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AGE AND DIETARY FACTORS AFFECTING ANTIBODY TRANSFER IN NEWBORN PUPPIES

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by

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INTRODUCTION

An important part of the resistance to disease in an animal is the presence of and the ability to produce antibodies. Antibodies are molecules of protein produced to react with specific foreign substances, or antigens, that have been introduced into the animal. Antibodies compose the gamma globulin fraction of blood serum proteins. The newborn mammal does not have the ability to produce antibodies to foreign protein at an early age; but, antibodies formed in the mother are transferred to the fetus or newborn individual.

The mechanism by which the immune mother transmits immunity to the newborn is of interest for several reasons. The most important of these is its influence on the welfare and viability of the newborn. The susceptible young animal must receive sufficient protection at an early age to enable it to survive and prosper in its natural environment. The immunity transferred by the mother is primarily against infectious agents common in its particular environment. If, for any reason, there is a failure of transmission of immunity the newborn is more susceptible to infectious agents and as such may succumb. The death of the newborn constitutes both an economic loss for the producer and an emotional loss for the pet owner.

Another important reason for studying the transmission of immunity to the newborn is its significance in later active immunization processes in the young individual. The presence of maternally derived antibody may

interfere with the efficiency of immunizing products administered at an early age, yet, an early loss of maternal immunity without prophylactic immunization results in a susceptible individual. The factors which determine the quantity of antibody transfer in the newborn are important in planning and conducting a satisfactory immunization program for young animals.

Factors which influence antibody transfer in the newborn are of interest in basic research. Knowledge of such factors not only contributes to an understanding of the complex phenomena of antibody transfer; but, it also enables a more complete understanding of the physiology of the newborn individual as a whole.

Extensive work has been conducted in several species on factors which influence antibody absorption in the newborn; however, the precise mechanism of antibody transfer in the newborn remains largely unknown. Very little of what is known has been done with the newborn puppy.

The purposes of this research were threefold: 1. to investigate the influence of diet on the postnatal period of antibody absorption in the puppy; 2. to investigate the influence of diet on the development of intestinal proteolytic activity in the newborn puppy; 3. to investigate the influence of intestinal proteolytic activity on the absorption of antibodies by the newborn puppy.

By knowing more of the dietary influence on antibody absorption the mechanism of transfer may be understood more fully. Knowledge of the development of intestinal proteolytic activity and its response to diet will be of value in neonatal dietetics.

LITERATURE REVIEW

The transmission of immunity from the mother to her offspring was verified by the experiments of Ehrlich, 1892. Mice actively immunized against ricin and abrin prior to conception transferred this immunity to the offspring through the milk and placenta. Ehrlich observed that young mice born of non-immunized mothers and allowed to suckle immunized lactating mothers acquired antibodies against ricin and abrin which persisted for 1 to 2½ months. Since the early experiments of Ehrlich much research has been done on the manner and mechanism of the transmission of antibodies from the mother to the offspring.

The transmission of immunity from the mother to the offspring may occur in two ways, prenatally across the placental membranes and postnatally by the intestinal absorption of antibodies contained in the colostrum. The amount of transmission before and after birth varies greatly among species.

Prenatal Transmission of Immunity

Brambell (1958a) has reported that antibody transmission is entirely prenatal in the rabbit with the yolk sac and vitelline circulation being the major route of absorption of antibodies from maternal plasma. Leissring and Anderson (1961) described the prenatal transfer of serum proteins from the mother to the fetus in the guinea pig. They found that the transfer of antibodies to the guinea pig fetus is primarily by way

of the vitelline circulation until 40 days; during later pregnancy and the early postnatal period the intestine is the predominant transfer organ. Bloch et al. (1963) determined that the ratio of $7S\gamma_1$ and $7S\gamma_2$ antibodies in the maternal serum of pregnant guinea pigs is similar to the ratio found in the serum of the offspring. This indicates that the fetal membranes of the guinea pig may not select certain maternal antibodies for transmission to fetal circulation and exclude other antibody fractions. However, the work of Kulangara and Schechtman (1963) indicates that there are species differences in the transmission of serum proteins across the fetal membranes. Human albumin was injected into pregnant cows, and human albumin, bovine albumin and human gamma globulin were injected into pregnant cats and pregnant guinea pigs. In cows near term no injected human albumin passed to the fetus. In cats both human and bovine albumins passed in large amounts into fetal blood and into the fetal fluids; but, human gamma globulin passed into the fetal serum in only small amounts and was virtually absent in the other fetal fluids. In guinea pigs, injected human albumin, human gamma globulin and bovine albumin were found in high concentrations in the fetal serum, but only traces of these three proteins were found in the other fetal fluids.

Mason et al. (1930) investigated the prenatal transfer of "L.D. bacillus" antitoxin in immunized pregnant ewes and did not demonstrate antitoxin in the serum of newborn lambs. They also actively immunized a pregnant cow against diphtheria toxin but did not detect antitoxin in

the serum of the newborn calf. The same workers passively immunized a pregnant bitch by subcutaneous injection of diphtheria antitoxin 4 days before parturition and did not demonstrate diphtheria antitoxin in the serum of the newborn pup. The results of Schneider and Szathmary (1939) in a study of bitches immunized against killed Salmonella typhi, and diphtheria toxoid, indicated that Salmonella typhi and diphtheria toxoid antibodies were present in the newborn puppy before suckling. Ott (1956) and Ott et al. (1957) detected neutralizing antibodies against canine distemper in the serum of the newborn puppy prior to suckling their distemper immune dams. Puppies born to distemper immune dams having a titer of 1:320 had distemper neutralizing antibody titers of less than 1:40.

Gillespie et al. (1957) conducted a study on 10 bitches immune to canine distemper and showed that transfer of antibodies occurred in utero to all progeny. They demonstrated a positive relationship between the antibody content of the mother and the amount of serum antibody in the newborn puppy. The average amount of antibody transferred in utero was found to be 2.9% of the mother's titer. Gillette and Filkins (1965) intravenously administered canine serum containing Salmonella pullorum antibodies to pregnant bitches and did not demonstrate antibody transfer to the fetus up to 48 hours after administration. Deutsch and Smith (1957) using ultracentrifugation did not detect the presence of gamma globulin in the serum of newborn calves or goats. Payne and Marsh

(1962a) using electrophoretic and immuno-diffusion techniques detected small amounts of gamma globulin in the starved newborn pig. In a study of three pregnant cats immunized against Salmonella montevideo, Harding et al. (1961) found that the in utero transfer of antibodies amounted to about 20% of the total concentration of antibodies in the suckling kitten. Kittens tested prior to nursing showed titers of approximately 1:200 while the serum titer of the mother reached as high as 1:3,000 prior to parturition.

Mason et al. (1930) actively immunized a pregnant mare against diphtheria toxoid and did not demonstrate diphtheria antitoxin in the newborn foal. Mason's work was supported in a study by Bruner et al. (1948) utilizing five pregnant mares immunized against Salmonella abortus equi in which they did not detect Salmonella abortus equi antibodies in the serum of newborn foals prior to suckling.

Since there is variation among species as to the amount of prenatal antibody transfer, the number of layers of tissue between the maternal and fetal circulation has been proposed by some authors as influencing the transmission of maternal antibodies to the fetus in utero.

Schneider and Szathmary (1939) classified the domestic animals according to the type of placentation present. Those animals possessing an epitheliochorial placenta, including the horse, pig, cow, and goat, were thought to not receive maternal antibody in utero due to the 7 tissue layers between the maternal and fetal circulation. Animals

possessing an endothelial chorial placenta, including the dog and cat were thought to receive a small amount of maternal antibody *in utero* due to the presence of only 4 layers between the maternal and fetal circulation. Man, rabbits, guinea pigs, mice, and rats possessing a hemochorial placenta with only 3 layers between the maternal and fetal placenta were described as receiving considerable maternal immunity *in utero*. This theory of a placental barrier to the *in utero* transfer of antibodies was also supported in the review by McGirr (1947). Research described by Brambell (1958a) indicates that absorption in rats, rabbits, guinea pigs, and mice is probably not via the placenta but rather that the antibodies are absorbed from the uterine lumen and transmitted to the vitelline circulation via the yolk sac splanchnopleur. The mode of prenatal transmission has not been established for the dog and cat.

Postnatal Transmission of Immunity

The transmission of immunity after birth occurs by the intestinal absorption of colostral antibodies. Antibodies are secreted in the colostrum and milk of the immune mother and when ingested by the newborn individual are absorbed from the intestine. Pierce and Feinstein (1965) have found that the bovine mammary gland has a highly selective preference for an electrophoretically fast immune globulin and is able to concentrate this immune globulin in the colostrum. The work of Schneider and Szathmary (1939) indicates that the pregnant bitch can

also concentrate immune globulins in the colostrum.

The ability of the intestine of the newborn to absorb the intact antibodies is time limited in all species that have been investigated. Payne and Marsh (1962a) have described antibody absorption as occurring for only 12 hours after birth in the colostrum fed pig. Deutsch and Smith (1957) found that the permeability of the intestine of the newborn calf and goat to protein molecules is largely lost within 24 hours following birth. The work by Bruner et al. (1948) in the foal indicates that the full complement of colostral protection is acquired within 24 hours of birth. In a comprehensive review by Brambell (1958a) it is reported that there is little or no postnatal transfer of immunity in the rabbit, guinea pig, or man. Brambell (1958a) gives the intestinal absorption of antibodies as occurring for 16 days in the mouse and 20 days in the suckling rat.

The work of Harding et al. (1961) indicates that maximal absorption of colostral gamma globulin by newborn kittens occurs during the first 24 hours of nursing. Kittens nursing queens having a titer of 1:3,000 against Salmonella montevideo at parturition developed a titer of 1:1,000 within 24 hours after birth and maintained this level for at least a week following birth. The titer of the queen milk against Salmonella montevideo antigen was found to be 1:400 before nursing and 1:200 at 24 hours postpartum. Milk titers declined to a titer of 1:10 1 month after parturition.

Ganghofner and Langer (1904) fed 6 day old puppies egg white and blood serum and reported demonstrating these substances in the blood by precipitin reactions. Research conducted by Bardelli (as incorrectly cited by Perla and Marmorston, 1941) indicated that puppies 2 or 3 days old would absorb agglutinins from milk in greater amounts than they would from ingested homologous serum. The ability to absorb antibodies from the intestinal tract was reported as ceasing after the tenth to twelfth day. Clinical evidence of the antibody titers in young puppies has been reported by Slanetz (1935). Seven day old puppies suckling distemper immune bitches were experimentally inoculated with distemper virus and were found to be immune to the disease, however, at 3 months of age uninoculated controls were susceptible to distemper. Mason et al. (1930) passively immunized a pregnant bitch by the subcutaneous administration of 14,000 units of diphtheria antitoxin 4 days prior to parturition. Antitoxin was demonstrated in the serum of the bitch (3-5 units/ml) and in the milk at parturition. At 24 hours of age 1 puppy measured had 0.2 to 0.5 units per ml of serum. A second pup was fed 5 ml of horse serum containing 16,000 units of tetanus antitoxin; 24 hours later the puppy's serum contained between 0.2 and 2 units of antitoxin.

The results of Schneider and Szathmary (1939) in a study of pregnant bitches immunized against Salmonella typhi and diphtheria toxoid indicated that colostral antibody titers were about 10 times higher than those of the maternal serum. They also demonstrated a marked increase

in the serum titer of the puppies following nursing.

Ott (1956) reported colostral transfer of distemper neutralizing antibodies in the puppy. Puppy titers were found to increase from birth to about 2 weeks of age. Distemper neutralizing antibody titers of 2 week old suckling puppies were in excess of 1:120 from dams having 1:320 serum titers. The puppy serum distemper neutralizing antibody titer was found to be positively correlated with the distemper neutralizing antibody titer of the maternal serum at parturition. In most litters studied there was a decline of serum neutralizing antibody in the puppies until a low level was reached at 6 weeks after birth.

Research conducted by Gillespie (1957) indicated that colostral antibody transfer showed a proportionate relationship between the maternal titer and that of the offspring. In a study utilizing 5 pregnant distemper immune bitches the average titer of 13 progeny 24 hours after birth was 77% of the average maternal serum titer. Colostral antibody titers were found to be generally higher than that of the maternal serum, however, the colostral titer fell markedly 2 days after whelping whereas the high maternal serum titer persisted during nursing. Distemper neutralizing antibody was demonstrated in the milk of the dam for at least 35 days after parturition, which was the last day tested. The average titer of puppies at age x weeks was expressed by the formula

$$\log_{10} \frac{\text{puppy titer}}{\text{mother titer}} = -.1026 - .2499 \text{ (age in weeks)}$$

On the basis of the mother's serum titer at parturition, it was suggested

that the age at which the antibodies in the puppy would no longer protect it against distemper could be predicted. Ott et al. (1957) have reported similar findings. Distemper neutralizing antibody was demonstrated in the colostrum of immune bitches and was found to persist in the milk for 25 days after parturition. Serum distemper neutralizing antibody was found in the serum of newborn puppies, however, the greatest quantity was received by the pups following the ingestion of colostrum.

Baker et al. (1959) have reported on a nomograph which may be used to predict the age at which puppies may be satisfactorily immunized against distemper. The nomograph is a clinical application of the correlation between the maternal titer at whelping and the degree of colostral immunity attained by the suckling puppy. Field trials using 53 puppies from 12 different litters indicated 98% immunity using a revised nomograph as compared to 82% immunity on puppies immunized according to the original nomograph. The mathematical relationship utilized in the revised nomograph to calculate the puppy age in weeks for 95% successful vaccination was 4 (mother's log titer) - 3.5488.

The transfer and decline of maternal infectious canine hepatitis (ICH) antibody in puppies have been studied by Carmichael et al. (1962). These workers found that the puppy antibody titers are linearly related to the antibody titer of the dam. Colostral antibody titers generally appeared to be higher than those of maternal serum; however, the correlation was poor. ICH antibody in the colostrum dropped rapidly during

the first week following parturition, but 1% of the initial amount of colostral ICH antibody persisted in the milk for at least 30 days. It was found that puppies had not attained their full complement of ICH antibody within 12 hours of birth but had received the full complement by 72 hours after birth. The relationship between puppy antibody titers and the maternal antibody titer was expressed as

$$\log_{10} \text{puppy titer} = -.16 + .92 \log_{10} \text{mother titer}$$

The half-life of maternally transferred ICH antibody was reported as 8.6 days which is in close agreement with the work of Dixon et al. (1957) who reported a half-life of 8 days following the injection of I^{131} labeled canine gamma globulin in mature dogs.

Gillette and Filkins (1965) demonstrated the absorption of Salmonella pullorum antibody when S. pullorum hyperimmune canine serum was fed to newborn puppies. S. pullorum was detected in the puppy serum 5 hours after the feeding of hyperimmune serum. Maximal absorption had occurred by 12-15 hours after feeding. Nursing puppies did not absorb Salmonella pullorum antibody when fed the hyperimmune serum after 36 hours of age.

Hemolytic anemia has been reported in newborn suckling puppies by Young et al. (1949) and by Christian et al. (1949). Their studies showed that Do-positive puppies regularly developed hemolytic anemia if they suckled Do-negative bitches that had been immunized by transfusions of Do-positive blood. Anemia was observed if the puppies suckled the bitch during the first or second day of life but was not observed in puppies

suckling after the second day. Hime (1963) successfully produced hemolytic disease in newborn puppies by immunizing a bitch with an A-negative blood type against an A-positive blood type. A-positive puppies born to the immunized bitch began to exhibit signs of hypoxia and developed marked jaundice the third day after birth. The antibodies to red cell antigens did not appear to cross the placental barrier and the disease was not manifested until after the ingestion of colostrum.

Non-specific and Selective Intestinal Absorption in the Newborn

The absorption of antibody from the intestinal tract of the newborn may be a selective process, however, absorption from the intestine of the newborn is not specific for homologous gamma globulin but rather, includes a wide variety of materials. Halliday and Kekwick (1960) demonstrated that the selection of antibodies by the intestine of the suckling rat was independent of the species in which the antisera was produced but was related to the antigen used. Through fractionation of antisera and titration of fractions they produced evidence that this selection of antibodies was related to their location in the serum proteins. The intestinal absorption of heterologous globulins by the newborn pig has been reported by Payne and Marsh (1962a) and Kaeberle and Segre (1964). The latter investigators found that orally administered antibodies of equine origin were absorbed as readily as those of porcine origin while antibodies of ovine origin were absorbed only one-half as

efficiently. Tetanus antibodies of ovine and porcine origin were more readily absorbed by newborn pigs than diphtheria antibodies from the same species. Diphtheria antibodies of equine origin were more efficiently absorbed than tetanus antibodies. This selective phenomenon has been further studied by Locke et al. (1964) in newborn pigs and the molecular weight of antibodies was found to be a significant factor in their absorption from the intestine. Low molecular weight (6.6S) diphtheria and tetanus antibodies of ovine origin were absorbed more efficiently by starved newborn pigs than were high molecular weight (18S) antibodies.

The absorption of dextran and albumin by the newborn pig has been demonstrated by Balfour and Comline (1959). Clark (1959) reported that proteins and colloidal materials administered orally to suckling rats and mice were absorbed by the intestinal epithelial cells. Evans blue dye, saccharated iron oxide, collidal gold, bovine gamma globulin and egg ovalbumin were absorbed by the intestinal epithelium. Lecce et al. (1961) and Lecce and Morgan (1962) have demonstrated the intestinal absorption of a synthetic non-proteinaceous compound, polyvinylpyrrolidone, and egg ovalbumin and conalbumin in the newborn lamb and pig. Hardy (1965) has shown that the newborn pig has the ability to absorb I^{131} labeled gamma globulin of bovine origin and radio-iodinated polyvinylpyrrolidone. The absorption of tetanus antitoxin of equine origin from the intestinal tract of a day old puppy has been reported by Mason et al. (1930). Pierce et al. (1964) have shown the absorption of orally

administered insulin in the newborn calf. McCance et al. (1949) have reported the presence of cholinesterase in colostrum of the bitch and its absorption by the newborn puppy. The pseudo-cholinesterase activity of puppy serum was found to increase up to 25 times its initial value within the first 72 hours after birth. This indicates that intestinal absorption of intact enzyme molecules occurs in the newborn puppy.

Mechanism and Route of Intestinal Absorption

Comline et al. (1951b) have investigated the histological changes in the epithelium of the small intestine during protein absorption in the newborn suckling calf. Following the feeding of colostrum, globules with staining properties similar to those of whey protein were present in the majority of the intestinal epithelial cells of the jejunum and ileum. In some sections the material was seen in the lacteals. The size and location of the protein globules within the cell were varied. In some sections discrete globules up to 2 microns in diameter were found within the lumen border of the cells. In others the protein material was present in the cytoplasm of the epithelial cells either as 1 or 2 large globules up to 10 microns in diameter. In another study, Comline et al. (1951a) utilized suckling or starved newborn calves in which the duodeum, cecum, and thoracic duct had been cannulated. They found that colostrum proteins appeared in the lymphatic system 60-120 minutes after introduction of whey into the duodenum.

No trace of colostrum proteins was found in either the lymph or blood after the introduction of whey into the stomach and large intestine when ligatures prevented entry into the small intestine. Their results indicate that colostral globulins are absorbed via the epithelial cells into the lacteals and do not enter the portal circulation in any appreciable amounts but rather are carried in the lymph to the peripheral blood. Clark (1959) using fluorescent microscopy confirmed the work of Comline and co-workers by demonstrating the presence of bovine gamma globulin in the lacteals of the intestinal villi, mesenteric lymph nodes and lymphatic ducts of the suckling rat.

Hill (1956) described numerous eosinophilic globules in the jejunal epithelial cells in suckling lambs up to 24 hours old. In older lambs the epithelial cells were mainly without globules although many cells contained very pink "ghosts". Hill and Hardy (1956) carried out a histological and histochemical study of the intestinal epithelium of young lambs and kids which were killed shortly after receiving colostrum. Results of histochemical tests showed that the globules present in the epithelial cells and lacteals were muco- or glyco-proteins. It was thought that the globules were mucous conjugated colostral proteins. Clark (1959) has done the most comprehensive morphological study in connection with antibody absorption by the intestinal epithelium. In an electron microscopic study of suckling rats and mice fed bovine gamma globulin and various colloidal materials, he described the absorb-

ed material as being segregated in membrane-enclosed vacuoles and tubes in the cellular cytoplasm. This system of vacuoles and tubes was found to be continuous in places with the apical cell membrane. It was suggested that ingestion of foreign material was accomplished by pinocytosis. In the suckling rat and mouse approximately 18 days after birth the columnar epithelial cells lost the ability to ingest proteins and colloids and no longer contained large vacuoles and tubules. The animals were still able to absorb particulate fat. Thus the capacity for pinocytosis may not be lost but rather may have become selective as to what substances may be ingested.

Brambell et al. (1964) have proposed a theoretical model of gamma globulin catabolism. These authors presented experimental evidence to suggest that there is a similarity between the process of passive transmission of immunity across the intestine of the newborn and passive anaphylactic sensitization. They maintained that a similar receptor mechanism is involved in both transmission and sensitization. Gamma globulin catabolism is proposed as occurring in the intestinal epithelium with degradation products being extruded into the intestinal lumen. Gamma globulin extruded into the lumen could be reabsorbed by attachment to the receptors and returned to the circulation whereas non-attached particles are degraded.

The authors further suggested that the change in the intestine which results in the termination of the transmission of immunity after

birth is due to the development of gastric and duodenal function. They proposed that the small intestine is still capable of transmission provided that the gamma globulin reaches it without having traversed the anterior portion of the digestive tract.

Influence of Diet on Antibody Absorption

It has been hypothesized by several investigators that the colostral whey contains components which accelerate the absorption of globulin from the newborn intestine. The influence of dietary regimen on the intestinal absorption of colostral proteins has been studied most extensively in the newborn pig. Barrick et al. (1954) reported that the oral administration of porcine gamma globulin, porcine and bovine serum solids, and cow's colostrum did not cause a marked or consistent response in the serum gamma globulin level of 1 day old pigs. Lecce et al. (1962) found that nursing pigs lost their capacity to absorb polyvinylpyrrolidone (PVP) when approximately 24-36 hours old while starved pigs were able to absorb PVP until at least 86 hours. Feeding newborn pigs cow's colostrum caused the same closure phenomenon as seen in nursing pigs. Similar results were obtained with lambs in that starved lambs retained the ability to absorb PVP for at least 48 hours while lambs fed cow's colostrum were unable to absorb PVP and egg proteins at 24 hours of age. Payne and Marsh (1962b) demonstrated uptake of fluorescent-tagged gamma globulin in pigs fed only water or starved for 106 hours after birth.

Pigs that were fed milk for 12 hours after birth failed to absorb tagged gamma globulin. Lecce et al. (1964) have fractionated and altered porcine colostrum and studied the influence of the various components on the intestinal absorption of proteins in newborn pigs. Their results indicate that absorption of large molecules and closure are two independent phenomena. Pigs fed salt solutions containing high molecular weight materials such as porcine and avian proteins absorbed the material without closure resulting. The feeding of protein-free diets such as boiled colostral whey and milk and colostral dialyzate induced closure. Their experiments also excluded carbohydrates, vitamins, and minerals as being responsible by themselves for closure. They suggested that a heat-stable, low molecular weight compound found in the dialyzate of colostrum or milk is responsible for the cessation of antibody absorption. Hardy (1965) reported negligible absorption of radio-labeled protein or polyvinylpyrrolidone administration to newborn pigs in an aqueous medium with an ionic composition similar to colostrum.

Deutsch and Smith (1957) did not demonstrate a milk factor as being responsible for closure in the newborn calf. Calves which were maintained by oral feeding of lactose-dextrose mixtures or intravenous fluid therapy did not have prolonged periods of intestinal permeability. Maintenance of the calf with blood transfusions from the dam was not successful in maintaining intestinal permeability. A mixture of amniotic fluid and milk did not maintain intestinal permeability to colostral

gamma globulin. The work of Schoenaers and Kaeckenbeeck (1964) is in agreement with that of Deutsch and Smith (1957). The former investigators have shown that the preliminary ingestion of milk, egg white, or glucose did not reduce the capacity of the newborn calf to absorb antibodies from colostrum. Preliminary ingestion of glucose in water did not appear to prolong the period of permeability of the intestine to colostral antibodies.

Balfour and Comline (1962) have investigated factors in colostrum which accelerate the absorption of gamma globulin in the newborn calf. When radio-labeled globulin was administered in a solution of chlorides with a similar cation composition to that of colostrum, very little globulin was absorbed. The addition of sodium chloride to test solutions or colostrum was found to delay absorption while filtrates prepared from milk whey after the removal of heat-coaguable proteins enhanced globulin absorption. These investigators using the methods of Laskowski and Laskowski (1951) separated a protein fraction of low molecular weight from colostrum whey which, in the presence of inorganic phosphate and glucose-6-phosphate, caused globulin absorption at the same rate as found in fresh whey. These three components had little or no effect when fed singly. The protein fraction isolated was thought to be similar to or identical to that isolated by Deutsch and Smith (1957).

The Development and Influence of Proteolytic Activity on Intestinal Absorption of Antibodies

The immune globulin molecule must be absorbed intact from the intestine in order that its immunological properties be maintained. It has been postulated by some investigators that the cessation of antibody absorption in the newborn individual is the result of proteolytic degradation of the globulin molecule with resultant loss of its identity and function. Hill (1956) has described gastric development and antibody transfer in the lamb, rat, and guinea pig. He found that there were numerous peptic cells containing pepsinogen granules in the abomasum of the newborn lamb. Parietal cells were very few in number at birth and increased in number rapidly during the first 48 hours of life. The pH of abomasal contents was found to decrease from 6-7 at birth to 3-4 in 36 hours. Observations on the young rat showed that the gastric glands were not fully developed until 3 weeks after birth while the newborn guinea pig had fully developed gastric glands. It was the conclusion of this author that there is secretion of gastric juice at birth in those species which receive antibodies in utero while gastric protein digestion is delayed in those species which receive most maternal immunity via the colostrum.

Smith and Erwin (1959), using duodenal cannulas, introduced colostrum directly into the duodenum of 48-60 hour old colostrum free calves and did not demonstrate intestinal absorption of colostral globulins.

They reported that gastric proteolytic activity was not the limiting factor in the cessation of antibody absorption in the newborn calf.

McCance and Brown (1953) reported that the empty stomach of a newborn puppy had a pH of 2-3, however, the stomach content of a recently fed day old puppy was found to have a pH of 5. Casein digestion by activated suspensions of pancreatic tissue indicated that pancreatic tryptic enzymes were present in the newborn puppy. In newborn puppies and suckled puppies acidophilic pancreatic secretory granules were abundant. Puppies maintained on boiled bitch's milk or evaporated milk had moderate numbers of secretory granules in the pancreas. Cholinesterase activity in the pancreas was found to increase from birth to 3-4 days of age. The authors postulated that the increase in pancreatic cholinesterase parallels the activity of pancreatic lipase and amylase. No function was proposed for the large quantities of cholinesterase found in colostrum or puppy serum.

Kryuchkova (1939) has reported on the composition of intestinal juice in newborn puppies. No pancreatic lipase was detected from birth to 12 days of age, however, small amounts of amylase were present. It was found that the intestinal juice of 1 day old puppies had the capacity to activate amylase.

Crystalline trypsin inhibitor has been isolated from bovine colostrum by Laskowski and Laskowski (1951). These authors suggested that action of the colostral trypsin inhibitor was to reduce intestinal pro-

teolytic activity thus allowing the absorption of intact immune globulins. Later work conducted by Barrick et al. (1954) have shown that the inclusion of trypsin inhibitor at the rate of 750 mg per 150 ml of milk did not prolong nor enhance gamma globulin absorption in the newborn pig. Hardy (1965) added trypsin inhibitor isolated from 1 liter of bovine colostrum to gamma globulin and observed increased gamma globulin absorption in the newborn pig. It may be that the compound reported by Balfour and Comline (1962) and Lecce (1964) was contained in the fraction along with the trypsin inhibitor. Deutsch and Smith (1957) have reported that the feeding of a pigeon crop extract containing desoxyribonuclease inhibitor did not prolong the period of antibody absorption in the calf. These authors concluded that pancreatic desoxyribonuclease does not play a role in the cessation of antibody absorption.

Hartman et al. (1961) have reported a comprehensive study of the digestive enzyme development in the young pig. The levels of proteinase in the stomach ingesta, when calculated on a body weight basis, were similar in animals at birth, and at 1 and 2 weeks of age. Tissue tributryinase activity in the pancreases of unweaned pigs was relatively high at birth and gradually increased with age. Levels of proteinase per gm wet weight of intestinal contents increased from birth through 7 weeks of age. Proteinase activities were generally lower in the first third of the intestine than in the 2 distal sections. Lloyd and Crampton (1957) have compared the digestibility of fats and oils by 10 day old

puppies, 14 day old pigs, and 3 day old guinea pigs. Neither mean molecular weight nor degree of saturation had any significant effect on the apparent digestibility of the fats or oils by 10 day old puppies. A species difference was reported in that the puppy was able to digest more completely fats or oils containing a large proportion of long chain fatty acids than was either the pig or guinea pig.

Snook and Meyer (1964a) have investigated the response of digestive enzymes to dietary protein. In a controlled study utilizing adult rats they found that the addition of dietary protein increased the secretion of proteolytic enzymes and retarded the rate of degradation of these enzymes within the intestine. In a latter study Snook and Meyer (1964b) further investigated the effect of diet and digestive processes on proteolytic enzymes in the adult rat. They found that the presence of dietary protein in the upper intestinal contents protected trypsin and chymotrypsin from proteolytic degradation and that the structural properties of trypsin and chymotrypsin were altered in the intestinal tract.

Influence of Hormones and Stress on Intestinal Absorption of Gamma Globulin

Deutsch and Smith (1957) have tested the effect of different hormones on the period of antibody absorption during the first 40 hours postpartum in the calf. They did not detect any change in intestinal permeability to intact proteins as the result of administering diethylstilbestrol, progesterone, cortisone or ACTH by either the intramuscular,

intraperitoneal or subcutaneous route.

Halliday (1959) has demonstrated that the administration of 1-5 mg of cortisone acetate to suckling rats caused a complete cessation of the absorption of antibody within 48 hours after administration. Aldosterone, progesterone, testosterone and stilbestrol, in the doses used, had no effect on antibody absorption. Concurrent with the cessation of antibody absorption following the administration of cortisone there was an increase in the alkaline phosphatase activity of the intestine. Clark (1959) found that following the administration of cortisone acetate to 8-10 day old rats there was a loss of permeability to intact proteins and that the morphology of the intestinal epithelial cells resembled that of an adult animal. Payne and Marsh (1962b) reported that starved pigs injected with cortisone acetate (25 mg/kg) ceased to absorb gamma globulin 48 hours after administration.

Morris and Steel (1964) have reported that the oral or intraperitoneal administration of cortisone acetate to the young hedgehog did not alter the normal 41 day period of antibody absorption.

Gillette and Filkins (1965) have found that the administration of hydrocortisone, progesterone, and adrenal anti-metabolites to newborn puppies did not significantly affect the period of antibody absorption. Treatment of pregnant bitches with hydrocortisone for 3-4 days prior to whelping or <u>in utero</u> administration of hydrocortisone in puppies was associated with a decreased antibody absorption in the newborn puppy.

Halliday (1965) has described the failure of some lambs to absorb maternal gamma globulin. Some lambs born following a severe winter during which the ewes experienced long periods of undernourishment and climatic stress during pregnancy were found to have little or no serum gamma globulin. The author postulated that the premature loss of absorption was induced by the mothers as a result of severe stress.

EXPERIMENTAL PROCEDURE

Procurement and Housing of Pregnant Bitches

Healthy, pregnant, mongrel bitches were purchased from local vendors and housed in departmental quarters. Each bitch was allowed approximately 45 square feet of floor space and was fed a commercial dry dog food[1] and water ad libitum. The rectal temperature of each bitch was taken 3-4 times daily and the mammary glands were examined for the presence of colostrum. A drop in body temperature of 1-2° F and the presence of colostrum in the mammary glands were assumed as evidence of impending parturition.

Procurement of Colostrum Free Puppies

Bitches which showed signs of imminent parturition were sedated with meperidine hydrochloride[2] intramuscularly at a dose of 5 mg per pound body weight. A 30 minute period was allowed for sedation to take effect. The bitch was placed on a standard surgical table and adequately restrained. The abdominal area was clipped, washed with soap and

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[1] Wayne Dog Food, Krumettes, Allied Mills, Chicago, Illinois.

[2] Demerol hydrochloride, Winthrop Laboratories, New York, New York.

disinfectant[1] applied. A local anesthetic, 2% lidocaine hydrochloride,[2] was infiltrated subcutaneously about the incision site. Using aseptic surgical procedures with sterile equipment and instruments the puppies were obtained via routine Cesarean section. In some instances where the bitch was observed whelping, the puppies were removed prior to the ingestion of colostrum. In other litters the puppies were allowed to be whelped naturally and were left to suckle the bitch.

Care, Housing and Feeding of Puppies

Immediately following birth the colostrum free puppies were dried, the umbilical cord tied, and the puppy weighed. Puppies were randomly assigned diets and were housed in groups of 2 or 3, according to treatment, in clean cardboard boxes in an isolated room. Room temperature was maintained in the range of 75-85° F through use of a heatlamp.

Dietary Regimens

Puppies were maintained on four dietary regimens: protein-free diet, protein diet, natural diet, and starvation. The composition of the diets was as follows:

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[1] Nolvasan, Fort Dodge Laboratories, Fort Dodge, Iowa.

[2] 2% Xylocaine HCl, Astra Pharmaceutical Products, Inc., Worcester 6, Massachusetts.

1. protein-free diet — 10 grams of sucrose[1] were dissolved in 100 ml of 0.85% NaCl aqueous solution.

2. protein diet — A dry commercial replacement[2] for bitch's milk was mixed by addition of 38 grams of the dry material to 100 ml of distilled water to give an approximately 12.5% protein solution.

Ingredients of the dry diet included soybean, cottonseed, and peanut oils, dried skimmed milk, alkaline caseinate, and dried egg yolk with vitamin and mineral additives. The guaranteed analysis of the dry product is as follows:

crude protein	min. 33.00%
crude fat	min. 40.00%
crude fiber	none
moisture	max. 5.00%

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[1] Cane sugar.

[2] Esbilac, Feed Supplement Division, Borden Company, New York, New York.

ash	max. 6.00%
	(per lb.)
Vitamin A	50,000 U.S.P. units
Vitamin D2	3,500 U.S.P. units
Thiamin	1.4 mg
Pantothenic acid	4.1 mg
Niacin	15.4 mg
Choline chloride	1300 mg
Riboflavin	3.2 mg

3. natural diet — Naturally whelped puppies were allowed to remain with and suckle their mother.

4. starvation

In several trials soybean trypsin inhibitor[1] was added to the protein diet at the rate of 125 mg of trypsin inhibitor to 10 ml liquid protein diet. This diet was fed until the feeding of hyperimmune serum which contained 100 mg trypsin inhibitor in 10 ml of hyperimmune serum. A limited number of trials were run in which puppies on the protein diet were fed hyperimmune serum containing 25 mg of crystalline trypsin[2] per

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[1] Soybean trypsin inhibitor lot 284-59, Armour-Baldwin Laboratories, Kankakee, Illinois.

[2] Tryptar, lot K 46409, Armour-Baldwin Laboratories, Kankakee, Illinois.

10 ml of serum. Three hours after the feeding of the trypsin and serum combination an additional dose of 25 mg of crystalline trypsin in 5 ml of distilled water was fed to each puppy.

Feeding Schedules and Procedures

All feeding was via stomach tube in order that a known quantity was ingested. PE100 polyethylene tubing[1] fitted over a 20 gauge needle and a 10 cc syringe proved to be an adequate dosing instrument. Puppies were fed every 4-6 hours or according to the design of the particular experiment. The protein-free and protein diets were fed at the rate of approximately 2 ml per 100 grams body weight or until mild abdominal distension was noted. No measure was made of the quantity of colostrum ingested by puppies suckling their mothers. Starved puppies were not fed or watered throughout the period of experimentation.

Pooled canine hyperimmune serum was fed via stomach tube at the rate of 3 ml per 100 grams body weight. The hyperimmune serum was not combined with the experimental diet and was fed either at birth or at least 4 hours after a feeding of the experimental diet.

All dietary materials were stored at 4-5° C between feedings.

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[1] Intramedic, Clay Adams Company, New York, New York.

Collection of Serum and Plasma Samples

Blood samples were drawn from the jugular vein using a 22-gauge needle and a 1 ml syringe or via cardiac puncture using a 20-gauge needle and a 5 ml syringe. Experience proved jugular vein puncture to be a satisfactory technique for obtaining 0.5-1.0 ml of blood while 2-3 ml of blood were obtained via cardiac puncture with little ill effect on the puppy as a result of the bleeding procedure.

Blood samples were allowed to clot for 4-6 hours at room temperature; the serum was collected and titrated or stored in a frozen state in sealed tubes until titration. Blood plasma rather than serum was used for electrophoretic separation. Hemolysis which occurred in many blood samples interfered with the electrophoretic analysis of serum proteins. Various procedures and techniques were utilized in an attempt to obtain serum free from hemolysis, but none were consistently successful with puppy blood. Heparinized microhematocrit tubes[1] were used to collect blood from the hub of the bleeding needle. The collected sample was then centrifuged for 5 minutes in a microhematocrit centrifuge. Following centrifugation the portion of the capillary tube containing the blood cellular elements was broken off and discarded. Plasma samples were stored at 4-5° C and were analyzed electrophoretically within 12 hours of collection.

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[1] Red Tip capillary tubes, Aloe Scientific, St. Louis, Missouri.

Production of Hyperimmune Serum

Healthy, adult mongrel dogs of both sexes were immunized against a phenolized suspension of Salmonella pullorum[1]. The dogs received weekly subcutaneous injections for a period of 6-8 weeks at which time the serum titer was determined and the dogs bled via cardiac puncture. All blood was allowed to clot at room temperature for 6-8 hours and the serum was collected, pooled, and stored in 100 ml aliquots at -20° C until used.

Titration of Salmonella Pullorum Antibody in Serum and Plasma

All puppy sera and hyperimmune sera were titrated using a tube agglutination procedure. Four-tenths ml of puppy serum was added to 0.6 ml of 0.85% NaCl solution and the tube agitated. Five-tenths ml of the serum and saline mixture in the first tube was added to a second tube containing 0.5 ml saline and dilutions were continued in this manner out to 8 dilutions. This procedure gave titrations of 5, 10, 20, 40, 80, 160, 320, and 640. Hyperimmune serum was titrated in the same manner with the exception that only 0.1 ml of hyperimmune serum was added to 0.9 ml of saline in the first tube and dilutions were then carried to give titers of 20, 40, 80, 160, 320, 1200, and 2560. In both instances 0.5 ml of the mixture of serum and saline in the last tube was

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[1] Salmonella pullorum culture supplied by Dr. R. A. Packer, College of Veterinary Medicine, Iowa State University.

discarded. The antigen for the agglutination procedure was a phenolized suspension of Salmonella pullorum diluted to a turbidity corresponding with tube 3 of McFarlands nephalometer. Antigen was stored at 3-5° C until used. Five-tenths ml of the standardized antigen was then added to each of the dilutions and the tubes were agitated. The tubes were incubated in a constant temperature water bath at 52° C for 4 hours and then for 12-16 hours at room temperature. Soft, "cottony" flocculations in the bottom of the tube with clear supernatant were evidence of a positive agglutination. In those samples in which excessive hemolysis was present the formation of protein precipitate precluded the definitive determination of positive agglutinations and such samples were discarded.

Electrophoretic Separation of Plasma Proteins

Cellulose polyacetate strips[1] (1" x 6 3/4") were immersed for 10 minutes or longer in a barbital-sodium barbital buffer[2] pH 8.6 (0.05 ionic strength) to produce a colloidal change in the polyacetate returning it to a gel-like structure. The strip was then removed from the buffer solution and blotted on clean absorbant paper. Three to four lambdas of blood plasma were applied approximately 2 inches from the

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[1] Sepraphore III, Gelman Instrument Company, Ann Arbor, Michigan.

[2] Buffer Salt (B-1), Hartman-Leddon Company, Philadelphia, Pennsylvania.

end of the strip at a right angle to the margin of the strip with a commercial sample applicator[1]. The strips were kept moist during the application procedure and immediately after streaking were placed in an electrophoretic chamber[2] and tensioned across chamber dividers. Each end of the strip was allowed to contact the barbital buffer. Experience showed that 6-8 strips could be satisfactorily separated at one time and that the electrophoretic chamber buffer was exhausted after 4 runs. A regulated power supply[3] was used to provide 200 volts for 90 minutes which effected a satisfactory separation. The strips were then stained with Ponceau S[4] (200 mg of Ponceau S in 100 ml 5% trichloroacetic acid) for 5 minutes. Following staining the strips were rinsed free of residual dye in several washes of 5% acetic acid. The strips were allowed to dry at room temperature and were cleared with light mineral oil for scanning. Strips were scanned with a electrophoretic densitometer[5] using a green filter and manually plotted on graph paper. Scanner patterns were quantitated with the use of a planimeter and the protein

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[1] Gelman Instrument Company, Ann Arbor, Michigan.

[2] Gelman rapid electrophoresis chamber, Gelman Instrument Company, Ann Arbor, Michigan.

[3] Heathkit regulated power supply, Model IP-32, Heath Company, Benton Harbor, Michigan.

[4] Hartman-Leddon Company, Philadelphia, Pennsylvania.

[5] Electrophoresis scanner, Model 39301, Gelman Instrument Company, Benton Harbor, Michigan.

fractions expressed as a percentage of total plasma protein.

Fluorescent Labeling of Globulin and Specimen Preparation

The conjugation of fluorescein dye and globulin was performed according to a procedure described by Cherry et al. (1960). Beta and gamma globulins of canine origin[1] were buffered by the addition of carbonate-bicarbonate buffer (0.5 M, pH 9.0) in an amount equal to 10% by volume of 1% globulin solution. The isothiocyanate powder was slowly added with constant stirring. The conjugated globulin was then dialyzed against buffered saline (0.01 M phosphate, pH 7.5) for 24 hours and was cleared of residual fluorescein by passage through a column of Sephadex G-25 (fine)[2] using 0.01 M phosphate buffer pH 7.5 as an eluent. The labeled globulin was stored at -20° C until used. Prior to usage the thawed solution was stirred in Sephadex G-25 and centrifuged to remove any diassociated fluorescein. Three to five ml of the conjugated globulin were fed via stomach tube to the puppies. One to three hours later each puppy was euthanized with sodium pentobarital and the intestinal tract removed. The intestine was flushed with 0.85% NaCl in distilled water and sections were frozen for tissue sectioning. A cryostat was used to prepare tissue sections which were fixed in absolute ethanol

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[1] Globulon, Pitman Moore, Indianapolis, Indiana.

[2] Pharmacia Fine Chemicals, New Market, New Jersey.

for 30 minutes and were then given four 15-minute rinses in 0.01 M phosphate buffered saline. Phosphate buffered glycerine was used as a mounting medium prior to application of cover slips. The preparations were then viewed microscopically using ultraviolet illumination. Tissues were viewed within 12 hours of preparation to avoid the development of autofluorescence.

Intestinal Proteolytic Activity Procedures

The proteolytic activity of intestinal ingesta was assayed using a modification of a procedure described by Cowgill and Pardee (1957). Puppies were fed regular amounts of their respective diets via stomach tube and were euthanized with sodium pentobarbital 2 hours after feeding. The first 12 inches of the small intestine were isolated and the intestinal contents flushed with 3 ml of chilled physiological saline into a chilled centrifuge tube. The samples were immediately centrifuged and placed in an ice bath. All samples were assayed immediately after collection and 2 determinations were made on each sample (for complete procedure see Appendix A). Two-tenths ml of the intestinal ingesta supernatant was added to 9 ml of a prewarmed casein solution. This solution was rapidly mixed and a 2 ml aliquot immediately withdrawn and placed in 3 ml of an aqueous 5% trichloroacetic acid solution. The remaining casein solution was placed in a 39° C constant temperature waterbath for 20 minutes. At the end of the 20 minute digestion period

a 2 ml aliquot was withdrawn and placed in 3 ml of 5% trichloroacetic acid. This was allowed to stand at room temperature for 30 minutes and then centrifuged. One-tenth ml of the supernatant was added to 1 ml of a 0.5 N aqueous solution of NaOH and to this quantity 5 ml of Folin's solution A were added. The solutions were well mixed and allowed to stand at room temperature. Five-tenths ml of Folin's solution B was added, the solution mixed, and allowed to stand for 30 minutes at room temperature. The resultant colored solution was read at 740 millimicrons in a spectrophotometer. A standard solution of casein was diluted to 0.03, 0.06, 0.09, 0.15, 0.31, and 0.30 mg/ml. The protein content of the standard solution was determined using the Folin's reagents A and B. The protein standard determinations were used to construct a standard curve which correlated spectrophotometric readings and protein concentration. The results from the digestion trials were quantitated using a standard curve determined the same day.

To standardize the experimental data the 2 determinations on each sample were averaged, and the measured quantity of casein digested was expressed per 100 grams of puppy weight. The following formula was used to correct data for dilution factors:

$$\text{mg casein digested per 100 gm body weight} = \frac{(\text{mg casein})(50)(4.6)(15)}{\text{puppy weight expressed in 100 grams}}$$

The dilution factors were as follows: Folin reagent, 50; digestion medium, 4.6; and intestinal ingesta dilution, 15.

RESULTS AND DISCUSSION

Influence of Diet on Salmonella Pullorum Antibody Absorption

The study of the influence of diet on antibody absorption was primarily conducted using the protein and protein-free diets. A limited number of observations was also made on puppies allowed to suckle their mother and on starved colostrum-free puppies. (See Tables 6 and 7 in Appendix B for individual observations on all diets). The largest number of observations was made on the protein and protein-free diets and a statistical analysis was performed on data from these two diets. A statistically significant difference in the slope of the two regression lines of the two treatments invalidated any analysis of covariance. In the pooled data the litter and treatment effects were confounded. To decrease the confounding of litter and treatment effect the data were analyzed in a randomized complete block design (see Table 1).

In this analysis the litter-age effects were statistically confounded because both litter and age were different in the various blocks. The influence of the protein and protein-free diets on antibody absorption was not significantly different ($p > 0.10$) in this analysis. Starved puppies were found to absorb antibody at 34 hours of age; however, starved puppies fed at 36 hours of age did not absorb antibody. Puppies on the protein diet failed to absorb hyperimmune serum antibody after 20 hours of age with the exception of one puppy fed at 30 hours of age.

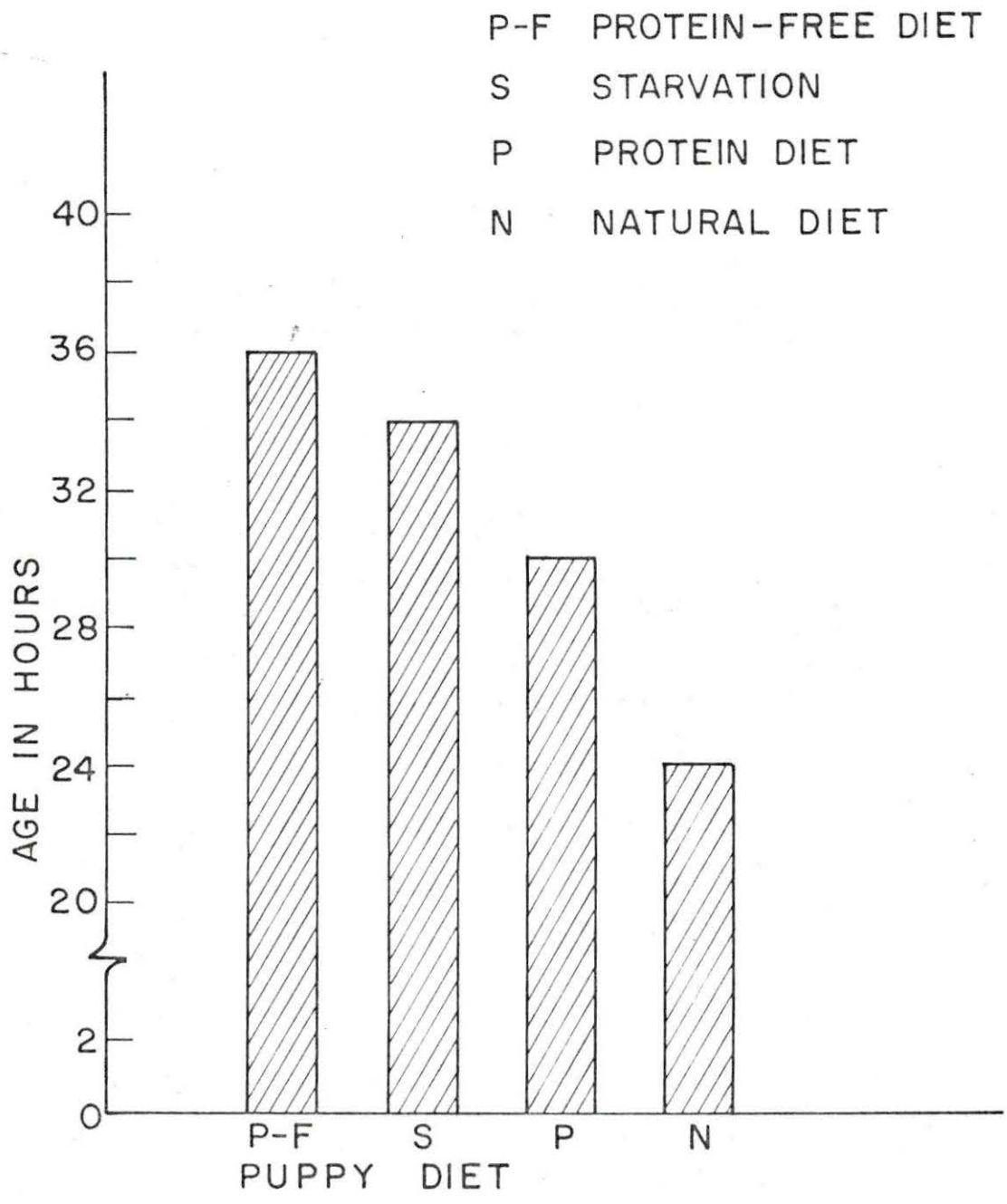
Table 1. Unweighted mean analysis of variance of influence of protein and protein-free diets on antibody absorption in the newborn

Source	Degrees freedom	Sum of squares	Mean square	F
Litter-age	10	82,841	8,282	3.91*
Treatment	1	2,101	2,101	.99
Interaction	10	8,412	841	.40
Error	5	10,603	2,120	

*Significant at 0.10 level.

The litter-age effect was found to be significant ($p < 0.10$). There was considerable variation in the quantity of antibody absorbed among litters at the same age; however, similarly treated puppies within a litter exhibited little variation in antibody absorption. Thus in the litter block design analysis the significant age-litter effect is probably due to litter. Visual examination of the data indicates that those puppies on the protein-free diet had a longer period of absorption than puppies on the protein diet. Thus, by deduction, it is probable that the protein-free diet significantly increased the period of antibody absorption. (See Figure 1). Even though there was not a statistically significant difference in the quantity of antibody absorption between puppies maintained on protein or protein-free diets there was generally greater antibody absorption in those puppies maintained on protein-free

Figure 1. Influence of diet on the period of *Salmonella pullorum* antibody absorption in the puppy


P-F PROTEIN-FREE DIET
S STARVATION
P PROTEIN DIET
N NATURAL DIET
AGE IN HOURS
40
36
32
28
24
20
2
0
P-F
S
P
N
PUPPY DIET

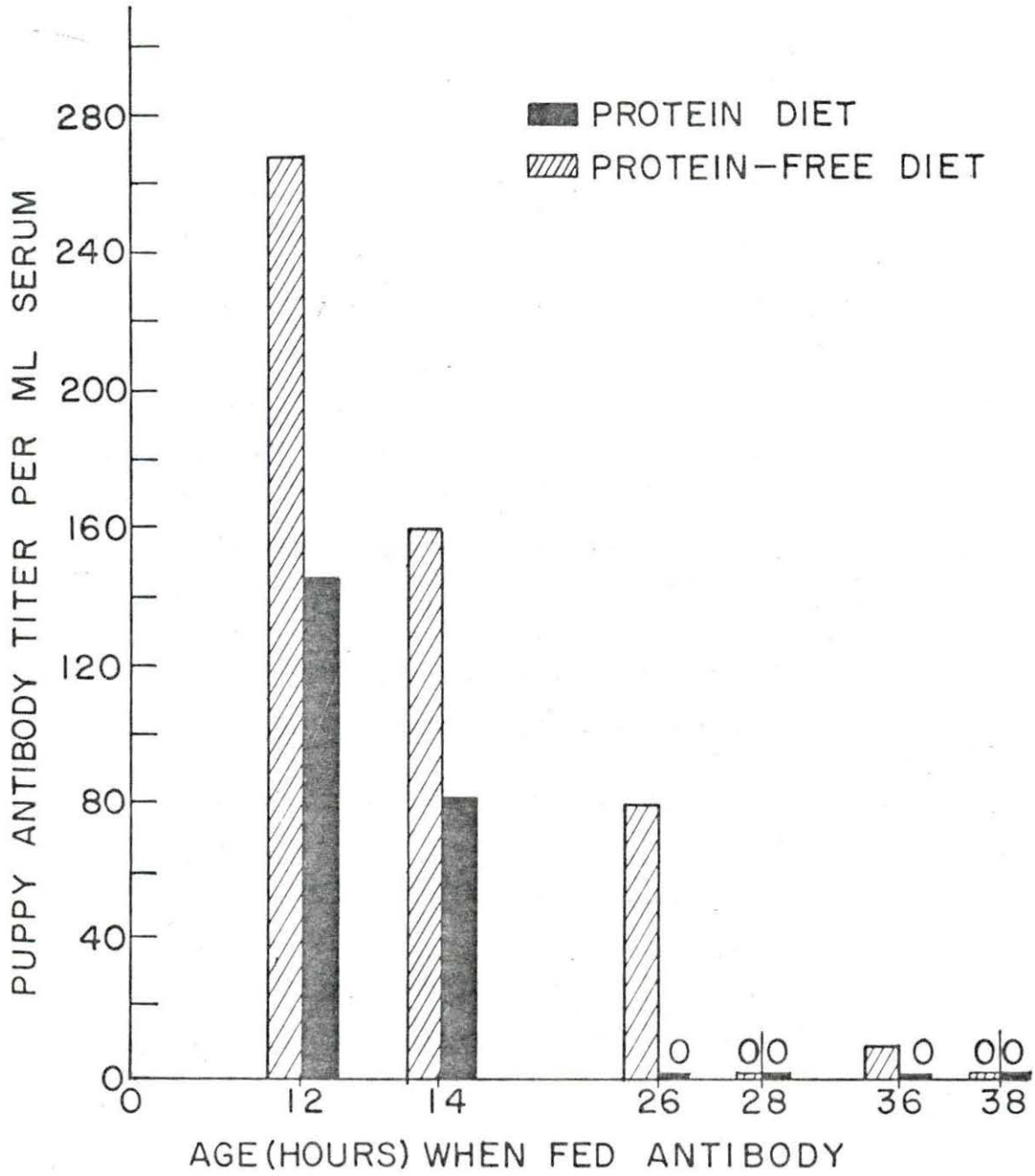
diets. (See Figure 2).

Some of the puppies on the protein-free diet absorbed antibody at 36 hours of age although several other puppies also fed hyperimmune serum at 36 hours did not absorb antibody. In those puppies suckling the bitch the absorption of hyperimmune serum antibody did not extend beyond 24 hours.

In the present study puppies maintained on the various diets were not tested for the ability to absorb serum antibody at each possible age. However, in pooling all of the data from 70 puppies on the four diets there were only 2 puppies which absorbed antibody at 36 hours of age or later. It can be said with a reasonable degree of assurance that the intestinal absorption of serum Salmonella pullorum antibody occurs only during the first 36 hours after birth.

In viewing the data obtained in these experiments the limitations of the present study should be kept in mind. Puppies were exposed to only one dose of Salmonella pullorum antibody. This is in contrast to the constant exposure to colostral antibody in naturally suckling puppies. Interference by serum proteins with antibody absorption and selective antibody absorption have been reported by Brambell et al. (1958) and Halliday and Kekwick (1960). It is possible that antibodies other than Salmonella pullorum antibody were absorbed for longer periods of time than reported in this study or that serum proteins interfered with antibody absorption. However, the fluorescent labeled gamma glob-

Figure 2. Influence of dietary protein on Salmonella pullorum antibody absorption in the puppy


PROTEIN DIET
PROTEIN-FREE DIET
PUPPY ANTIBODY TITER PER ML SERUM
280
240
200
160
120
80
40
0
0
12
14
26
28
36
38
0
0
0
0
0
AGE (HOURS) WHEN FED ANTIBODY

ulin studies indicated that there was no absorption of gamma globulin in a 24-hour old suckling puppy and two 50-hour old puppies maintained on the protein and protein-free diets, respectively.

The failure of starved puppies to absorb antibody after 36 hours of age may indicate a species difference in antibody absorption. Payne and Marsh (1962a) reported that 106-hour old starved baby pigs retained the ability to absorb gamma globulin. The ingestion of saliva, meconium, and paper bedding or the occurrence of self-suckling by starved puppies may have complicated this experiment. However, in natural circumstances somewhat similar to this study, it is doubtful if the starved puppy retains the ability to absorb gamma globulin after 36 hours of age.

The results of this study indicate that even though a protein-free diet or starvation prolonged the period of antibody absorption, cessation of antibody absorption still occurred. Protein contact with the intestinal epithelium must not be the primary factor which regulates the period of antibody absorption.

Intestinal Absorption of Fluorescent Labeled Gamma Globulin

The absorption of fluorescent labeled gamma globulin occurred primarily in the jejunum and ileum. The absorption of gamma globulin by intestinal epithelial cells was evidenced by marked intracellular fluorescence 1 to 2 hours after feeding of the labeled material. In puppies studied 2 to 3 hours after feeding of labeled globulin the fluorescence

was located in the submucosa and appeared as a band of fluorescing globules. In most preparations examined the fluorescence was either in the epithelial cells or in the submucosa; but rarely found in both areas. This indicates that the epithelial cells absorb their capacity of gamma globulin before any is released into the submucosa. The lack of definition in the unstained preparations studied precluded any further definition of the area in the submucosa in which the fluorescence was concentrated.

Absorption of the labeled gamma globulin was detected in two 28-hour old puppies maintained on the protein and protein-free diets, respectively. There was no absorption of labeled globulin in two 50-hour old puppies maintained on the protein and protein-free diet, respectively, or in a 24-hour old suckling puppy.

The failure of gamma globulin absorption by the older puppies indicates that the absence of Salmonella pullorum antibody in the serum of puppies 36 hours of age or older was due to a cessation of antibody absorption and not due to interference or selective absorption.

Electrophoretic Analysis of Puppy Serum Proteins

The presence of hemolyzed erythrocytes in most puppy serum samples caused difficulty in the analysis of the serum proteins. Hemoglobin and other blood pigments appeared to migrate near the gamma globulin band and extremely large gamma globulin determinations resulted. To decrease

the degree of hemolysis, plasma samples were used in the electrophoretic analyses. Although fibrinogen would be expected in blood plasma it did not migrate as a distinct band. This factor caused confusion in the interpretation of the plasma protein profiles. Generally 4 to 5 distinct bands were seen on the stained cellulose polyacetate strips. The separations were distinct with little or no tailing of fractions. On the basis of comparison with adult plasma samples the components of puppy plasma were identified and measured as albumin, alpha, beta, and gamma globulins. In some samples the alpha globulin fraction and in others the beta globulins were divided into two bands. This inconsistent separation of fractions into bands caused difficulty in determining the gamma globulin band. For the comparative purposes of this study the plasma proteins were measured as albumin, alpha globulin and combined beta and gamma globulins.

No difference was detected between plasma samples taken prior to feeding of hyperimmune serum and those taken 12 hours later. (See Table 2). Even though Salmonella pullorum antibody titers were detected following the feeding of hyperimmune serum, there did not appear to be a consistent increase in the percentage of gamma globulin present in the plasma proteins.

Small amounts of gamma globulin were detected in the plasma of colostrum-free newborn puppies; however, no quantitative studies were undertaken due to the difficulty of separation of gamma globulin and blood pigments.

Table 2. Plasma proteins of newborn puppies before and 12 hours after the feeding of hyperimmune serum expressed in percent of total plasma protein

	Litter 1		Litter 2		Litter 3	
	Before (av. of 3 puppies)	After	Before (av. of 2 puppies)	After	Before (1 puppy)	After
Albumin	62.5	65.8	71.3	65.7	56.4	61.5
Alpha globulin	15.6	17.8	14.8	19.6	22.3	21.5
Beta and gamma globulin	21.9	16.4	14.0	14.7	21.2	16.9

Influence of Diet on the Development of Intestinal Proteolytic Activity

Gastric and intestinal proteolytic activities have been proposed by some authors as being responsible for the cessation of antibody absorption in the newborn. A study of dietary influence on antibody absorption must take into consideration dietary influence on the development of proteolytic activity in order for valid conclusions to be made. There may be no real dietary effect but rather a secondary proteolytic effect induced by the diet.

Intestinal proteolytic activity was satisfactorily measured without the addition of exogenous enterokinase. The addition of enterokinase may have enhanced intestinal proteolytic activity; however, the results obtained in this study are valid on a comparative basis and reflect the

effective proteolytic activity of the intestinal ingesta at the time of sampling.

The data obtained on the influence of the protein and protein-free diets on intestinal proteolytic activity were statistically analyzed. (For individual observations see Tables 6 and 7 in Appendix B).

The analyses of the unweighted means in a randomized complete block design showed the influence of diet on proteolytic activity among littermates. (See Table 3).

In the unweighted mean analysis of variance in the block design the litter and age effects were confounded; however, the combined effect was barely significant ($p < 0.10$). Those littermates on a protein diet had significantly ($p < 0.025$) more proteolytic activity than littermates on the protein-free diet. There was no significant interaction between the litter-age factor and treatment.

In the analysis of covariance of the pooled data (see Table 4) the effect of treatment and age on the development of proteolytic activity was demonstrated. Figure 3 graphically portrays the results of this analysis. There was a significant increase ($p < 0.005$) in the intestinal proteolytic activity in those puppies maintained on the protein diet when compared with those on a protein-free diet. In both diets there was a statistically significant ($p < 0.005$) increase in proteolytic activity with age. There was not a significant difference ($p > 0.10$) in the slope of the regression lines fitted by the method of least

Table 3. Unweighted mean analysis of variance of the influence of diet on intestinal proteolytic activity (2-way factorial, treatment x litter-age)

Source	Degrees freedom	Sum of squares	Mean square	F
Litter-age	10	10,639	1,064	2.04*
Treatments	1	4,071	4,071	7.82**
Interaction	10	6,568	657	1.26
Error	23	11,982	520	
Total	44			

*Significant at 0.10 level.

**Significant at 0.025 level.

squares for the protein and protein-free diets. This indicated that there was an increase in proteolytic activity with age irrespective of diet.

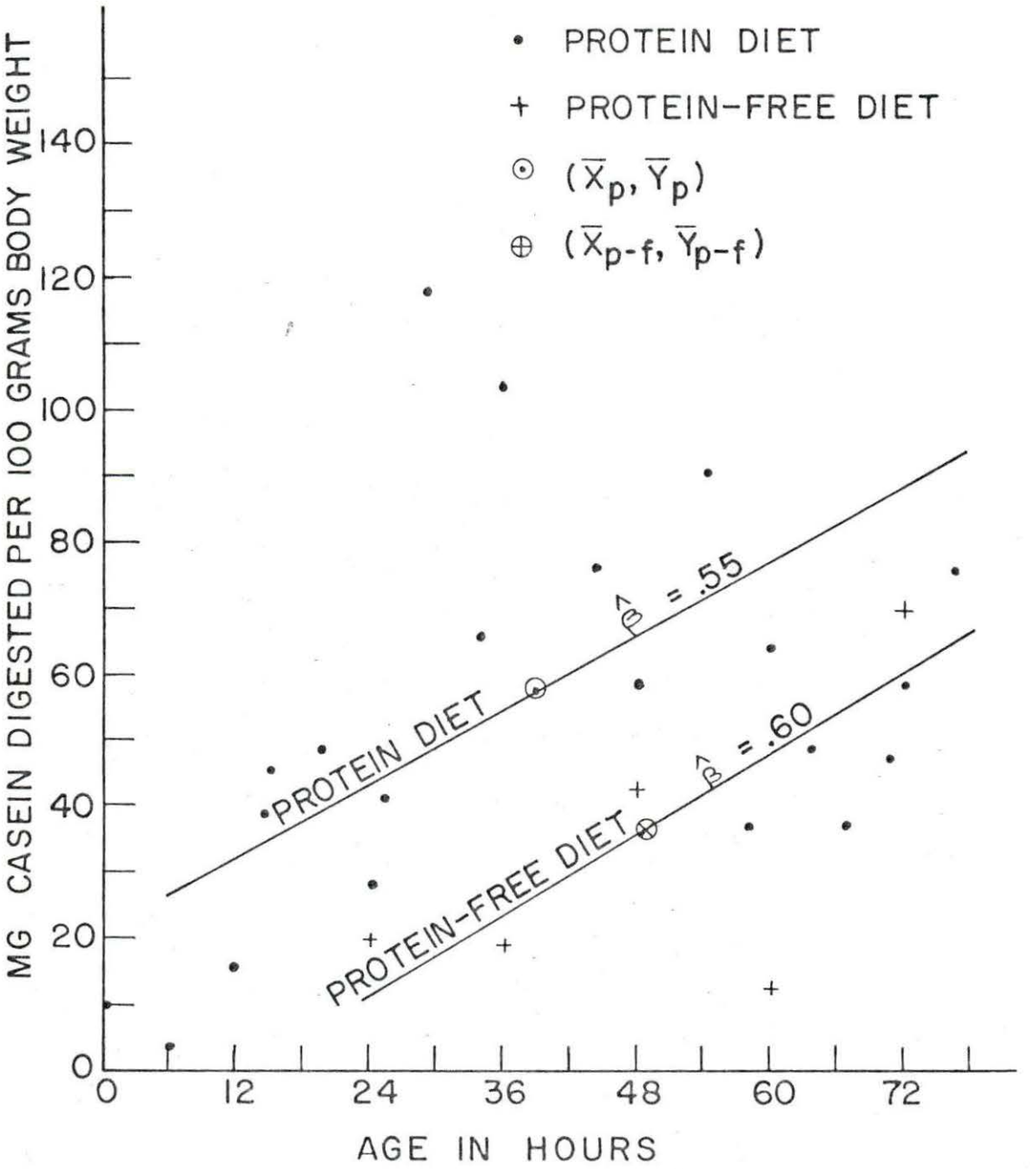
The significant increase in intestinal proteolytic activity found with the protein diet is in agreement with the work of Snook and Meyer (1964a and 1964b). The precise mechanism in which this increase is mediated was not determined in the present study; however, the work of Snook and Meyer indicates that the presence of protein in the intestine protects the enzymes from degradation and causes an actual increase in enzyme secretion.

Table 4. Analysis of covariance of pooled data on influence of diet on the development of proteolytic activity

Source	Degrees freedom	Σx^2	Σxy	Σy^2	Reg. coef.	Deviations from regression			
						Degrees freedom	$\Sigma y^2 - (\Sigma xy)^2/\Sigma x^2$	Mean square	F
Protein diet	61	21,270	11,678	77,981	.60	60	71,569	1,192	
Protein-free diet	27	10,270	6,192	11,721	.55	26	7,987	3,072	
Within						86	79,557	925	
Reg. coef.						1	30	30	.03
Common	88	31,541	17,871	89,702	.57	87	79,587	914	
Adj. means						1	12,724	12,724	13.75*
Total	89	33,519	13,819	98,998		88	92,311		

*Significant at the 0.005 level.

Figure 3. Influence of dietary protein on the development of intestinal proteolytic activity in the puppy


PROTEIN DIET
PROTEIN-FREE DIET
$(\bar{X}_p, \bar{Y}_p)$
$(\bar{X}_{p-f}, \bar{Y}_{p-f})$
MG CASEIN DIGESTED PER 100 GRAMS BODY WEIGHT
0
20
40
60
80
100
120
140
0
12
24
36
48
60
72
AGE IN HOURS
PROTEIN DIET
$\hat{\beta} = .55$
PROTEIN-FREE DIET
$\hat{\beta} = .60$

The presence of proteolytic activity in the newborn puppy is consistent with the histological findings of McCance and Brown (1953). These workers described abundant secretory granules in the pancreas of the newborn and suckling puppy. Fewer granules were found in the puppies maintained on boiled bitch's milk or on evaporated goat's milk. Thus agreeing with the present finding of dietary influence on proteolytic activity in puppies.

There appeared to be an increase in intestinal proteolytic activity at about 30-36 hours of age in those puppies maintained on a protein diet. This occurred shortly after the cessation of antibody absorption. Although the data were well fitted to a regression line, it is possible that this is a true increase and is related to cessation of antibody absorption. It has been shown that protein in the diet stimulates the development of proteolytic activity. Following the cessation of the protein absorption, the presence of non-absorbable protein may stimulate the secretion of proteolytic enzymes.

Influence of Proteolytic Activity on Antibody Absorption

Influence of trypsin inhibitor on proteolytic activity and antibody absorption

Trypsin inhibitor was included in the diet of 15 puppies in 5 litters (see Tables 10 and 11 in Appendix B for individual observations). An unweighted mean analysis of variance (see Table 5) was used to evaluate the data.

Table 5. Unweighted mean analysis of variance of the influence of trypsin inhibitor on intestinal proteolytic activity

Source	Degrees freedom	Sum of squares	Mean square	F
Litter-age	4	7,839	1,960	3.67*
Treatment	1	3,251	3,251	6.09*
Interaction	4	394	99	.18
Error	5	2,673	534	
Total	14			

*Significant at the 0.10 level.

The addition of trypsin inhibitor at the rate of 125 mg per 10 ml of liquid protein diet caused a significant decrease ($p<0.10$) in intestinal proteolytic activity. However, there was residual intestinal proteolytic activity in puppies fed trypsin inhibitor. Not all of the proteolytic activity in intestinal ingesta is due to the action of trypsin and as such it would not be expected that all proteolytic activity would be inhibited. The residual proteolytic activity found in trypsin inhibitor fed puppies at 24 to 36 hours was equivalent to that found in 14 hour old puppies on the protein diet. Thus proteolytic activity was reduced to a level which was found in those puppies that demonstrated the ability to absorb antibody.

Trypsin inhibitor in the diet and hyperimmune serum given orally to 18 puppies in 5 litters did not elicit a significant increase in either the quantity of antibody absorbed or the period of antibody absorption (for individual observations see Table 11 in Appendix B). In puppies maintained on the trypsin inhibitor diet and fed hyperimmune serum at either 24, 36, or 48 hours there was no antibody absorption. In two litters fed trypsin inhibitor and hyperimmune serum at 3 and 30 hours, respectively, there was absorption of antibody; however, there was no significant difference in antibody titers between treated and control puppies. This indicates that reduction of intestinal proteolytic activity to a level found in younger puppies that did absorb antibody did not enhance antibody absorption nor prolong the period of absorption in older puppies. This is not in agreement with the work of Hardy (1965) who found that trypsin inhibitor isolated from bovine colostrum accelerated antibody absorption in newborn pigs. However, the trypsin inhibitor fraction fed by Hardy may have contained the low molecular weight protein described by Balfour and Comline (1962) and Lecce (1964). These investigators reported that presence of this protein enhanced antibody absorption. The crystalline soybean trypsin inhibitor used in this study probably did not contain this factor. The results of this study are supported by the work of Barrick et al. (1954). These workers reported that inclusion of trypsin inhibitor in the milk did not prolong or enhance gamma globulin absorption in the pig.

It is possible that proteolytic degradation of antibody may occur in the stomach and that such digestion contributes to the cessation of antibody absorption. However, the work of Smith and Erwin (1959) indicated that antibody absorption did not occur in 48-60 hour old calves when antibody was administered directly into the duodenum. Assays of gastric proteolytic activity were not carried out in this study.

Influence of trypsin on antibody absorption

A limited study was conducted in which 6 puppies from 2 litters were fed crystalline trypsin at the rate of 25 mg tryptic activity per 10 ml serum and later fed trypsin in the diet at the rate of 50 mg per 10 ml liquid protein diet (for individual observations see Table 11 in Appendix B). These puppies were fed the trypsin-hyperimmune serum combination at 1 or 7 hours of age. No significant decrease in the quantity of antibody absorbed was observed in puppies fed trypsin when compared with puppies fed the protein diet.

The results of this study indicate that proteolytic activity does not, within the parameters studied, play a significant role in the cessation of antibody absorption. Further evidence supporting this conclusion was demonstrated in the fluorescent labeled gamma globulin study. Within 1 to 2 hours after feeding of the fluorescent labeled globulin there was marked fluorescence within the intestinal epithelial cells and intestinal submucosa. The rapidity of absorption would not seem to allow complete degradation of antibody by proteolytic enzymes if they were present.

SUMMARY AND CONCLUSIONS

The influence of diet on the intestinal absorption of Salmonella pullorum antibody of canine serum origin was studied in newborn puppies. Four dietary regimens were utilized in the study: 1. protein diet (12.5% protein liquid solution); 2. protein-free diet (10% sucrose in 0.85% NaCl); 3. natural diet with puppies allowed to suckle; and 4. starvation. Dietary regimens did not significantly influence the quantity of serum Salmonella pullorum antibody absorption. The protein-free diet significantly increased the period of antibody absorption in puppies as compared with those on the protein diet. Puppies on the protein-free diet were found to absorb antibody until 36 hours of age while puppies on the protein diet were found to absorb antibody up to 30 hours after birth. Starved puppies retained the ability to absorb antibody for 34 hours after birth while antibody absorption ceased in naturally suckling puppies at 24 hours of age.

Intestinal proteolytic activity was significantly increased in puppies on the protein diet when compared with puppies on the protein-free diet; however, there was an increase in intestinal proteolytic activity with age regardless of the dietary regime. The regression lines of proteolytic activity on age were parallel for puppies on the protein and protein-free diets. The feeding of trypsin inhibitor in the diet caused a significant decrease in intestinal proteolytic activity. Studies with trypsin inhibitor or trypsin in the diet and hyper-

immune serum indicated that proteolytic activity in the intestine was not a significant factor in the cessation of antibody absorption.

Studies with fluorescent labeled gamma globulin indicated that absorption occurred primarily in the jejunum and ileum within 1 to 2 hours after the feeding of the labeled globulin. Fluorescent globules were present in either the epithelial cells or submucosa; but, were rarely seen in both areas in the same section. Absorption of fluorescent labeled globulin was not detected in puppies over 36 hours of age.

Small amounts of gamma globulin were demonstrated in the plasma of newborn colostrum-free puppies. The presence of hemoglobin and other blood pigments precluded a quantitative determination of the plasma gamma globulin content.

The factors which are responsible for the cessation of antibody absorption in the newborn puppy are still unknown. The influence of protein contact and the development of proteolytic activity are not primary factors in the cessation of absorption. Thus, some unknown factor or factors, possibly hormonal may regulate the postnatal absorption of antibody in the newborn puppy.

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APPENDIX A

Proteolytic Activity Determination

Reagents:

Trichloroacetic acid: 5 grams per 100 ml distilled water

Sodium hydroxide: 0.5 N

Sodium potassium tartrate: 2.7 grams/100 ml distilled water

Sodium carbonate: 2 grams/100 ml distilled water

Casein[1]: digestion solution (5 mg/ml distilled water)
standard solution (0.3 mg/ml 0.5 N sodium hydroxide)

Folin-Ciocalteu reagent[2]

Folin's solution A: 1 ml 2.7% sodium potassium tartrate
1 ml 1% cupric sulfate
100 ml 2% sodium carbonate

Folin's solution B: commercial Folin-Ciocalteu reagent diluted with water to 1 N acid

Procedure:

1. Digestion

Add 0.1 ml of intestinal ingesta supernatant to 9 ml of the casein digestion solution prewarmed to 39° C. Mix well and immediately withdraw 2 ml control sample and place in 3 ml of trichloroacetic solution. Incubate remaining casein solution for 20 minutes at 39° C.

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[1] De-vitaminized casein (94% protein), Sheffield Chemical, Norwich, New York.

[2] Phenol reagent - Folin Ciocalteu, Hartman-Leddon Company, Philadelphia, Pennsylvania.

Following the digestion period a 2 ml aliquot was removed from the digestion medium and added to 3 ml of trichloroacetic acid solution and allowed to stand at room temperature for 30 minutes and then centrifuged.

2. Protein determination on control and digestion samples

0.1 ml of supernatant is added to 1 ml of sodium hydroxide solution and to this is added 5 ml Folin's solution A. The solutions are mixed and allowed to stand for 10 minutes at room temperature. 0.5 ml of Folin's solution B is added, mixed and allowed to stand at room temperature for 30 minutes.

The resulting color solution is read at 750 millimicrons in a spectrophotometer[1].

Protein Standard Determination

The standard solution of casein was diluted with 0.5 N sodium hydroxide to give concentrations of 0.03, 0.06, 0.09, 0.15, 0.21, and 0.30 mg/ml. Protein determinations were done on 1 ml of each dilution by adding 5 ml of Folin's reagent A, waiting 10 minutes and adding 0.5 ml of Folin's reagent B. After 30 minutes the color solution is read at

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[1]Model B spectrophotometer, Beckman Instrument Company, Fullerton, California.

750 millimicrons in a spectrophotometer. A standard curve was drawn to correlate spectrophotometric readings and protein concentration.

APPENDIX B

Individual Puppy Data

Table 6. Influence of diet on Salmonella pullorum antibody titers per ml puppy serum 12-15 hours after feeding of hyperimmune serum. Pooled data

Protein diet		Protein-free		Natural diet		Starvation	
Age fed (hrs)	Serum titer	Age fed (hrs)	Serum titer	Age fed (hrs)	Serum titer	Age fed (hrs)	Serum titer
1	160,120,40, 40,40,60, 40,80,60, 160,40,80, 160,120,40, 80	10	80	24	120,80	34	10,30,60
		12	240,240, 320	36	0,0,0,0,0	36	0,0,0,0
				38	0,0	38	0,0
		14	160,160	48	0,0	43	0,0,0
		24	80,80,80, 60,60	56	0		
				72	0,0,0		
2	80	26	80,80				
3	20,30	28	0,0,0,0				
12	240,120,80	32	0,0,0,0				
14	80,80	36	10,20,0,0, 0,0,0,0				
15	120,160,40, 40	38	0,0				
19	40,20	45	0,0				
20	10,20	47	0,0				
24	0,0,0	48	0,0				
26	0,0	72	0,0,0				
28	0,0	84	0,0				
30	30						
32	0,0						
34	0,0						
36	0,0,0,0,0						
38	0,0,0,0,0, 0,0						
43	0						
45	0						
47	0,0						
48	0,0						
56	0,0						
60	0,0						

Table 7. Influence of diet on Salmonella pullorum antibody titers per ml puppy serum 12-15 hours after feeding of hyperimmune serum. Littermate comparison

Litter	Age fed (hours)	Diet		
		Protein-free	Protein	Starvation
22	12	240, 240, 320	240, 120, 80	
14	14	160, 160	80, 80	
14	26	80, 80	0, 0	
48	28	0, 0	0, 0	
48	32	0, 0	0, 0	0, 0
26	34		0, 0	10, 30, 60
13	36	10, 20	0, 0	
17	36	0, 0	0	
18	36	0, 0	0	
19	36	0, 0	0	
20	38	0, 0	0, 0	0, 0
31	43		0, 0	0
34	45	0, 0	0	0, 0, 0
27	47	0, 0	0, 0	

Table 8. Influence of diet on proteolytic activity of intestinal ingesta in mg casein digested per 100 grams puppy body weight. Pooled data (average of two determinations per puppy)

Age in hours	Diet			
	Protein	Protein-free	Natural	Starvation
0	14.95, 5.75			
6	3.69			
12	18.07, 12.81		41.07, 53.48, 35.78	
14	47.55, 46.88, 23.61			
15	40.66, 50.27			
20	49.83			
24	27.18, 17.75, 56.88, 28.34, 9.86	13.78, 22.29, 10.50, 16.50, 34.50		
25	23.66, 29.32, 42.83, 70.38			
28	118.23			
34	62.10, 74.56, 62.10			
36	55.91, 70.01, 155.25, 77.62, 113.85, 148.35	25.87, 21.56, 9.58	72.45, 85.34, 86.25, 84.33	
44	94.01, 62.24, 72.09			
48	30.04, 35.03, 10.84, 23.46, 130.81, 41.07, 82.14, 84.87, 66.35, 72.15, 84.09, 103.47, 141.31, 58.65	24.35, 30.90, 63.69, 35.78, 62.10, 53.31, 15.58, 44.36, 20.39, 51.13, 33.95, 51.75, 41.14		66.11, 25.87, 78.86
55	83.27			
56	10.00, 63.25			
60	66.91, 9.58, 59.59, 58.63, 71.30	17.76, 6.47		13.80, 18.65, 32.68

Table 8. (Continued)

Age in hours	Diet			
	Protein	Protein-free	Natural	Starvation
64	21.56, 56.93, 84.82, 34.36			
72	46.76, 58.46	69.86, 69.00, 69.00	69.00	
76	75.15			
98		72.45, 35.56		

Table 9. Influence of diet on proteolytic activity of intestinal ingesta in mg casein digested per 100 grams puppy body weight. Littermate comparison (average of two determinations per puppy)

Litter	Age in hours	Diet	
		Protein-free	Protein
14	24	13.78, 22.29	27.18, 17.25
22	24	10.50, 16.50, 34.50	56.88, 28.34, 9.86
14	36	63.69, 6.69	70.01, 55.91
13	48	24.35, 30.35	30.04, 35.03, 10.84
17	48	35.78, 62.10	23.46
18	48	53.31, 15.58	130.81
19	48	44.36, 20.39	41.07, 82.14
20	48	51.13, 55.95	84.87, 66.35
27	48	51.75, 41.14	103.45, 141.31
34	60	17.76, 6.47	58.63, 71.30
28	76	69.86, 69.00, 69.00	75.15

Table 10. Influence of trypsin inhibitor on intestinal proteolytic activity in mg casein digested per 100 grams puppy body weight. Littermate comparison (average of two determinations per puppy)

Litter	Age in hours	Protein diet + trypsin inhibitor	Protein diet
37	28	139.86, 59.70	118.23
40	72	24.35, 15.53, 6.00	58.46
42	15	16.98, 14.27	45.66, 50.27
41	48	27.97	58.65
43	55	27.60	83.27

Table 11. Influence of trypsin inhibitor or trypsin on Salmonella pullorum antibody titers in puppies. Littermate comparison

Litter	Age fed (hours)	Protein diet + trypsin inhibitor	Protein diet	Protein diet + trypsin
37	30	40, 20	30	
40	36	0, 0, 0	0	
41	48	0, 0	0	
42	3	40, 40	30, 20	
43	24	0, 0	0, 0	
38	7		40	20, 40, 80
39	1		240	80, 120, 160