relationship between worm and lesion must remain tentative.

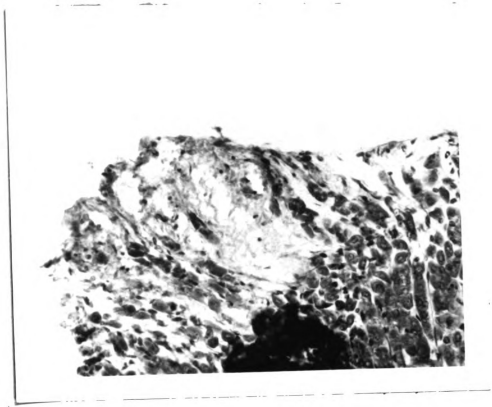

Figure 59. Section of stomach of a juvenile cottontail showing a typical ulcer. HE. X188.

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Appendix M

NEOPLASMS

These findings have already been reported (Lopushinsky and Fay, 1967), but are summarized here since some of the neoplasms were found during the research for the thesis. Neoplasms were found in 19 of 170 cottontail rabbits necropsied at the Wildlife Pathology Laboratory, 1950-1966. Nine tumors were benign; eight fibromas and one lipoma. Six malignant lymphomas, one nephroblastoma and three undiagnosed but apparently malignant tumors, were also found. Of particular interest was the high ratio of malignant to benign neoplasms which contrasted to other reports of much lower frequencies for malignant tumors in wildlife. Gross and microscopic characteristics for the neoplasms were discussed and a case history involving a malignant lymphoma was discussed.

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