STUDIES OF GAMMA-GLOBULIN METABOLISM
IN THE FOETAL AND NEONATAL LAMB

A Thesis
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by
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Statement

With the exception of some of the surgical operations which were done by Dr. M.W. Simpson-Morgan, the experiments described in this thesis were done by myself.
During the course of their evolution, mammals have been endowed with elaborate mechanisms for defence against invasion by foreign noxious agents. They are able to synthesize special proteins, the antibodies, which can render many of these noxious agents harmless. The number of different antibodies which an individual animal can make is prodigious, but any given antibody is made only in response to invasion by the agent against which it is directed specifically.

During the time between their conception and the beginning of their independent existence, mammals develop in an environment free of noxious agents. When such an animal is born, it must mount responses against the multitude of noxious agents it encounters. The time required to mount effective responses might well lead to the animal succumbing to this first encounter, if it were not otherwise protected. However, most animals are protected during this early period of independent existence, by virtue of their mothers' experience of encounters with noxious agents, which indeed are those most likely to be encountered by the young. This protection is accomplished by the transfer of maternal
antibodies to the young, either during gestation, or soon after birth.

The experiments reported in this thesis were carried out to study aspects of this process in sheep. In this species, maternal antibodies are transmitted in the first milk of the ewe to the lamb which is able to absorb them intact, for a short period after birth. The need for colostrum antibodies by lambs, the extent to which the antibodies were absorbed, and the period during which the absorption was possible, were studied in newborn lambs. In order to avoid some of the difficulties associated with carrying out these experiments in newborn lambs, and in order to gain a better insight into mechanisms involved in the absorption of maternal antibodies, techniques were devised so that the process could be studied in foetal lambs in utero. Quantitative and morphological aspects of the absorption in utero were then studied and the results of these studies compared with the results of similar studies with the newborn lamb.
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CHAPTER 1.

INTRODUCTION

THE NATURE OF ANTIBODIES

The discovery of antibodies

It has been known for a long time that after an animal recovers from certain diseases, it is immune to that particular disease. Jenner (1798) discovered that vaccinia had a protective action against smallpox, and this led to "vaccination" against this disease. Schwann (1835) and Pasteur (1860) established that putrefaction was caused by micro-organisms, and associated many disease processes with micro-organisms. Pasteur began the use of attenuated organisms for producing immunity against virulent forms of the organism (Pasteur, 1885). Panum (1874) and Metchnikoff (1883) showed that white blood cells could engulf micro-organisms, and certain forms of immunity could be related to the phagocytic activity of white cells. Lister (1881) noted that shed blood did not putrefy as rapidly as did other organic materials, and Grohmann (1884) showed that blood plasma inhibited the activity of certain bacteria. Buchner (1889) found that blood serum also contained
bactericidal activity which was attributed to both a heat-labile component (complementary body) and a stable component (immune body). This was confirmed by Pfeiffer (1894a). Passive immunization by transfer of blood or serum from an immune to a susceptible animal was demonstrated by von Behring and Kitasato (1890) in rabbits, and provided evidence for immune bodies being present in serum. The immune bodies were first described in terms of their activity in vitro. Pfeiffer (1894b) measured the "bacteriolysins", Gruber and Durham (1896) the "agglutinins", and Kraus (1897) the "precipitins" present in immune sera. These immune bodies came to be known as "Antikörper" (Ehrlich, 1900), "anticorps" (Deutsch, 1899), and finally the term antibody came into use about 1903 (Walker, 1903). In an attempt to classify the antibodies in chemical terms, Ehrlich (1900) introduced his Side-Chain Theory of immunity in which cells released specific receptors in response to antigen. The receptors or antibodies combined specifically with the antigen. This and later work led to the Unitarian Theory described by Zinsser (1921) in which all the diverse group of precipitins, agglutinins, etc., were considered as antibodies with basically similar
properties. All the reactions observed in vitro resulted from the formation of an antigen-antibody complex, the observed haemolysis, agglutination, or precipitation being determined by the other conditions in the system.

The discovery and isolation of γ-globulin

Panum (1851) and several other workers (cf. Putnam, 1960) observed that a precipitate formed when acidified serum was diluted with water, and Schmidt (1862) named this precipitate "globulin". Later Hofmeister (1888) introduced the use of neutral salts for the precipitation of proteins by "salting-out". This method was used extensively for the fractionation of serum and Gürber (1894) obtained crystalline serum albumin by ammonium sulphate precipitation. Atkinson (1900) precipitated a globulin fraction from horse serum using magnesium sulphate, and found that the antitoxin activity of the serum was associated with the globulin fraction. However Pick (1901) and Gibson and Collins (1907) were unable to attribute antibody function to a single specific fraction obtained by salt precipitation. Howe (1921) using sodium sulphate separated serum into four components which he called euglobulin, pseudoglobulin I and II, and albumin, but no further separation was achieved by this method alone.
Tiselius (1937) introduced moving boundary electrophoresis for the analysis of blood serum proteins, and identified four fractions which he designated albumin and α-, β-, and γ-globulin. He observed that serum protein fractions obtained by salt precipitation contained some of each of the four fractions, explaining the failure of some earlier workers to relate antibody function to the salt-precipitated fractions. Tiselius and Kabat (1939) showed that when antibody from immune serum was removed by precipitation with the antigen, there was a marked decrease in the concentration of γ-globulin. Wright (1942) demonstrated that antibodies to either serum γ-globulin or to a specific antibody would cross-react, and the antibody activity could be precipitated by anti-γ-globulin serum. Thus there was shown to be a close association between γ-globulin and antibody activity. Subsequent work using purified protein fractions obtained by a wide variety of techniques (cf. Sober, Hartley, Carroll and Peterson, 1965) has confirmed that antibodies are γ-globulins.

The characterization of γ-globulin

The molecular weight of purified γ-globulin has been estimated by various means. An average figure of
150,000 for the 7S γ-globulin and $10^6$ for the 19S macroglobulins are given by Cohen and Porter, (1964). The amino acid composition of the γ-globulins has been determined by Smith, McFadden, Stockell and Buettner-Janusch (1955) and Fleischer, Hardin, Horowitz, Zimmerman, Gresham, Turner, Burnett, Stary and Haurowitz (1961).

Since even the purest samples of γ-globulin from normal serum contain a large number of slightly different protein molecules, no positive correlation between the amino acid composition and a particular antibody activity has been found.

Chemical reduction of the disulphide bonds in the γ-globulin molecule yielded two components which could be separated by gel filtration (Edelman and Benacerraf, 1962; Cohen, 1963; Cohen and Porter, 1964). The larger and smaller components were named the heavy or A chain, and the light or B chain respectively. Papain and pepsin digestion (Porter, 1959) of purified γ-globulin preparations, and subsequent analysis of the peptides liberated by these enzymes, have provided further evidence for the structure of antibodies (Porter and Press, 1962; Cohen and Porter, 1964). From this work a reasonable picture of the structure of the γ-globulin
molecule has been built up, as depicted in Fig. 1 (Cohen and Porter, 1964). Of the three fragments obtained by papain digestion, all of which have a molecular weight of about 45,000, two are identical, FI and FII (Fab), and the third fragment, FIII (F′ab'), contains most of the antigenic determinants of γ-globulin.

No homogeneous antibodies have yet been obtained since, although antibodies produced in response to a single antigen might have broadly similar physical chemical properties, some heterogeneity can be inferred from the way in which they react with the antigen.

Work on the structure of γ-globulin has been concentrated on the γ-globulin produced by myeloma cells, which for a strain of myeloma is believed to be homogeneous and characteristic of that strain.

The amino acid sequence of at least part of both the A and B chains in these proteins has been elucidated (Huebner, 1968).

The site of synthesis of γ-globulin

Early studies by Deutsch (1899) demonstrated that the spleen and lymphoid tissue were important in antibody production.
molecule has been built up, as depicted in Fig. 1 (Cohen and Porter, 1964). Of the three fragments obtained by papain digestion, all of which have a molecular weight of about 45,000, two are identical, FI and FII (Fab), and contain the antigen-binding sites. The third fragment, FIII (Fc), contains most of the antigenic determinants of \( \gamma \)-globulin.

No homogeneous antibodies have yet been obtained since, although antibodies produced in response to a single antigen might have broadly similar physico-chemical properties, some heterogeneity can be inferred from the way in which they react with the antigen. Hence recent work on the structure of \( \gamma \)-globulin has been concentrated on the \( \gamma \)-globulin produced by myeloma cells, which for a given strain of myeloma is believed to be homogeneous and characteristic of that strain. The amino acid sequence of at least part of both the A and B chains in these proteins has now been elucidated (Haber, 1968).

The metabolism of \( \gamma \)-globulin

The site of synthesis of \( \gamma \)-globulin

Early studies by Deutsch (1899) demonstrated that the spleen and lymphoid tissue were important in antibody
production. Later, systems were developed using fragments of spleen, bone marrow and lymph nodes, which could produce antibody in vitro for periods of several days (Carrel and Ingebrigtsen, 1912; Salle and McOmie, 1937; Stavitsky, 1961). Askonas and Humphrey (1958) found that tissue slices from the spleen, lymph nodes, bone marrow and lung of rabbits taken after immunization of the animal would synthesise specific antibody. Isolated cell preparations from lymph nodes have also been shown to synthesise antibody in vitro (Nossal, 1958; Attardi, Cohn, Horibata and Lennox, 1959).

Therefore γ-globulin synthesis differs from that of the other major plasma protein fractions which are produced by the liver (Miller, Bly, Watson and Bale, 1951; Miller and Bale, 1954).

The catabolism of γ-globulin

The site of catabolism of all the plasma proteins including γ-globulin is still obscure. Evidence for the use of plasma proteins as a source of energy by the fasting dog when they are given intravenously, suggests that most cells of the body might metabolize these proteins (Daft, Robscheit-Robbins and Whipple, 1938). Specific organs have been proposed as sites
for albumin catabolism, viz., the liver (Gitlin, Klinenberg and Hughes, 1958; Cohen and Gordon, 1958), the kidney (Katz, Rosenfeld and Sellers, 1960) and the small intestine (Wetterfors, Gullberg, Liljedahl, Plantin, Birke and Olhagen, 1960). The use of iodinated albumin, which is partially denatured during preparation, in these experiments may have influenced the results since it has been shown that the kidney catabolises biologically screened iodoalbumin to a less extent than iodoalbumin which has not been screened (Katz et al., 1960). Albumin catabolism in the mouse was inhibited when the reticulo-endothelial cells of the liver were blocked with Indian ink (Gitlin et al., 1958), suggesting that these cells are involved in plasma protein breakdown.

The turnover of γ-globulin

Most of the studies of the rates of γ-globulin metabolism in the intact animal have made use of labelled plasma proteins injected into the animal. An understanding of the continual turnover of the plasma proteins developed after the fractionation of plasma yielded relatively pure proteins, and isotopic tracers became available. Although used as tracers in plants
earlier (Hevesy, 1923), heavy isotopes were only used in turnover experiments in animals from 1938 (Schoenheimer and Rittenberg, 1940). Using $^{15}$N-glycine, Schoenheimer and his colleagues established that there is a continual turnover of body proteins in the normal animal. Immune globulin was used as a case of a specific body protein, and it was established by following the isotopic concentration of specific antibodies to type III polysaccharide, that there is a continual turnover of antibodies (Schoenheimer, Ratner, Rittenberg and Heidelberger, 1942).

Two methods using radioactive isotopes have been used for measuring the rate of turnover of plasma proteins. Proteins have been labelled in vivo by the injection of radioactively labelled amino acids, and the time course of the level of various radioactive proteins present in the plasma has been followed (Fink, Enns, Kimball, Silberstein, Bale, Madden and Whipple, 1944; Campbell, Cuthbertson, Matthews and McFarlane, 1956; Cohen, Holloway, Matthews and McFarlane, 1956; Webster, Laver and Fazekas de St. Groth, 1962). This method is wasteful of isotopes since only a very small proportion of the administered
amino acids is incorporated into plasma proteins, and re-incorporation into the plasma proteins of labelled amino acids liberated by the catabolism of labelled proteins can cause errors in the estimation of rates of turnover.

*In vitro* labelling of isolated proteins with $^{131}$I and $^{125}$I has been used by many workers using a variety of labelling methods (Dixon, Talmage, Maurer and Deichmiller, 1952; Francis, Mulligan and Wormall, 1951; McFarlane, 1956, 1958; Hunter and Greenwood, 1962; Katz and Bonorris, 1968). The turnover rate has been estimated from total body radioactivity, iodide excretion curves, and plasma radioactivity decay curves (Cohen, Freeman and McFarlane, 1961; Beeken, Volwiler, Goldsworthy, Garby, Reynolds, Stogsdill and Stemler, 1962). Several workers have compared the half-lives for $\gamma$-globulin obtained by *in vivo* and *in vitro* labelling methods, and have found reasonable agreement (McFarlane, 1956; Campbell et al., 1956; Cohen et al., 1956).

The rate of turnover of $\gamma$-globulin varies from one species to another. Dixon *et al.* (1952) found the half-life of $\gamma$-globulin to be 1.9 days in the mouse,
4.6 days in the rabbit, and 20.3 days in man. The results of turnover studies suggest that despite the fact that, unlike other plasma proteins, it is produced by lymphoid tissue in response to specific antigenic stimulation, in some other respects γ-globulin behaves similarly in vivo to other plasma proteins.

PASSIVE IMMUNIZATION OF THE NEWBORN ANIMAL

Agammaglobulinemia in the newborn

Low levels of circulating γ-globulin in germfree animals (Luckey, 1963) are a result of the absence of antigenic stimuli for antibody production. Similarly, since the number of antigens encountered by the foetus in the uterine environment is very limited during normal gestation, the newborn animal is lacking in autologous specific antibodies to pathogenic organisms. In the serum of the newborn piglet (Payne and Marsh, 1962a), calf (Pierce, 1955) and lamb (McCarty and McDougall, 1953), there is almost no detectable γ-globulin. In other species the presence of maternal γ-globulin transferred to the foetus before birth masks the lack of autologous antibody in the newborn animal. In some species maternal antibody is transferred from mother...
to offspring during the first few weeks of life, giving passive immunity to many diseases to which the mother is immune. The manner in which the antibody is transferred and the time at which transfer takes place in various species are discussed below.

Transfer of maternal antibody to the offspring

Birds

In birds where the embryo develops within an egg quite separate from the mother, maternal antibodies present in the yolk are absorbed by the yolk-sac epithelium and transferred to the vitelline and hepatic circulation. In chick embryos this occurs after 10 days incubation by which time the pliae or folds of the yolk-sac epithelium are functional and absorb most of the contents of the yolk (Romanoff, 1960).

Mammals

In eutherian mammals the fertilized ovum develops into a new individual within the uterine environment. Antibody can pass to the young either before birth from the maternal plasma to the foetus or after birth through the milk ingested by the newborn offspring. The colostrum or milk produced by the mammary gland immediately after birth is rich in antibodies which can be absorbed intact by the intestinal epithelium of some young animals.
(i) Prenatal transfer of antibody

Of those species in which maternal antibody is transferred to the foetal circulation before birth, the rabbit, guinea-pig, primates and rodents will be considered.

In the rabbit, transfer of antibody appears to be almost exclusively by way of the uterine lumen and the yolk-sac epithelium (Brambell, Hemmings, Henderson, Parry and Rowlands, 1949). Plasma proteins at a level of 50% of that in maternal serum were found in rabbit yolk-sac fluid at 9 days post-coitum, showing that the uterine epithelium was able to release antibodies into the uterine cavity. The yolk-sac persists until term in the rabbit (Brambell, 1966) though the distal wall breaks down by the 15th day, exposing the inner surface of the yolk-sac to the uterine lumen (Brambell et al., 1949).

Another species in which antibody transfer is almost entirely prenatal is the guinea-pig. Leissring and Anderson (1961) showed that transfer of Brucella abortus antibodies occurred by way of the yolk-sac epithelium up to about the 35th day of gestation, analogous to the route of transfer in the rabbit. During the last three
weeks of gestation, the foetal intestine was responsible for most antibody transfer, demonstrated by a cessation of uptake after ligation of the mouth of the foetus. Ligation of the vitelline vessels at this stage had no effect on the uptake of antibody.

Direct transfer of antibody across the trophoblast membrane from maternal to foetal circulation appears to be negligible in the lower animals, but the reason for this is not clear. Morphological differences in placentation do not explain the differences in γ-globulin transfer between these animals and the primates.

In man and other primates with a haemochorial placenta, the trophoblast can transfer antibodies to the foetus. Bangham, Hobbs and Terry (1958) and Bangham (1960) demonstrated that labelled γ-globulin injected intravenously into pregnant Rhesus monkeys was transferred to the foetal circulation, but this did not happen when the γ-globulin was injected into the amniotic fluid. These workers inferred that transfer from mother to foetus was via the placenta.

Prenatal transfer in the rat contributes a smaller proportion of the maternal antibody to the young than postnatal absorption by the gut. The route of entry to
the foetus has been investigated by Brambell and Halliday (1956) and Quinlivan (1964) and several different paths appear to contribute to the transfer. Antibody is absorbed from the uterine lumen and enters the foetal fluids by way of the yolk-sac epithelium and the amnion. Oral injection of antibody showed some absorption from the foetal gut was possible (Brambell and Halliday, 1956). Also, some antibody was transferred via the allantoic vessels draining the placenta (Quinlivan, 1964). Therefore the rat differs from the other species considered in that antibody is transferred by several routes at the same time.

(ii) Postnatal transfer of antibody

Among those species in which transfer of antibodies through the intestinal wall of the newborn occurs after birth there are two groups, those which are born with some circulating antibody, e.g., the rodents, and those which are born almost agammaglobulinemic and rely entirely on postnatal transfer, e.g., the pig, the horse, and the ruminants.

Antibody absorption from the intestine after birth has been studied extensively in rats and mice since the pioneer work of Ehrlich (1892), in which he showed that
ricin antitoxin was transferred to the offspring of immunized mice both before and after birth. In newborn mice, the period during which antibody is absorbed from the intestine is 14 days (Halliday, 1959). In the newborn rat the intestine remains permeable to protein for 18 days, after which there is a rapid decline in permeability (Halliday, 1955). Antibody absorption ceased rapidly in young rats after removal from the mother at less than 18 days of age (Halliday, 1959), yet when 17 day old rats were fostered on to rats in early lactation absorption of protein ceased at the normal time. This result suggested that the rapid change in intestinal permeability was not associated with a change in the milk, but that the stress of removing the young from their mother could cause the change in permeability to occur before 18 days of age.

In the piglet, Connaway (1921, reported by Nelson, 1932) found that temporary immunity to swine fever was transferred to sucking piglets via the colostrum. Transfer of antibody to vaccinia virus was shown by Nelson (1932). Earle (1935), using sodium sulphate fractionation, showed that the total globulin in piglet serum increased six times after ingestion of colostrum,
due mainly to an increase in euglobulin. Electrophoretic examination of piglet serum before and after the feeding of colostrum showed an increase in concentration of \(\gamma\)-globulin from about 1 mg/ml. to 20 mg/ml., representing about 35% of the total protein, during the first 24 hours post-partum (Foster, Friedell, Catron and Dieckmann, 1951).

The period during which the intestine of the piglet remains permeable to large molecules is normally 24-36 hours, but this period has been extended to 86 hours by starvation (Lecce and Morgan, 1962). When piglets were given only water to drink, absorption from a feed of colostrum \(\gamma\)-globulin was observed up to 106 hours of age (Payne and Marsh, 1962b), but feeding of milk or colostrum caused the cessation of protein absorption at the usual time.

Early reports (Mason, Dalling and Gordon, 1930; Earle, 1935) established that the foal at birth had a low level of serum globulin which rose rapidly after the ingestion of colostrum. Further work on this species was stimulated by the finding that absorption of antibody from colostrum was the cause of haemolytic icterus in foals born to mares which were sensitized to the foetus'
red blood cells (Bruner, Edwards and Doll, 1948; Caroli and Bessis, 1947; Bessis and Caroli, 1947). Bruner, Doll, Hull and Kinkaid (1950) were able to produce the disease by the intravenous injection of sensitized mare's serum into foals. They showed also that intestinal absorption of antibody from an oral dose of adult horse serum ceased from 24 to 36 hours after birth. Recent electrophoretic studies suggested absorption might continue for five days (Kudryashov and Sergeeva, 1965). These workers measured a 20% increase in total serum protein within the first 24 hours after birth, and showed that a mature plasma protein profile was not reached until 180 days of age.

Absorption of \(\gamma\)-globulin by young ruminants has been investigated extensively. It was found that calves deprived of colostrum sickened and died within two weeks with evidence of gross infection of the tissues (Smith and Little, 1922). Howe (1922b, 1924) and Orcutt and Howe (1922) found that specific antibodies which could be precipitated by low concentrations of sodium sulphate appeared in the serum of newborn calves after they ingested colostrum. Hansen and Phillips (1947) reported that 90% of colostrum-deprived calves, raised on a skim milk diet, survived when infectious diseases were
prevented by sulfathiazole although body weight gains were less than those for normal calves.

No γ-globulin could be detected electrophoretically in the serum of newborn calves until after they had ingested colostrum (Jameson, Alvarez-Tostado and Sortor, 1942; San Clemente and Huddleson, 1943; Hansen and Phillips, 1947). The period during which the gut remained permeable to antibodies was found to be 24-30 hours (Hansen and Phillips, 1947; Pierce, 1955; Deutsch and Smith, 1957). Studies on the time of cessation of antibody absorption in the calf have shown a marked difference from the piglet. Whereas starvation prolonged the absorptive period in the piglet, calves maintained by intravenous feeding or transfusion of maternal blood were unable to absorb protein from the intestine after the normal 24 hours (Deutsch and Smith, 1957). Moreover, lactose-dextrose given orally, or progesterone, stilboestrol, or cortisone given parenterally, did not alter the time at which absorption ceased. Smith and Erwin (1959) introduced colostrum directly into the duodenum of calves of various ages previously maintained on mature milk, and found that absorption still ceased at the usual time of 24 hours. Steck (1962) found intact antibody in the faeces of calves 6 days old, suggesting
that there was still intact protein present in the intestine after absorption had ceased.

Smith, Reed and Erwin (1964) found that calves delivered by Caesarean section 14-19 days before term, were able to absorb \( \gamma \)-globulin, and that absorption ceased in less than 38 hours after delivery. These findings suggest an association between the start of independent existence and the time at which antibody absorption ceases.

The route taken by the \( \gamma \)-globulin absorbed by the intestinal epithelium has been elucidated in the calf, and presumed to apply to other species. Comline, Roberts and Titchen (1951a) using anaesthetized calves showed that colostrum antibody infused into the duodenum could be collected in thoracic duct lymph, and cannulation of the lymphatic vessel prevented a rise in blood serum titre which occurred in control animals.

Although Chauveau (1888) observed that lambs from ewes immune to anthrax appeared to be immune to the disease, it was not until later that the appearance of maternal globulin in the serum of the lamb after the ingestion of colostrum, and its importance in the passive transfer of immunity to the lamb, were described (Mason, Dalling and Gordon, 1930). These workers established
that no lamb dysentery antitoxin reached the lamb through the placenta, and that the titre of colostrum which initially was higher than that of maternal serum, fell rapidly after parturition. Antitoxin was absorbed by a newborn lamb, but not by a four day old lamb.

Using a combination of electrophoresis and salting-out techniques, McCarthy and McDougall (1953) studied the changes in the serum proteins of lambs either sucking from their mothers, or deprived of colostrum for various periods. Their findings supported those of Mason et al. (1930) and showed that absorption of globulin ceased between 29 and 48 hours.

Selectivity in γ-globulin transport

**Birds**

In the hen the process of antibody transfer by the ovary is selective, though absorption by the chick embryo across the yolk-sac endoderm is apparently non-selective, since most of the yolk has been absorbed by the time of hatching. Antibody is concentrated in the yolk by the ovary of the hen during production of the ovule. By a process not understood, globulin molecules are transported across the follicular epithelium. Buxton (1952) measured the transfer of antibodies to **Salmonella**
pullorum to the yolk, and his results suggested that some of the antibody was produced locally by the follicular epithelium, although most of it was derived from the circulating plasma. No hypothesis has been proposed for the mechanism which selects \(\gamma\)-globulin from other plasma proteins in the hen.

**Mammals**

In all species of mammals so far examined, there is a mechanism specific for \(\gamma\)-globulin at some stage in the transfer of antibody from the maternal circulation to that of the young animal. This enables the yolk-sac epithelium, the trophoblast, the intestinal epithelium, or the mammary gland to select and accumulate antibodies for the passive immunization of the newborn.

(i) **Selective transfer by the yolk-sac epithelium**

Batty, Brambell, Hemmings and Oakley (1954) used antibodies to the toxins of several organisms to demonstrate that antibody transfer by the yolk-sac epithelium of the rabbit was selective. The rate of transfer varied according to the species in which the antibody was raised, and decreased in the order rabbit, man, guinea-pig, dog, horse, and cow. Hemmings (1958) found that antibodies injected into the uterine cavity...
of the pregnant rabbit were almost completely removed, yet only a small proportion of the antibodies injected could be found in the foetal circulation. This result suggested that the uptake of protein by the yolk-sac epithelium was not selective, but there was selection during transfer to the foetal circulation. Hemmings (1961) showed that there was selection between different rabbit proteins; \( \gamma \)-globulin was transported more readily than albumin, and \( \beta \) - and \( \alpha \) -globulin less readily than either albumin or \( \gamma \)-globulin. Rabbit macroglobulin was transferred almost as readily as 7S \( \gamma \)-globulin (Hemmings and Jones, 1962), providing further evidence that the transfer of protein across the rabbit yolk-sac epithelium involves active selection of immune globulins rather than molecular sieving.

Following the finding that pepsin digestion of antitoxin prevented its transport across the trophoblast in man (Hartley, 1951) the effect of protein digestion on the transfer of antitoxin in the rabbit was investigated by Brambell, Hemmings, and Oakley (1959). Only very low antibody titres could be found in the foetal plasma after the injection of pepsin-digested antitoxin into the uterine cavity. Brambell, Hemmings
Oakley and Porter (1960) showed that $^{131}$I-labelled FIII fragments produced by the papain digestion of $\gamma$-globulin (Porter, 1959) were transmitted to the foetus almost as readily as the intact $\gamma$-globulin. Since Cohen and Porter (1964) reported that pepsin digestion destroys the FIII fragment of $\gamma$-globulin, this finding and those discussed above suggest that selective transfer of $\gamma$-globulin across the rabbit yolk-sac epithelium is somehow dependent on the portion of the $\gamma$-globulin molecules which gives rise to the FIII fragment.

(ii) Selective transfer by the trophoblast membrane

In the Rhesus monkey $\gamma$-globulin was transported across the placenta to the foetus 15-20 times more rapidly than albumin while $\alpha_2$- and $\beta$-globulin were not transported at all (Bangham, Hobbs and Terry, 1958). Together with the observation that pepsin digestion prevented the transfer of diphtheria antitoxin across the human placenta (Hartley, 1951) the above result indicated that the trophoblast membrane of the haemochorial placenta had similar selective properties to those of the yolk-sac epithelium in the rabbit.

(iii) Selective transfer by the small intestine

Halliday (1955) observed that the young rat absorbed
rabbit γ-globulin less readily than rat or mouse γ-globulin, and neither bovine nor avian antibody could be detected in the serum after ingestion of these proteins. A smaller proportion of $^{131}\text{I}$-labelled rat albumin than $^{131}\text{I}$-labelled rat γ-globulin was absorbed from an oral dose of serum (Bangham and Terry, 1957a). About 7.5% of the globulin dose was present in the circulation several hours after feeding compared with 0.2% of the albumin. In the same experiments the circulating half-life of γ-globulin given intraperitoneally was compared with that absorbed from the gut, and no difference was detected in the period from 5 to 15 days following the doses (Bangham and Terry, 1957b). Nevertheless Brambell, Halliday and Hemmings (1961) showed that some $^{131}\text{I}$-labelled γ-globulin was modified during absorption, and this resulted in a loss of antibody activity. The modified protein was found to be more readily absorbed when fed to other rats.

As well as demonstrating preferential absorption of homologous antibodies, Halliday and Kekwick (1961) reported that rats were able to absorb relatively less antibody to Salmonella pullorum from mothers lactating shortly after a primary immunization than from mothers lactating during a period of hyperimmunity. The
predominance of antibody in the slower-moving globulin fraction during hyperimmunity was proposed as an explanation for this selectivity.

Interference in the transfer of antibodies by \(\gamma\)-globulin of the same or different species (Brambell, Halliday and Morris, 1958) or by the FIII fragment from papain-hydrolysed \(\gamma\)-globulin (Morris, 1963) was observed in rats and mice. At the same relative molecular concentration the effect of the FIII fragment was greater than that of \(\gamma\)-globulin, suggesting that recognition of \(\gamma\)-globulin by the absorptive cells was dependent on this part of the molecule. This was a similar result to that obtained in the rabbit (Brambell et al., 1960). Other serum proteins had no effect on transfer. Since there was almost quantitative removal of protein radioactivity from the intestinal lumen of the rat during the first 4-6 hours after feeding (Brambell, Halliday and Hemmings, 1961), interference was thought to be a result of competition for a membrane site which enabled the attached protein molecules to be transported.

A hypothesis to explain this phenomenon has been put forward (Brambell, Halliday and Morris 1958; Morris 1967). It is postulated that during pinocytosis, when
protein is absorbed non-selectively into the cell, portions of the cell wall form the membrane surrounding the vesicles thus formed. Homologous \( \gamma \)-globulin is preferentially attached to receptor sites on the cell membrane via the FIII portion of the molecule. During transport through the cell, free protein in the vesicles is digested and only the intact protein attached to the membrane remains to be released into the tissue space. The apparent concentration of antibodies at the rim of vesicles both within cells (Clark, 1959) and outside cells (Anderson, 1964) supports this hypothesis.

A change in selectivity of absorption with time was recently observed in the rat (Jordan and Morgan, 1968). From their results, it appeared that the ability to absorb rat albumin was rapidly lost and then successively transferrin, \( \beta \)-globulin, and finally \( \gamma \)-globulin. An increase in catheptic digestion with time could explain this change in terms of Brambell's hypothesis. Further clarification of the mechanisms involved in selective transport is required, since the present hypothesis in no way identifies the carrier molecules, or explains the change in absorptive capacity of the epithelium at the time of cessation of absorption.
Non-selective transfer by the small intestine

In the larger domestic species, the pig, the horse and the ruminants, the absorptive process in the intestine of the newborn is less selective than in the species considered above. In the piglet, Barrick, Matrone and Osborne (1954) reported that lyophilized bovine serum proteins were not absorbed from the intestine when fed with cow's milk. However Olsson (1959) showed that bovine serum γ-globulin was absorbed by the intestine, the amount of absorption depending on the concentration of γ-globulin in the intestine. Lecce, Matrone and Morgan (1961) reported that unaltered nonporcine protein, ovalbumin and polyvinylpyrrolidone (PVP) were absorbed. Some selectivity was reported by Pierce and Smith (1967a) who found that more porcine than bovine γ-globulin was transferred by the intestine of the piglet when both were presented in equal concentrations.

Absorption of protein by the intestine of the newborn calf has been shown to be non-selective by several workers (Deutsch and Smith, 1957; Pierce, 1961a, 1961b). Most or all of the proteins of colostrum whey are absorbed. Subsequently the low molecular weight proteins, predominantly β-lactoglobulin, are excreted in
the urine, resulting in the transient proteinuria observed by earlier workers (Howe, 1924; Smith and Little, 1924). Similar results were found using \(^{131}\text{I}\)-labelled serum and colostrum proteins all of which appeared to be absorbed with equal facility (Bangham, Ingram, Roy, Shillam and Terry, 1958). Balfour and Comline (1959) found that bovine and human serum albumin and dextrans were all absorbed from the intestine when they were infused into the duodenum.

In the lamb, Mason et al. (1930) showed that heterologous antitoxin either present in the colostrum as a result of prior injection into the circulation of the mother or fed directly to the lamb was absorbed by the intestine. Lecce and Morgan (1962) found that PVP and egg proteins were absorbed by the intestine up to 48 hours after birth, suggesting that absorption was non-selective as in the calf. Proteinuria in the lamb followed the ingestion of colostrum (McCarthy and McDougall, 1953).

(v) Selective transfer by the mammary gland

In some of the species described above, the offspring are passively immunized before birth. Yet in all the animals studied, the first milk produced by the
mother after birth is rich in γ-globulins. Recent findings indicate that there may be qualitative differences between the colostrum γ-globulins of the animals where antibody transfer is prenatal and those where it is predominantly postnatal.

The process of colostrum formation was studied in the rabbit (Campbell and Work, 1951, 1952) and amino acids rather than the plasma proteins were found to be the precursors of most milk proteins. Further work showed that the immune globulins of colostrum whey were derived from the blood (Askonas, Campbell, Humphrey and Work, 1954) and it was inferred that antibodies were transported intact by the secretory epithelium of the mammary gland. The characterization of the predominant immune globulin of rabbit colostrum has established that it is similar to the IgA of human colostrum (Feinstein, 1963; Cebra and Robbins, 1966; Sell, 1967).

The human infant only absorbs trace amounts of antibody after birth yet human colostrum is rich in γ-globulin, predominantly IgA (Chodirker and Tomasi, 1963; Tomasi and Zigelbaum, 1963). The IgA appears to be a large molecule (11S) compared with the 7S slow IgG globulin which is largely excluded from the
secretion. It has not been confirmed whether the IgA is derived from the plasma, or produced locally, but the presence of IgA in other secretions such as tears, saliva and intestinal fluid suggests that the IgA present in human colostrum might be synthesised in the mammary gland. A hypothesis for such a secretory system has been proposed (South, Cooper, Hong and Good, 1967). The role of IgA in human colostrum may be that of an inhibitor of microbial growth in the lumen of the intestine of the infant (Ammann and Stiehm, 1966).

There has not been much work done on the secretion of γ-globulin by the mammary gland of the rodents and the guinea-pig, though Fahey and Barth (1965) have found three immunoglobulins in mouse colostrum corresponding to IgA, and slow and fast IgG.

The antibodies present in porcine colostrum have been examined by immunoelectrophoresis (Rejnek, Kostka and Travnicek, 1966). Although a protein fraction similar to IgA was present in the colostrum whey, antitoxin activity was only present in the IgG and IgM fractions. Studies on the milk of the sow later in lactation showed that plasma proteins form the major part of the whey proteins throughout lactation (Morgan and
Lecce, 1964) unlike the cow in which mature milk has a very low plasma protein content.

Famulener (1912) found that immediately after parturition bovine colostrum had a higher antibody titre than plasma from the same animal, suggesting an accumulation of antibody by the mammary gland. Crowther and Raistrick (1916) and Woodman (1921) showed by chemical methods that colostrum globulin had an amino acid content similar to that of plasma proteins, whereas other milk proteins did not. Woodman and Hammond (1922, 1923) and Asdell (1925) made a detailed study of the mammary gland secretion in cows and goats, and concluded that during the last few weeks of gestation, a fluid rich in globulin was produced by the gland. This fluid contributed the bulk of the colostrum globulin at parturition.

Howe (1922a) showed that the precipitation characteristics of colostrum globulin were the same as those of serum globulin. Using electrophoretic analysis, Smith (1946, 1948) and Pierce (1955) were able to show that immune lactoglobulin was electrophoretically similar to but not identical with the T (fast IgG) component of serum globulin.
Intravenous injection of $^{131}$I-labelled bovine $\gamma$-globulin into a cow close to term resulted in the accumulation of radioactivity in the colostrum (Blakemore and Garner, 1956). Larson (1958) also reported a significant loss of circulating plasma protein into the mammary secretion near term. The circulating levels of $\beta_2$-(IgM) and $\gamma_1$-(fast IgG) globulin decreased near parturition, but $\gamma_2$-(slow IgG) globulin levels did not (Larson and Kendall, 1957; Larson, 1958). Pierce and Feinstein (1965) by a combination of electrophoresis, ultracentrifugation, and immunological precipitation demonstrated that three colostrum globulins were related to three serum globulins. The mammary gland selected the electrophoretically fast $\gamma$-globulin and discriminated against the slower $\gamma$-globulin component.

Earlier, Campbell, Porter and Petersen (1950) reported an accumulation of plasma cells around the alveoli of the bovine mammary gland near parturition and after the cessation of milking. These workers inferred that the lymphoid cells produced a high local concentration of antibody which was transferred to the milk. This histological finding was not confirmed by Dixon, Weigle and Vazquez, (1961) and is at variance with the physiological studies discussed above.
Colostrum formation in the goat and sheep has not been studied as extensively as in the cow. Famulener (1912) showed there was a higher antibody titre in goat colostrum than in the serum at the same time, and Askonas, Campbell, Humphrey and Work, (1954) showed that γ-globulin was transferred from the plasma to the colostrum in the goat. McCarthy and McDougall (1953) reported an increase in the antibody titre of colostrum in a ewe from 1 in 25 to 1 in 1,000 in the 12 days prior to lambing, suggesting an accumulation of γ-globulin in this species. The levels of γ-globulin present in samples of colostrum varied from 1.9 to 25 g/100ml., the latter value being 77% of the total whey protein.

Richards and Marrack (1962), and Sullivan and Tomasi (1964) showed that the predominant immune globulin in ovine colostrum was fast IgG. The identity of fast and slow IgG was suggested by comparison with immuno-electrophoretic data from other species (Silverstein, Thorbecke, Kraner and Lukes, 1963) and confirmed by Aalund, Osebold and Murphy (1965). Mackenzie and Lascelles (1968) studied the transfer of fast and slow IgG from the blood into the milk of lactating ewes, and demonstrated that fast IgG was transferred more readily
than slow IgG. Since the two molecules are of similar size some selective mechanism must be able to discriminate between the two proteins.

Following infection of the udder of the ewe there was local production of antibody in the gland (Lascelles, Outteridge and Mackenzie, 1966). This may be part of a specific mechanism for protecting the mammary gland from infection, and is probably a separate process from normal antibody transfer from the plasma.

**Morphological characteristics of γ-globulin transfer**

The processes of γ-globulin transfer associated with passive immunization of the young animal can be classified into two types. First, a relatively long-lived phenomenon which results in a gradual accumulation of antibody in the foetus or mammary gland over some weeks, and secondly a transient phenomenon in which there is a large quantity of protein transferred in a short time, such as happens in the intestine of the newborn of several species. The first group are not so amenable to microscopic study since at any one time the concentrations of γ-globulin present in the absorptive cells are likely to be low. However, Dixon et al. (1961) and Feldman (1961) have studied the histology of the
bovine mammary gland during colostrum formation and lactation. Immunofluorescent studies showed an accumulation of bovine \( \gamma \)-globulin in the secreting cells of the alveoli during colostrum formation, apparently in the form of vesicles associated with the Golgi region. No vesicles similar to those found in the intestinal epithelium of the sucking calf were evident.

Several studies have been made of the morphological aspects of intestinal absorption of \( \gamma \)-globulin. The histological changes in the intestine of the calf after birth were examined by Smith (1925) who described "dropsical" vesicles in the epithelium at birth which disappeared within three days. Only a few of these vesicles would stain with eosin and methylene blue, the rest being apparently empty of protein material.

Comline, Roberts and Titchen (1951b) examined the intestine of the calf after \( \gamma \)-globulin had been infused into the duodenum, and found a rapid appearance of large numbers of vesicles in the epithelium which could be stained with iron-haematoxylin. Hill (1956) and Hill and Hardy (1956) found similar vesicles in the intestine of the lamb during \( \gamma \)-globulin absorption. The vesicles stained positive for both protein and carbohydrate.
The process of absorption of antibody and colloidal materials in the intestine of the young rat was studied by Clark (1959). A variety of materials entered the cell although absorption is selective in this species. He concluded that the large vesicles formed in the cytoplasm were a product of pinocytosis at the cell border.

Payne and Marsh (1962b) identified the contents of vesicles formed in the epithelium of the intestine of the piglet by feeding fluorescent γ-globulin. The vesicles fluoresced under ultra-violet light providing substantial evidence for the contents of the vesicles being γ-globulin.

More recent studies with the electron microscope support Clark's original hypothesis concerning the origin of the vesicles (Clark, 1959). A network of tubular and vesicular profiles can be seen in the apical region of epithelial cells in the newborn rabbit and rat (Kraehenbuhl, Gloor and Blanc, 1967; Wissig and Graney, 1968) and the piglet (Staley, Jones and Marshall, 1968; Staley, Jones and Corley, 1969). Studies with ferritin have shown that this protein enters the cells by the route postulated for γ-globulin (Kraehenbuhl, et al., 1967) and is concentrated in large vesicles.
After they are formed in the supranuclear region of the cells, the large densely-staining vesicles migrate past the nucleus to the base of the cell. The protein is released from the cell by a process not yet understood. The apical region of the cells changes in appearance during the first few days of life in the piglet (Staley et al., 1969), synchronous with the cessation of absorption of γ-globulin.

**SUMMARY**

It is clear from the evidence presented above that the offspring of numerous species of mammals are protected from infection for several weeks after birth by maternal antibodies which reach the circulation of the young animal by a variety of routes, and at a time which is characteristic of the species. Many aspects of the transfer of antibodies from mother to young have been described, but the mechanisms involved in the process are not understood. The experiments described in this thesis were carried out to assess the importance of maternal antibodies in the development of the young lamb, and to characterize further the process by which antibodies are transported across the intestinal epithelium of newborn lambs.
CHAPTER 2.
MATERIALS AND METHODS

MATERIALS

Experimental animals

The pregnant sheep used in these experiments were either Merino or Cross-bred Merino ewes. They were mated to either Merino or Border Leicester rams and varied in age from 2-6 years. These animals were obtained from commercial flocks on the tablelands of New South Wales and the Australian Capital Territory, and from the Trangie Agricultural Research Station flocks in northern New South Wales. They were maintained on irrigated clover pasture in small holding paddocks in the University. Pregnant ewes were housed indoors in individual pens prior to surgery and fed lucerne chaff and oats. After surgery they were kept in metabolism cages (Fig. 2) and fed lucerne chaff, lucerne hay and oats. The foetal lambs carried by these ewes were judged to be from 120-150 days post-conception at the time of operation. After surgery they were carried by the ewes until they were either born spontaneously or delivered by Caesarean section. Those surviving birth were fed by hand with colostrum and reconstituted cow milk.
Fig. 2  Two pregnant ewes in metabolism cages approximately 24 hours after surgery in which thoracic duct fistulae were established in their foetal lambs. Lymph is collected into the two fraction collectors shown on the right which are covered during experiments.
The lambs used in the experiment described in Chapter 3 were obtained from the C.S.I.R.O. Division of Plant Industry and were the male offspring of eighty Merino and Cross-bred ewes mated to Border Leicester rams. They were removed from the ewes at birth and reared by hand as described below.

Rabbits used in the production of antisera were obtained from the outbred colony maintained by the laboratory. They were housed in individual cages and fed a diet of commercial rabbit pellets.

**Sheep blood**

Sheep blood for the production of serum and serum \( \gamma \)-globulin was obtained from the local abattoir. The blood was collected into stainless steel buckets as cleanly as possible and allowed to clot at room temperature after which it was kept at 4°C while the clot contracted. The serum expressed was removed with a siphon, centrifuged to remove red blood cells, and sterilized by Seitz filtration when required.

**Sheep colostrum**

Colostrum was milked from ewes which had just lambed and the colostrum kept at 4°C until it was centrifuged free of fat and used for the production of colostrum whey.
Anticoagulant

Heparin (158.5 I.U./mg, pyrogen-free; Evans Medical) was used as an anticoagulant for all lymph samples collected.

Antibiotics

To inhibit bacterial growth in lymph during collection, crystalline penicillin (Crystapen, C.S.L.) was added to the fluid. Penicillin powder was also dusted into the foetal fluids and the abdominal cavity of ewes during surgery. Daily intramuscular injections of 2 ml. of procaine penicillin and streptomycin (Streptopen, Glaxo-Allenbury's) were given to ewes following surgery as a prophylactic measure to avoid sepsis of the foetus. A veterinary preparation of oxytetracycline hydrochloride (Terramycin, Pfizer) was added to milk fed to newborn lambs deprived of colostrum to prevent enteric infection.

Antigens

Swine influenza virus (SIV) which had been harvested after culture in living chick embryos was obtained from Dr. R.G. Webster as a stock solution of the virus in saline containing sodium azide as a preservative. The solution contained 10,000 haemagglutinating units/ml., one haemagglutinating unit
being equivalent to $6 \times 10^6$ virus particles (Fazekas de St. Groth and Webster, 1966).

Rabbit red blood cells were also used as an antigen. Rabbit blood was collected in Alsever's fluid and the cells separated by centrifugation. They were resuspended in fresh Alsever's fluid and centrifuged again. This procedure was repeated three times and the washed cells finally made up to a volume such that $10^9$ cells were present in 0.25 ml. The cells were counted by means of an electronic particle counter (Model B, Coulter Electronics).

**Chemicals**

All chemicals used in the preparation of solutions were of analytical or laboratory reagent grade.

**Solutions**

Physiological saline - a solution of NaCl (0.15M) in distilled water.

Alsever's fluid - a sterile solution of sodium citrate (27mM), NaCl (69mM), and glucose (120mM), brought to pH 6.1 with a 10% citric acid solution.

Haemagglutination diluent - a sterile solution containing NaCl (0.15M), CaCl$_2$ (0.25mM), and MgCl$_2$$\cdot$6H$_2$O (0.39mM) was used for serial dilution of the serum to be titrated for antibody.
Trypsin solution - trypsin (1:250, Difco Laboratories), 4 mg/ml., was dissolved in 0.1M sodium phosphate buffer pH 8.0 and stored frozen in 10 ml. portions.

Glycerol-saline - a 4% (v/v) solution of glycerol in physiological saline.

Guinea-pig complement solution - lyophilized complement (equivalent to 2.2 ml. of guinea-pig serum, C.S.L.) was dissolved in 10 ml. of sterile water and kept in ice until used.

Glycine buffer - (McFarlane, 1963). Glycine (0.8M) and NaCl (0.2M) were dissolved in water and brought to pH 8.5 with NaOH.

Tris-HCl buffers - (i) 0.1M Tris (Tris(hydroxymethyl)aminomethane) solution was brought to pH 8.4 with concentrated HCl. (ii) 0.5M Tris was brought to pH 8.5 with concentrated HCl.

Scintillation fluids

Dioxane scintillator - naphthalene (60g), 2,5-diphenyloxazole (4.0g; PPO, scintillation grade, Packard) and methanol (100 ml.) were dissolved in dioxane to a final volume of 1 litre.

Toluene scintillator - PPO (5g) was dissolved in 1 litre of toluene.
Radioactive materials

Carrier-free Na$^{125}$I (10-15 mc/µg; Amersham) was used for the labelling of γ-globulin.

Radioiodinated human serum albumin ($^{131}$I-HSA, 25 µc/mg; Amersham) was obtained as an isotonic solution in NaCl (0.75% w/v) and benzyl alcohol (0.9% w/v).

Histological fixatives

Light microscopy

Ethanol (95%), used at 4°C.

Zenker's formol - a stock solution containing HgCl$_2$ (5%), K$_2$Cr$_2$O$_7$ (2.5%) and Na$_2$SO$_4$ (1%) was prepared. Formalin (5 volumes) was added to the stock solution (95 volumes) just before use.

Electron microscopy

A 1% solution of osmium tetroxide in 0.1M sodium phosphate buffer was prepared by adding 1 volume of a 4% OsO$_4$ solution in water to 1 volume of water and 2 volumes of 0.2M sodium phosphate buffer at pH 7.4. The solution was kept on ice before and during fixation.

GENERAL METHODS

Lamb-rearing methods

Of thirty-seven lambs used in the experiment described in Chapter 3., five were allowed access to
colostrum during their first day of life. Two were left with their mothers for 36 hours during which time they consumed an unknown amount of colostrum. Three were taken from their mothers at birth and fed 500 ml. of colostrum obtained from other ewes at parturition. From the second day, these lambs were fed the same diet as colostrum-deprived lambs. The remainder of the lambs were deprived of colostrum and reared from birth on a diet of fresh cow milk with chaff and water *ad libitum*. Three lambs, one of which had received colostrum, died during the early weeks of the experiment of undetermined cause and are not included in the analysis of the results.

**Blood sampling**

Routine blood samples were taken by syringe from the external jugular vein of sheep and lambs. Rabbits were bled from the posterior marginal vein of the ear and the blood collected in a beaker. Fowls were bled from a wing vein and the pooled blood pipetted into Alsever's fluid. The red blood cells were washed three times before being made up to a 5% solution for haemagglutination inhibition titrations.

**Preparation of antiserum**

Antiserum to whole adult sheep serum was obtained from hyperimmunized rabbits. Two injections each of
0.5 ml. of serum emulsified in 0.5 ml. of complete Freund's adjuvant were given subcutaneously, the second injection following three weeks after the first. The rabbits were bled one week after the second injection. Further collections of blood were made seven days after a subcutaneous injection of sheep serum alone. The rabbit blood was allowed to clot and centrifuged at 3,000 rpm for 30 minutes to separate the serum which was pipetted off and stored frozen in 5 ml. portions until required for the immuno-electrophoretic analysis of lymph or serum.

**Statistical methods**

Standard statistical procedures as described by Mather (1951) were used for analysing the experimental data. The various statistics calculated are defined as follows.

The mean $\bar{x}$ of a series of observations $x_1$, $x_2$, $x_3$ ... $x_n$ is given by $\bar{x} = \frac{S(x)}{n}$ where $S(x)$ is the sum of all the values of $x$ and $n$ is the number of observations.

The standard deviation of the mean (standard error) $s_{\bar{x}}$ is given by $s_{\bar{x}} = \sqrt{\frac{V_x}{n}}$ where $V_x = \frac{S(x-x)^2}{n(n-1)}$, i.e., $\frac{1}{n}$ of the variance of a single observation.

The variance of the difference between two means $V_d$ when $\bar{x}_1$ and $\bar{x}_2$ were calculated from $n_1$ and $n_2$ independent
observations is given by:

\[ V_d = \frac{(n_1+n_2)}{n_1 \cdot n_2} \cdot \frac{[S(x_1-x_1)^2 + S(x_2-x_2)^2]}{n_1+n_2-2} \]  \hspace{1cm} (d.f. = n_1+n_2-2)

**Linear regression.** When pairs of observations \( x_1 y_1, x_2 y_2, x_3 y_3 \ldots x_n y_n \) were made, the linear relationship between \( x \) and \( y \) was calculated by finding the values of \( a \) and \( b \) in the equation \( Y = a + b (x-x) \) by the method of least squares.

\[ a = \frac{\bar{y}}{n} \] \hspace{1cm} (d.f. = n-2)

\[ b = \frac{S(y-x)(x-x)}{S(x-x)^2} \] \hspace{1cm} (d.f. = n-2)

\[ V_y = \frac{1}{n-2} \cdot \frac{[S(y-x)^2 - S^n (-y(x-x))]}{S(x-x)^2} \] \hspace{1cm} (d.f. = n-2)

**'t'-test.** The significance of the various statistics calculated from the experimental data was tested by the null hypothesis using a 't' test. The value of 't' was calculated for each statistic as the ratio of the statistic to its standard error. The probability of finding a value of 't' at least as large by chance was determined from standard tables of 't'.

**Analysis of variance**

When the total sum of squared deviations of a series of observations from their mean could be partitioned into
independent sums of squares attributable to items of interest, the significance of an item was tested by comparing the mean square of the item to that of the residual or error mean square. The mean square of an item was calculated by dividing the sum of squares attributable to the item by its number of degrees of freedom. The significance of the item was tested by the null hypothesis to determine whether the degrees of freedom removed by the item removed a proportionately greater amount of variation from the total than the degrees of freedom attributable to error variation, within the limits of random sampling.

DETAILED METHODS

Estimation of antibodies

Haemagglutination inhibition titrations

The method used for estimation of the serum antibody to SIV was the haemagglutination inhibition titration used by Fazekas de St. Groth and Webster (1966). The SIV solution was titrated on the morning of each day that tests were to be carried out in order to standardize both the virus and the chicken red cell suspension being used. The stock solution was diluted 1 in 50 and serial 2-fold
dilutions made in haemagglutination diluent on standard WHO titration plates with a Takátsy-loop. After the addition of 0.025 ml. of freshly prepared 5% suspension of fowl red cells, the plates were left for 35 minutes at room temperature and the end point (+) of partial agglutination was evaluated. According to the result of this preliminary titration, the virus stock solution was diluted to give a solution containing 160 haemagglutinating units/ml., i.e., 4 units/0.025 ml.

To inactivate the non-specific haemagglutination inhibiting activity of the serum, samples were treated with trypsin and periodate before titration (Jensen, 1961). Equal quantities of serum (0.2 ml.) and trypsin solution in phosphate buffer were incubated at 56°C for 30 minutes, then potassium periodate solution (0.6 ml.) was added and the samples left for 15 minutes at room temperature. Then one ml. of glycerol-saline was added to give a final dilution of the serum of 1 in 10.

For the haemagglutination inhibition assay, duplicate serial dilutions from 1 in 20 to 1 in 10,240 of the serum were made in the diluent, 0.025 ml. of the diluted virus solution was added, and the plates left to stand for 30 minutes at room temperature. Then 0.025 ml.
of the 5% suspension of fowl red cells was added and the plates shaken before being left to stand for 35 minutes. The endpoints of the titrations were read from the pattern of clumped red cells at the base of the wells. From the endpoints the antibody titre of the serum was calculated.

**Haemolysin titrations**

Duplicate dilutions, from 1 in 5 to 1 in 5,120 of the lamb sera were made, then 0.025 ml. of guinea-pig complement solution and 0.025 ml. of a 1% solution of rabbit red blood cells were added to each dilution. The plates were shaken, incubated at 37°C for 30 minutes and left overnight at 4°C. The endpoint of each titration was taken as the highest dilution showing any haemolysis. Rat serum containing antibodies to rabbit red blood cells was used to test the method.

**Surgical methods**

**Anaesthesia**

Merino ewes judged to be within the last three weeks of pregnancy were deprived of food and water for 24 hours before surgery. Anaesthesia was induced with Thiopentone sodium ("Intraval" sodium, May and Baker Ltd.), the ewe intubated and maintained with Halothane ("Fluothane", I.C.I. Ltd.) and oxygen administered with
a closed circuit anaesthetic machine. All operations were carried out under strict aseptic conditions.

**General surgical procedures**

The ewe is tied out on her back and the abdomen closely clipped, scrubbed clean and treated with a solution of Hibitane (I.C.I. Ltd.). The peritoneal cavity is opened through a mid-line incision extending posteriorly from the umbilicus as far as the udder. The subcutaneous mammary veins lying on each side of the mid-line and their anastomotic connections are avoided as far as possible, as at this stage of pregnancy the blood supply to the udder is well developed. The pregnant uterus is then mobilized and delivered through the abdominal incision and positioned on the belly of the ewe in such a way that no undue tension is placed on the uterine vessels. A thin polythene bag is then drawn over the uterus to enclose it completely and prevent desiccation during the manipulations which follow.

The plastic bag and the uterus are incised over the appropriate area as near as possible to that part of the foetus required for surgery. The uterine incision is best made over the area where the amnion and allantois are fused to avoid the loss of allantoic fluid and to facilitate the subsequent repair of the membranes. Care
must be taken to avoid any cotyledons or major blood vessels when incising the uterus, and any bleeding vessels are cauterized at once. The incision is continued through the wall of the uterus until the foetal membranes are visible. Any vessels present in the exposed foetal membranes are cauterized along the line of the incision.

The fused amnion and allantois are picked up with forceps and incised, and the cut edges of the membranes retracted. In most lambs near to term the amount of fluid in the amniotic cavity is small and if the foetus is left in the uterus it is usually possible to carry out the operation without removing any of the foetal fluid. Operations on foetal lambs of 130-150 days gestation are best done without removing them from the uterus. At this late stage of pregnancy the uterus contracts strongly once the foetus is delivered to the outside and it is difficult to return the lamb without damaging the foetal membranes.

Once the operation on the foetus has been completed the edges of the foetal membranes are held up with blunt forceps and tied off with a series of purse-string sutures. Any plastic cannulae inserted into the foetus are included in these sutures. The membranes are tested
for leaks by applying pressure to the uterus, and provided none are present, the uterine incision is inverted and closed with chromic cat-gut sutures. The uterus is returned to the peritoneal cavity and positioned to avoid any tension of the uterine vessels. The peritoneal incision is then closed with interrupted mattress sutures and the skin joined with Michel clips. During these last stages, the depth of anaesthesia is reduced so that the ewe is usually standing and eating within 3-4 hours of the completion of the operation.

**Thoracic duct cannulation**

With the foetus on its left side within the uterus, an incision is made through the skin over-lying the eighth rib on the right side extending ventrally from the costal articulation with the vertebra to the sternum. The eighth rib is exposed, freed of its periosteum and removed. Then the costal pleura is incised through the periosteum, care being taken to avoid the intercostal blood vessels. The incision is retracted and the diaphragmatic lobe of the right lung is packed off. The thoracic duct is usually seen at once lying beneath the parietal pleura on the dorsolateral aspect of the thoracic aorta. Occasionally the thoracic duct pursues
an anomalous course and is transposed with the hemiazygos vein; if this is so, the duct cannot reasonably be cannulated from the right side.

The pleura is incised over the duct and the duct tied off cranially with a 4/0 silk suture. A length of duct is dissected free from the surrounding tissues for a distance of about 1 cm behind the occluding tie and a second thread passed around the duct and left loose. One end of a length of polyvinyl tubing, about 2 metres long of I.D. 0.5 mm and O.D. 1.0 mm (Dural Plastics) filled with a heparin-saline solution, is introduced into the pleural cavity of the foetus through a stab wound in the chest wall near to the spine and cranial to the point at which the duct is to be cannulated. The duct is opened with scissors and the tube introduced into it for a distance of 5-10 mm and tied in place. Two anchoring sutures are placed around the tubing after it is correctly aligned and lymph is flowing freely from the tube. The foetal chest wall is then reconstituted with silk sutures.

A short length of the plastic tube is left inside the amniotic cavity and the purse-string suture is tied around the foetal membranes and the tube to make a leak-proof seal. A length of the plastic cannula is left
loose inside the peritoneal cavity of the ewe so that any subsequent movements of the foetus or the uterus do not place stresses on the cannula or the thoracic duct.

**Cannulation of the duodenum**

After the thoracic duct of the foetal lamb has been cannulated successfully, it is necessary to insert a cannula into the duodenum if solutions containing γ-globulin are to be infused into the intestine. The foetus is re-positioned within the uterus by pulling it forward with an Allis clamp fastened around the last rib, and a small laparotomy incision is made posterior to the last rib and as far ventrally as possible. A cotton wool swab is used to locate the greater omentum which is used to exteriorize the pyloric region of the abomasum and the proximal end of the duodenum. A small area of the abomasal wall is cauterized and a 2-metre length of plastic tubing similar to that used for the thoracic duct cannulation is introduced through the cauterized area and passed about 5 cm down the duodenum through the pylorus. The cannula is then tied securely in place with a purse-string suture at the point of entry through the abomasal wall. The abomasum and duodenum are returned to the abdominal cavity and the cannula is brought to the
exterior of the foetus through the laparotomy incision which is then closed. It is important to minimize haemorrhage during the duodenal cannulation. When excessive haemorrhage occurs, red cells rapidly appear in the lymph in the thoracic duct cannula, and the lymph clots. The duodenal cannula is brought to the exterior of the ewe in the same way as the thoracic duct cannula. In one series of animals in which morphological aspects of protein absorption were studied, only the duodenum of the foetuses was cannulated.

**Post-operative procedures**

The ewes were allowed to recover from the anaesthetic and were then placed in metabolism cages. The lymphatic cannula was connected to a covered fraction collector (Towers Model A Automatic Fraction Collector) so that hourly collections of lymph could be made automatically throughout the subsequent experiments. The duodenal cannula was sealed by heat until required for infusion of solutions into the foetal intestine.

The foetus and foetal fluids were found to be very susceptible to bacterial infection post-operatively. Although all operations were carried out under standard aseptic conditions, antibiotics were administered
prophylactically to minimize the risk of sepsis in the foetus and foetal fluids. Crystalline penicillin (Crystapen, C.S.L.) was dusted into the foetal fluids and the abdomen of the ewe during the operation, and procaine penicillin and streptomycin (Streptopen, Glaxo-Allenbury's) were given intramuscularly to the ewes following surgery.

Incubation of the foetal intestine with $^3$H-thymidine

The rate of renewal of the epithelial cells of the intestine of the foetal lamb was measured by labelling the dividing cells in the crypts and following their subsequent movement up the intestinal villi by autoradiography. In order to produce high localized levels of radioactivity in the intestinal epithelium, $^3$H-thymidine was introduced into the lumen of a segment of the small intestine isolated between two clamps.

Foetal lambs were exposed as described above and a portion of the small intestine was brought to the exterior through a small laparotomy incision made in the right flank. The intestine was clamped gently in two places approximately 5 cm apart so as to occlude the lumen and isolate that portion of the intestine. Two loose silk ties were placed around the gut beside the right flank, and a segment of the small intestine was
clamps to identify the segment later. Approximately 100 μc of $^{3}\text{H-}\text{thymidine}$ in 1 or 2 ml. of sterile saline was injected into the lumen of the isolated segment, and the area was covered with moist gauze and a plastic sheet to prevent desiccation and cooling of the gut. After one hour, several milligrams of unlabelled thymidine was injected into the segment and the clamps removed. The intestine was returned to the peritoneal cavity and the incision closed with silk sutures. The foetal membranes, the uterus and the abdomen of the ewe were reconstituted as described previously and the ewe allowed to recover. At prescribed times following the incubation with $^{3}\text{H-}\text{thymidine}$ the animals were killed and the segment of intestine between the identifying ties was fixed in Zenker's formol for subsequent autoradiography.

Incubation of the intestine of the newborn lamb with $^{3}\text{H-}\text{thymidine}$

Newborn lambs were anaesthetized with Nembutal sodium (Abbott) and an area on the right flank closely clipped and treated with Hibitane. The whole animal was covered with a plastic sheet which had been soaked in Hibitane solution. An incision was made through the plastic sheet over the appropriate area of the lamb's right flank, and a segment of the small intestine was
isolated and incubated with $^3$H-thymidine as described above for the foetal lamb. After the incubation, the intestine was returned to the peritoneal cavity and the abdominal incision closed. The lambs were then allowed to recover. At given times after the incubation, the lambs were killed and samples of the incubated portion of the intestine were taken for examination by autoradiography.

**Preparative methods**

**Solutions for infusion**

In several early experiments non-sterile solutions were infused into the intestines of foetal lambs. After 2-3 days, the lambs were delivered by Caesarean section and found to be grossly infected, showing that the foetus and foetal fluids were very susceptible to bacterial sepsis. Therefore in subsequent experiments all solutions were either sterilized by Seitz filtration, or, in the case of colostrum whey which was too viscous to pass through a Seitz filter, the whey was filtered twice through filter paper.

(i) **Solutions of γ-globulin**

Sheep serum for the extraction of γ-globulin was obtained from sheep blood and centrifuged free of cells and debris. The serum was then warmed to 25°C, and to it
was added with stirring a sufficient quantity of solid anhydrous Na$_2$SO$_4$ to give a concentration of 13% (Kekwick, 1940). The solution was allowed to stand for one hour and the precipitate spun down in an MSE centrifuge at 1,000 rpm for 10 minutes. The supernatant was discarded and the approximate volume of the precipitate was measured and presumed to contain 13% Na$_2$SO$_4$. The precipitate was then dissolved in a total volume of saline equal to 3/5 of the original volume of serum. Sodium sulphate was added to the solution to bring the concentration of the salt to 13% for the second precipitation. The precipitate was spun down as before and then reprecipitated from a volume approximately 40% of the original serum volume. This precipitate was dissolved in a minimal amount of either (a) physiological saline or (b) a solution containing Na$^+$ (56.7 mM), K$^+$ (44.8 mM), Cl$^-$ (81.5 mM), and PO$_4$$^{3-}$ (20 mM) (Balfour and Comline, 1962). The dissolved γ-globulin was dialysed at 4°C against 5 changes of the appropriate electrolyte solution to remove Na$_2$SO$_4$ and then spun at 8,000 rpm (8,000 x g) for 30 minutes (Sorvall RC-2 Refrigerated Centrifuge) to remove any particulate matter. The clear supernatant was passed through a sterile Seitz filter and stored at -20°C until use.
(ii) **Colostrum whey**

Sheep colostrum was centrifuged at 4°C for 45 minutes at 8,000 x g to remove the fat. The fat-free colostrum was then warmed to 37°C and incubated with rennin for 15 minutes with continuous stirring. This caused the casein to be precipitated as a finely broken curd and the whey was expressed from the curd immediately. The whey was filtered twice through Whatman No. 1 filter paper on a Buchner funnel and stored in 100 ml. portions in sterile bottles at -20°C. Although this colostrum whey was not sterilized, no sepsis of the foetuses occurred during prolonged infusion with the colostrum whey.

**Infusion Procedure**

For infusion of protein solutions into the duodenum of the foetus, the duodenal cannula was connected by a hypodermic needle to a sterile glass cylinder. The protein solution was placed in the cylinder and overlaid with liquid paraffin. A reciprocating syringe pump (Braun) or an infusion pump (Palmer) filled with paraffin was connected to the cylinder and used to expel the protein solution from the cylinder into the duodenum. By this means a constant rate of infusion could be maintained
for long periods without exposing the protein solution to contamination.

**Purification of serum γ-globulin**

A purified preparation of γ-globulin was required for iodine-labelling of the protein. Therefore the crude γ-globulin prepared for infusion was used as starting material and 2 ml. applied to a DEAE-Sephadex (Pharmacia, Sweden) column (Gelotte, Flodin and Killander, 1962). The column was prepared by first allowing the DEAE-Sephadex to swell in 0.1 M Tris-HCl buffer, pH 8.4, and then pouring a column of approximate dimensions 0.7 cm diameter x 15 cm length. The column was then washed thoroughly with the buffer before application of the protein solution. A flow rate of about 1 ml./min was maintained. One ml. fractions of the effluent were collected manually and their optical density at 280 m\(\mu\) was determined. The first 8 fractions contained a single protein peak (I) which was examined by immunoelectrophoresis and found to contain only fast and slow IgG globulin (Fig. 3). This solution was either dialysed against glycine buffer before being used for iodine-labelling, or kept in 0.1 M Tris buffer and stored frozen.
Fig. 3 Agar gel immunoelectrophoretograms of successive stages in the purification of γ-globulin for infusion into the intestine of the foetal lamb and for labelling with iodine.

(a) Normal sheep serum.
(b) One precipitation with 13% Na₂SO₄.
(c) Two precipitations with 13% Na₂SO₄.
(d) Three precipitations with 13% Na₂SO₄. This solution was used for the infusions.
(e) Purified γ-globulin after treatment with DEAE-Sephadex (Peak I), used for labelling with iodine.
(f) The protein removed from the DEAE-Sephadex column by 0.5M Tris-HCl (Peak II), probably fast IgG.
The recovery of protein from the column in the first peak was calculated suggesting that protein-protein y-globulin applied to the column. Therefore the column was washed with buffer, and the protein eluted was measured by its optical density at 280 nm, and characterized by immunelectrophoresis. This peak (a) appeared to be a single protein (Fig. 3), probably fast IgG, and represented a final washing of the column with sodium acetate buffer. A further 10% of the original protein giving a total recovery of 90% was added to the sample. The volume of each hourly sample was measured and the cells present counted in an electronic cell counter (Countelec). The lymph was then centrifuged and the cells preserved for future analysis. Measure of the cells from some samples were stained by the method of Lowry, Rosebrough, Farr and Randall.
The recovery of protein from the column in the first peak was only 20%, suggesting that protein-protein interaction might inhibit the movement of some of the γ-globulin applied to the column. Therefore the column was washed with 0.5 M Tris-HCl buffer and the protein eluted was measured by its optical density at 280 m\( \mu \) and characterized by immunoelectrophoresis. This peak (II) appeared to be a single protein (Fig. 3), probably fast IgG, and represented about 60% of the total. A final washing of the column with a sodium acetate buffer removed a further 10% of the original protein giving a total recovery of 90%.

**Analytical methods**

**Treatment of lymph samples**

The volume of each hourly lymph sample from the foetal lambs was measured and the cells present counted in an electronic particle counter (Coulter Electronics, Model B). The lymph was then centrifuged free of cells and kept frozen for further analysis. Smears of the cells from some samples were stained with Leishman's stain and examined under oil.

**Protein estimations**

The total proteins in lymph and plasma were measured by the method of Lowry, Rosebrough, Farr and Randall,
(1951). To 0.5 ml. of an appropriate dilution of the sample to be measured was added 2.5 ml. of the alkaline copper reagent. Ten minutes later 0.25 ml. of Folin-Ciocalteu reagent was added, the solution was stirred immediately and the colour developed after 40 minutes was read at 780 mµ against a reagent blank. Protein levels were estimated from a standard curve prepared on each occasion by treating samples of bovine serum albumin (Fraction V, Armour) containing 0-100 µg in a similar manner to the diluted samples (Fig. 4).

**Estimation of protein precipitated by 18% Na₂SO₄**

To 1 ml. of lymph was added 4 ml. of 13.75% Na₂SO₄ the solution was stirred and left for 1 hour at room temperature. The fibrin precipitated by this treatment was centrifuged down (1500 rpm for 15 min) and 2 ml. of the supernatant transferred to a 5 ml. graduated test tube. Three ml. of 22.5% Na₂SO₄ solution was added and the solution stirred. The tube was then stoppered and left for 1 hour at 37°C. The precipitate formed was spun down and the supernatant removed by suction. The precipitate was resuspended in three ml. of 18% Na₂SO₄ and centrifuged again. After removal of the supernatant, the precipitate was dissolved in a total volume of 5 ml.
of saline, stirred, and its protein content estimated as described above. Normal foetal lymph before the infusion of protein into the intestine gave virtually no precipitate with this treatment, after the fibrin was removed.

Estimation of Evans Blue dye in lymph and serum

Lymph and serum samples from a foetal lamb infected with a solution of Evans Blue (T-1824) dye were treated according to the method of Allen (1952). One ml. of lymph or 0.05 ml. of serum was mixed with 1 ml. of 25% trichloroacetic acid (Shell) and left for 10 minutes. The solution was then sucked through a cotton column of cellulose prepared from pulped Kleenex tissue (Lascelles, 1962) packed into Pasteur pipettes. The column was washed with 15 ml. of saline and the adsorbed dye was then eluted with acetone/water (1:1) to a volume of 5 ml. The optical density of the solution at 660 mµ was measured and the concentration of the dye in the original lymph or serum estimated from a standard curve prepared from a 1% solution in water (Fig. 3).

Fig. 4 A standard curve for the measurement of protein concentration by the Folin-Phenol method (Lowry et al., 1951).

Serum proteins were separated on cellulose acetate membranes in barbiturate buffer, pH 8.6, ionic strength 0.075M, in a Microzone electrophoresis cell (Beckman, Model R-101) using the method standardized for the
of saline, stirred, and its protein content estimated as described above. Normal foetal lymph before the infusion of protein into the intestine gave virtually no precipitate with this treatment, after the fibrin was removed.

**Estimation of Evans Blue dye in lymph and serum**

Lymph and serum samples from a foetal lamb injected with a solution of Evans Blue (T-1824) dye were treated according to the method of Allen (1951). One ml. of lymph or 0.05 ml. of serum was mixed with 1 ml. of 25% Teepol (Shell) and left for 10 minutes. The solution was then sucked through a 2 cm column of cellulose prepared from pulped Kleenex tissue (Lascelles, 1962) packed into a Pasteur pipette. The column was washed with 15 ml. of saline and the adsorbed dye was then eluted with acetone:water (1:1) to a volume of 5 ml. The optical density of the solution at 620 mµ was measured and the concentration of the dye in the original lymph or serum estimated from a standard curve prepared from a T-1824 solution in water (Fig. 5).

**Cellulose acetate electrophoresis**

Serum proteins were separated on cellulose-acetate membranes in barbiturate buffer, pH 8.6, ionic strength 0.075M, in a Microzone electrophoresis cell (Beckman, Model R-101) using the method standardized for the
apparatus. Electrophoresis was carried out for 20 minutes at 250 volts, and the membrane was then stained in Ponceau-S dye solution for 10 minutes. Excess dye was removed with 3 changes of 5% acetic acid. The membrane was dehydrated, cleared, dried at 110°C for 15 minutes and then stored in a plastic envelope.

**Fig. 5** A standard curve for the estimation of T-1824 dye in serum or plasma.

Serum and lymph samples from lambs and foetuses were analysed by immunoelectrophoresis in agar gel, using commercial apparatus (LKB 6802). Cleaned microscope slides were coated with adhesive agar (0.1% agar containing 0.05% glycerol) and allowed to dry. They were then placed in a plastic frame and coated with a 1% solution of agar in veronal buffer (I=0.1, pH 6.6, LKB). After 5 minutes the coated slides were placed in a humid chamber for 30 minutes. When the edges were cut in the agar and the wells sucked out. The wells were then filled with serum or lymph and the frames put in the electrophoresis apparatus. A voltage (10 volts/cm) was applied along the gels for 1 hour, after which the agar was removed from the troughs which were then filled with antiserum to adult sheep serum. The frames were placed in a humid chamber for 20 hours after which...
Electrophoresis was carried out for 20 minutes at 250 volts, and the membrane was then stained in Ponceau-S dye solution for 10 minutes. Excess dye was removed with 3 changes of 5% acetic acid. The membrane was dehydrated, cleared, dried at 110°C for 15 minutes and then stored in a plastic envelope.

**Immunoelectrophoresis**

Serum and lymph samples from lambs and foetuses were analysed by immunoelectrophoresis in agar gel, using a commercial apparatus (LKB 6800A). Cleaned microscope slides were coated with adhesive agar (0.1% agar containing 0.05% glycerol) and allowed to dry. They were then placed in a plastic frame and coated with a 1% solution of agar in veronal buffer (I=0.1, pH 8.6, LKB). After 15 minutes the coated slides were placed in a humid chamber for 30 minutes. Wells and troughs were cut in the agar and the wells sucked out. The wells were then filled with serum or lymph and the frames put in the electrophoresis apparatus. A constant voltage (10 volts/cm) was applied along the gels for 1 hour, after which the agar was removed from the troughs which were then filled with antiserum to adult sheep serum. The frames were placed in a humid chamber for 20 hours after which
any protein not precipitated in antigen-antibody complexes was removed by two washes in 1% NaCl for 6 and 16 hours respectively. The NaCl was removed by a one-hour wash in distilled water and the gels were dried under strips of blotting paper. The dried agar films were stained for 10 minutes in 1% Amido Black 10B (Gurr) in rinsing solution (water:methanol:acetic acid = 9:9:1) and the excess stain removed by 4-5 washes of 10 minutes each in the rinsing solution. The stained slides were covered with coverslips mounted on DePeX (Gurr) and photographed.

Radioisotope methods

Labelling of purified γ-globulin

The protein was labelled with $^{125}$I using the iodine monochloride method (McFarlane, 1956, 1958). Iodine monochloride (ICl) solution was prepared by injecting 2 ml. of a solution containing 108 mg of NaIO$_3$.H$_2$O into 8 ml. of 6N HCl containing 150 mg of NaI. All the free iodine in the solution was removed by shaking with carbon tetrachloride. A portion of this solution (0.01 ml.) was pipetted into an acid-washed test tube in an ice bath. Approximately 200 $\mu$C of carrier-free Na$^{125}$I (Amersham) was added to the ICl solution resulting in the labelling of a small proportion of the ICl molecules. Cold glycine buffer (0.5 ml.) was squirted into the $^{125}$ICl solution
to release the hypoiodite ion, the solution was sucked into a Pasteur pipette which had been drawn to a fine tip, and squirted steadily into a test tube containing 0.25 ml. of purified γ-globulin and 0.25 ml. of glycine buffer. The protein solution was stirred in a Vortex mixer during the labelling procedure to promote even iodination of the molecules. The mixture was dialysed for 24 hours against 4 changes of glycine buffer (250 ml. each) to remove any non-protein-bound radioactivity. Portions of the dialysates (0.1 ml.) were counted in 10 ml. of dioxane scintillator and from these counts and those for the dialysed protein solution the percentage of the 125I activity incorporated into protein was estimated to be from 40-50%. The dialysed protein solution was kept at -4°C and suitable quantities added when required to infusion solutions and to colostrum fed to newborn lambs. The labelled γ-globulin was rarely used more than 6 weeks after preparation.

Scintillation counting of 131I-HSA radioactivity in lymph

Labelled human serum albumin present in lymph samples was separated from other lymph proteins using a modification of the method for measuring serum albumin concentrations
described by Debro, Tarver and Korner (1957). The proteins in 1 ml. of each lymph sample were precipitated with 9 ml. of 7.5% trichloracetic acid (TCA), the mixture was centrifuged, and the supernatant which contained most of the radioactivity not bound to protein was removed. The precipitate was washed once with 7.5% TCA and was then resuspended in 96% ethanol to dissolve the albumin present in the precipitate. The insoluble material was spun down, and one ml. portions of the supernatant were counted in 10 ml. of dioxane scintillator and from these counts the radioactivity in the lymph attributable to $^{131}$I-HSA was calculated.

**Scintillation counting of $^{125}$I-γ-globulin**

The efficiency of counting of Na$^{125}$I was found to be ca. 55% in dioxane scintillator. However lymph samples containing $^{125}$I-γ-globulin were counted at an efficiency of approximately 30%, due to the quenching effect of the water and protein present in the vial.

A spectrum of the radioactivity emitted by $^{125}$I-γ-globulin was obtained on the scintillation counter by plotting the cumulative counts/minute in a sample against the upper limit of the variable window discriminator. This spectrum (Fig. 6) falls between the spectra for $^3$H
Fig. 6 Integrated spectra for $^{125}$I (x), $^{3}$H (●) and $^{14}$C (○) as determined with the Beckman liquid scintillation counter. The cumulative counts per minute for each isotope in windows counting from a lower limit of zero are plotted against the upper limit of the window. The window limits are arbitrary machine settings.

... and $^{14}$C. A window was set up to count the major part of the $^{125}$I radioactivity and eliminate the lower part of the scale in which most of the counts due to scintillation occurred. The lymph was pipetted into a scintillation vial which contained 10% of dioxane scintillator added from a small scintillator layer 2-3 mm thick on the bottom of the vial, and the vial was centrifuged at 1,000 rpm for 10 minutes to compress the precipitated material onto the scintillator layer. The vials were counted in the scintillation counter (Automatic 3-counter; Beckman) after several hours had elapsed for the $^{14}$C to equilibrate in the counter. This method was suitable for routine preparation of a large number of samples and gave a reasonable counting efficiency. The cumulative counts for each isotope were made on each lymph sample.
and $^{14}$C. A window was set up to count the major part of the $^{125}$I radioactivity and eliminate the lower part of the scale in which most of the counts due to chemiluminescence occurred.

**Scintillation counting of the total radioactivity in lymph and infusion solutions**

One ml. of lymph was pipetted into a scintillation vial and 10 ml. of dioxane scintillator added from a burette. The vial was centrifuged at 1,000 rpm for 10 minutes which compressed the precipitated material into a flat white layer 2-3 mm thick on the bottom of the vial, and then counted in the scintillation counter (Automatic 3-channel counter; Beckman) after several hours had elapsed for the vials to cool to 10°C in the counter. This method was suitable for routine preparation of a large number of samples and gave a reasonable counting efficiency (ca. 30%). Portions of the infusion solutions were made to 1 ml. with unlabelled foetal lymph and counted in dioxane scintillator in a similar manner to the lymph samples.

The method was tested over a range of protein concentrations and although the presence of 5 mg of protein caused a considerable decrease in the efficiency of counting of a small quantity of $^{125}$I-γ-globulin, the
counting efficiency did not change much over the range of protein levels found in lymph, i.e., 15–25 mg/ml. (Fig. 7).

Only a relatively low number of counts due to chemiluminescence resulted from this method of sample preparation and these were avoided by choosing an appropriate counting window. Other methods reported in the literature (Chen, 1958; Brown and Badman, 1961) were tested and found to be inferior to the method above. Hyamine hydroxide-10X (Packard) dissolved proteins only with difficulty, usually causing yellow discolouration of the solution. High counts due to chemiluminescence were a problem, reappearing whenever the vials were exposed to light and taking several days to disappear completely.

Estimation of protein-bound: non-protein-bound \( ^{125}I \) radioactivity

In the infusion solutions counted as described above, more than 95% of the radioactivity was precipitated by the scintillator, but this was not the case with some lymph samples. These observations formed the basis for a method for estimating protein-bound and non-protein-bound \( ^{125}I \) radioactivity in lymph. After the total radioactivity in a sample was determined, half the supernatant fluid (5.5 ml.) was transferred to an empty vial and the radioactivity counted. From these counts an estimate of
protein-bound to non-protein-bound radioactivity was calculated.

Counting of intestinal segments

One foetuses which had been operated on to produce a duodenal fistula were infused with $^{125}\text{I}}\gamma$-globulin at a constant rate for periods of 2-11 hours. The animals were killed, the small intestine was removed of mesentery and at regular intervals (30 cm or cm lengths were removed, slit open, and rinsed in ). Each piece was then placed in the bottom of a scintillator vial and fixed in absolute alcohol. Two solution washes of absolute alcohol were used to denature the tissue, then a 12-hour wash with toluene removed most of the alcohol and cleared the tissue. Toluene scintillator (5 ml.) was added to each segment in the vial and the radioactivity present in the segment was measured. The results are expressed as a percentage of the relative activity along the gut so that this might be related to the histological appearance of the gut.

Fig. 7 The effect of increasing amounts of protein on the counting efficiency for $^{125}\text{I}}\gamma$-globulin in a liquid scintillation system.
protein-bound to non-protein-bound radioactivity was calculated.

**Counting of intestinal segments**

The foetuses which had been operated on to produce only a duodenal fistula were infused with $^{125}$I-γ-globulin solution at a constant rate for periods of 2-11 hours. Then the animals were killed, the small intestine was freed of mesentery and at regular intervals (30 cm or 1 m) 5 cm lengths were removed, slit open, and rinsed in saline. Each piece was then placed in the bottom of a scintillator vial and fixed in absolute alcohol. Two 12-hour washes of absolute alcohol were used to dehydrate the tissue, then a 12-hour wash with toluene removed most of the alcohol and cleared the tissue. Toluene scintillator (5 ml.) was added to the gut segment in the vial and the radioactivity present in the segment was measured in the scintillation counter. It was difficult to assess the efficiency of counting in this system. The counts measured were used only as a crude estimate of the relative distribution of radioactivity along the gut so that this might be related to the histological appearance of the gut.
Histological methods

Light microscopy

(i) Fluorescent antibody localization
Segments from different regions of the small intestines of foetal lambs were slit open and fixed in cold 95% alcohol (Sainte-Marie, 1962). After 1 hour the segments were cut transversely into pieces 1-2 mm thick and left in the fixative for a further 24 hours. The tissue was dehydrated in cold absolute alcohol (3 x 2 hr), cleared in cold xylene (2 x 2 hr), and embedded in paraffin. Transverse sections of the intestine were cut at 5 µ and examined by immunofluorescent techniques.

The sections were taken rapidly through baths of cold xylene, alcohol and saline to remove the paraffin and hydrate the tissue. Fluorescein-conjugated rabbit antibody to sheep γ-globulin to which rhodamine-tagged albumin had been added was centrifuged at 2,000 rpm for 10 minutes to remove any particulate matter and one drop was placed over each section and left for 20 minutes in a humid atmosphere. The sections were then washed in saline to remove any unbound fluorescent antibody and mounted in glycerol:water (1:1). The sections were examined under U-V light to study the localization of the fluorescent antibodies.
(ii) Examination of the intestine by other staining techniques

Sections of intestine from foetal and neonatal lambs were fixed in 95% alcohol and embedded in paraffin as described above and then stained with haematoxylin-eosin and Mallory's stain (Richardson, Jarett and Finke, 1960). Pieces of intestine were also fixed in Zenker's formol for 8-12 hours, washed for 12-24 hours and embedded in paraffin as above. Before the sections were stained, the mercurial precipitate from the fixative was removed with Lugol's iodine, followed by sodium thiosulphate which was removed by running tap water. Sections were stained with haematoxylin-eosin, Mallory's stain and Dominici's stain (orange G and toluidine blue) and photographed on Pan-F film (Ilford) using a photomicroscope (Zeiss) with appropriate filters.

Electron microscopy

Two fixatives were tested for electron microscopic examination of the intestine of the lamb, a 1.25% glutaraldehyde solution in Tyrode's buffer and 1% osmium tetroxide in phosphate buffer. The osmium tetroxide method was preferred since it gave better preservation of the cytoplasm and intracellular structures. The method
used was as follows. Representative segments of duodenum, jejunum, and ileum of anaesthetized foetal and neonatal lambs were isolated by haemostats and small pieces of the tissue immediately immersed in a pool of fixative (1% OsO₄ in 0.1M phosphate buffer, pH 7.4). The tissue was cut into pieces 1-2 mm across and placed in 2-3 ml. of fixative for 2 hours. The fixation and embedding procedures are described in Table 1.

Table 1. Fixation and embedding procedures for electron microscopy.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>No. of changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% OsO₄ in 0.1M PO₄ buffer</td>
<td>2 hr</td>
<td>1</td>
</tr>
<tr>
<td>Cold physiological saline</td>
<td>5 min</td>
<td>3</td>
</tr>
<tr>
<td>30% acetone</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>50% acetone</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>1% uranyl acetate in 50% acetone</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>70% acetone</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>90% acetone</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>Dry acetone</td>
<td>30 min</td>
<td>3</td>
</tr>
<tr>
<td>1:3 Durcupan:acetone</td>
<td>1 hr</td>
<td>1</td>
</tr>
<tr>
<td>1:1 Durcupan:acetone</td>
<td>1 hr</td>
<td>1</td>
</tr>
<tr>
<td>3:1 Durcupan:acetone</td>
<td>1 hr</td>
<td>1</td>
</tr>
<tr>
<td>Durcupan at 47°C</td>
<td>1 hr</td>
<td>3</td>
</tr>
</tbody>
</table>

The tissue was then embedded in fresh Durcupan (Fluka) in gelatin capsules (Parke, Davis & Co.) and the resin polymerised by heating at 75°C for 24 hours.
Sections were cut from the resin blocks on an ultramicrotome (Riekert), stained with lead citrate according to the method of Reynolds (1963), and examined in a Siemens Elmiskop I microscope using an accelerating voltage of 80 kV and a plate magnification in the range 2,200 to 26,000 times. Photographs of the sections were taken on photographic plates (Ilford N.50, 6.5 x 9 cm) using an automatic exposure meter (Siemens) attached to the microscope.

**Autoradiographic methods**

Epithelial cells of the intestine which had been labelled with $^3$H-thymidine during a one-hour incubation in vivo were identified by autoradiography. The rate of migration of labelled cells from the crypts of the intestine up the walls of the villi was estimated by killing the animals at different times after the incubation up to a maximum of 72 hours, and comparing the autoradiographs obtained from sections of their intestines.

The segments incubated with $^3$H-thymidine were fixed in Zenker's formol, then washed for 12-24 hours before being dehydrated with alcohol and cleared in chloroform. The cleared specimens were embedded in
paraffin and 5 μm transverse sections of the intestine were mounted on glass slides and taken through xylene and graded solutions of alcohol to remove the paraffin and hydrate the tissue. The mercury precipitate in the tissue from the Zenker's formol fixation was removed and then the slides were immersed in adhesive solution (0.5% gelatin, 0.05% chrome alum in distilled water) and dried in front of a fan in a vertical position. The sections were coated with pieces of stripping film (Kodak AR-10) by first floating the film on water with the emulsion face downward and then bringing the slide up underneath the film. The film was dried on to the slides with a fan and the slides kept at 4°C in a light-tight box containing a Petri dish of anhydrous CaCl₂ as a desiccant. All manipulations with the stripping film were carried out in a darkroom under a safelight (Wratten Series 1, 25 watt).

After 14-16 days the stripping film was developed by immersing the slides in D19 developer (Kodak) at 16°C for 10 minutes, rinsing in water and fixing in acid fixer for 10 minutes. The film was washed in running tap water for 20 minutes and then stained for histological examination. Since the film tended to
winkle, all staining procedures were carried out at 4°C with increased staining times. A schedule for the staining procedures is given in Table 2.

Table 2.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harris' alum haematoxylin</td>
<td>40</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>1</td>
</tr>
<tr>
<td>1% HCl in 70% alcohol</td>
<td>2-3</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>1</td>
</tr>
<tr>
<td>Dilute Lithium Carbonate solution</td>
<td>5</td>
</tr>
<tr>
<td>Rinse in distilled water</td>
<td>1</td>
</tr>
<tr>
<td>1% eosin in water</td>
<td>10</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>2</td>
</tr>
<tr>
<td>70% alcohol</td>
<td>2</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>5</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>15</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>30</td>
</tr>
<tr>
<td>Xylol</td>
<td>30</td>
</tr>
<tr>
<td>Xylol</td>
<td>60</td>
</tr>
</tbody>
</table>

The slides were then covered with coverslips mounted on DePeX after the edges of the film had been trimmed. To avoid lifting of the emulsion during staining, some of the last slides examined were dried following development of the film and fixed for five minutes in 10% formalin. After two washes in water to remove the formalin, the slides were stained as usual with good results.
CHAPTER 3.

THE EFFECTS OF COLOSTRUM DEPRIVATION AND SERUM SUPPLEMENTATION ON THE GROWTH AND DEVELOPMENT OF YOUNG LAMBS

Lambs are born with virtually no detectable antibodies in their blood. Normally, during their first day of life, the lambs ingest colostrum which is rich in maternal antibodies. A considerable proportion of these antibodies is absorbed intact by the small intestine of the lamb and transferred to the blood. In this way maternal antibodies can protect lambs from certain infections during the first few weeks of life when they have low levels of circulating autologous antibodies.

Hand-reared lambs are often stunted and of poor quality. Many of these lambs are orphaned at birth and are thus deprived of colostrum. It is not known whether their failure to thrive when hand-reared is related to their being deprived of colostrum or to inadequate nourishment. The problems met by other workers in rearing calves deprived of colostrum (Smith and Little, 1922; Ingram, Lovell, Wood, Aschaffenburg, Bartlett, Kon, Palmer, Roy and Shillam, 1956; Steck, 1962) and the delayed maturation of the plasma protein profile observed in
colostrum-deprived piglets (Lecce and Matrone, 1961; Miller, Harmon, Ullrey, Schmidt, Luecke and Hoefer, 1962) suggest that in other species colostrum might have a role in the onset of normal growth and immunological development, over and above a temporary passive protection against infectious diseases.

In order to investigate whether or not maternal antibodies have any effects other than protecting young lambs from infectious diseases, an experiment was designed to test the effects of colostrum deprivation, and supplementation with adult serum by various routes at different ages, on the rate of growth of lambs and their subsequent ability to respond to antigenic challenge.

**Experimental procedures**

Lambs were removed from their mothers at birth and were kept indoors in pens with slatted wooden floors. Later they were left out on irrigated pasture during the day and housed indoors at night. They had access to lucerne chaff and water *ad libitum* throughout the experiment. Two lambs were allowed to suck their mothers for 36 hours and were then removed from their mothers and reared in the same way as the other lambs.
Feeding methods

For a period of two weeks after birth all lambs were fed four times daily with 250 ml. of fresh cow milk. During the third week the lambs were fed three times daily, and from four to eight weeks of age they received two feeds of 500 ml. each per day. During the ninth week only one feed of 500 ml. per day was given, after which the lambs were weaned on to pasture. In this manner all lambs were fed equal amounts of milk for a period of nine weeks, and any differences in performance were not due to differences in the amount of milk consumed. At first all the lambs were fed individually, but later a bucket fitted with six teats ("Lam-Bar", Baker) was adapted so that each of the teats led to a bottle containing the measured ration of milk, and by this means all the lambs could be fed within 45 minutes.

To protect the lambs from infection, a veterinary preparation of Terramycin (Pfizer) was added, according to directions, to the milk fed during the first five days of life. Chlorodyne (Tinctura Chloroformi et Morphinae, B.P.) was given to individual lambs which showed signs of scouring during their first two weeks of life. At weaning all the animals were dosed with an anthelminthic,
"Thibenzole", (Merck, Sharpe and Dohme) and Pulpy Kidney antitoxin (C.S.L.).

All lambs were weighed daily at approximately the same time from birth to 12 weeks of age.

**Experimental design**

A 2 x 2 x 2 factorial experiment was designed to study whether the administration of adult sheep serum affected the performance of colostrum-deprived lambs. Lambs were given 50 ml. of physiological saline or serum, orally or intraperitoneally, on the day of birth or at 4 days of age. The ages at which the serum was given were chosen to compare the effect of serum given orally at a time when antibodies can be absorbed intact from the gut, with that when the gut is impermeable to intact proteins (McCarthy and McDougall, 1953; Lecce and Morgan, 1962). The experimental design is given in Table 3., together with the scheme for analysing the treatment effects.

The lambs were obtained from a limited number of Merino and Cross-bred Merino ewes, and only the male lambs were available for the experiment. It was hoped initially that two replicates of the eight treatment combinations could be made of lambs born to both Merino and Cross-bred Merino ewes, so that possible variation due to breed
Table 3. The design of the experiment to test the effect of serum supplementation on the growth of colostrum-deprived lambs.

<table>
<thead>
<tr>
<th>Treatment combination</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment administered</td>
<td>Route of administration</td>
</tr>
<tr>
<td>Saline</td>
<td>Oral</td>
</tr>
<tr>
<td>Saline</td>
<td>Oral</td>
</tr>
<tr>
<td>Saline</td>
<td>I-P</td>
</tr>
<tr>
<td>Saline</td>
<td>I-P</td>
</tr>
<tr>
<td>Serum</td>
<td>Oral</td>
</tr>
<tr>
<td>Serum</td>
<td>Oral</td>
</tr>
<tr>
<td>Serum</td>
<td>I-P</td>
</tr>
<tr>
<td>Serum</td>
<td>I-P</td>
</tr>
</tbody>
</table>

The scheme for partitioning the treatment sum of squares to analyse the treatment effects.

<table>
<thead>
<tr>
<th>Item</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main effects</td>
<td></td>
</tr>
<tr>
<td>Saline vs. serum (1)</td>
<td>100</td>
</tr>
<tr>
<td>Oral vs. I-P (2)</td>
<td>010</td>
</tr>
<tr>
<td>Day 1 vs. day 4 (3)</td>
<td>001</td>
</tr>
<tr>
<td>First order interactions (1) x (2)</td>
<td>110</td>
</tr>
<tr>
<td>(1) x (3)</td>
<td>101</td>
</tr>
<tr>
<td>(2) x (3)</td>
<td>011</td>
</tr>
<tr>
<td>Second order interaction (1) x (2) x (3)</td>
<td>111</td>
</tr>
</tbody>
</table>
differences could be tested and allowed for in analysing the experimental results. As the lambs were born they were assigned at random to treatment combinations in the appropriate replicates. Two complete Cross-bred replicates and one complete Merino replicate were filled. A fourth replicate containing the progeny of both Merino and Cross-bred ewes was filled. However two lambs in this fourth replicate died during the experiment and the results for the lambs remaining in the replicate were omitted from the subsequent analysis. Thus although the groups contained lambs of different breeds a comparison of these groups does not necessarily reflect a comparison of breeds, but is confounded with the order in which the lambs were born.

Administration of treatments

Sterile sheep serum (50 ml.) was administered orally by means of an oesophageal tube. Intraperitoneal injections of 50 ml. of sterile serum were made through a small incision in the skin of the flank so that the needle entered the peritoneal cavity with a minimum of exertion. The tip of the needle used for the injection was fitted with a short piece of polythene tubing to avoid entering any internal organs. Control lambs were given 50 ml. of sterile saline in a similar manner to the above.
Antigenic challenge

The lambs were challenged with two antigens (SIV and rabbit RBC) to assess their immune response. A dose of 1 ml. of SIV containing 10,000 haemagglutinating units was injected subcutaneously into each lamb at six weeks of age and a secondary challenge given in the same manner 15 days later. Washed rabbit RBC, $10^9$ cells per lamb, were given subcutaneously at the same time as SIV but on the opposite side. Primary and secondary challenges were given as for the SIV.

Blood samples were taken from each lamb at the time of primary challenge and 7, 11 and 15 days later, when the lambs were given their secondary challenge. Further blood samples were collected 5, 10 and 15 days after the secondary challenge. The blood was allowed to clot at room temperature and then centrifuged at 3,000 rpm for 10 minutes. The serum was collected and kept frozen until the antibodies to the two antigens were estimated.

Results

The effects of treatments on serum protein profiles

Agar gel immunoelectrophoresis of serum from the lambs showed that the dose of 50 ml. of serum given orally on the day of birth and intraperitoneally on the first or
Fig. 8 Serum protein profiles obtained by agar gel immunoelectrophoresis of serum from a lamb deprived of colostrum, and given 50 ml. of adult serum orally on the day of birth, treatment code 100. The serum samples were taken from the lamb 1, 2, 14, 27, 59, and 74 days after birth.
Serum protein profiles obtained by agar gel immunoelectrophoresis of serum from a lamb deprived of colostrum, and given 50 ml. of adult serum intraperitoneally on the fourth day after birth, treatment code 111. The serum samples were taken from the lamb 1, 5, 14, 28, 49, and 62 days after birth.
Fig. 10  Serum protein profiles obtained by agar gel immunoelectrophoresis of serum from a lamb deprived of colostrum and given saline orally on the day of birth, treatment code, 000. The serum samples were taken from the lamb 2, 13, 27, 44, 59, and 74 days after birth.
Day 2

Day 13

Day 27

Day 44

Day 59

Day 74
fourth day after birth resulted in a detectable precipitin line corresponding to fast IgG globulin (Figs. 8, 9). The fainter line resulting from oral administration of the serum suggested that only a proportion of the γ-globulin fed reached the circulation intact. Serum fed orally on the fourth day did not produce any γ-globulin precipitin arcs in the serum profile. In the lambs which did not receive any serum there was virtually no γ-globulin present in the serum up to 4 weeks after birth yet with 2 exceptions these lambs remained quite healthy. These lambs attained a mature serum protein profile by the age of eight weeks, but even at 4 weeks, fast IgG was present in the serum, and slow IgG and IgM were detectable at 6 weeks of age (Fig. 10). These results showed that the treatments used in the experiment succeeded in producing different circulating levels of γ-globulin.

The effects of treatments on body weight and rate of growth

To assess the overall rate of growth in these lambs, the means of the logarithms of the body weights were plotted against age in weeks (Fig. 11), and from this graph it appears that during the first week of life and during and after weaning the rate of growth was less than
The mean log body weight for all colostrum-deprived lambs plotted against age in weeks. Between the ages of 1 and 8 weeks, mean log body weight is related linearly to age and the solid line is the regression of mean log body weight on time, fitted by the method of least squares, between these ages.
in the intervening period. From 1 to 8 weeks, the mean log body weight increased linearly with time, as demonstrated by the close correspondence between the log body weight curve and the regression of mean log body weight on time during this period. Therefore an analysis of variance was applied only to this period during which growth appeared to be in a log phase and there were no apparent restrictions imposed by the feeding regimen.

The analysis of variance for the body weight data from 1 to 8 weeks of age is given in Table 4. The significant variation between replicates (P<0.001) is worthy of comment in that it cannot be attributed simply to differences in breed of the mothers of the lambs. The mean log body weights of the three replicates were 1.2171 (Replicate 1, Cross-bred), 1.1701 (Replicate 2, Cross-bred) and 1.1965 (Replicate 3, Merino), which suggests that the time at which the lambs were born might have been more important than the breed of the lambs' mothers in determining body weight.

The mean square for the residual sum of squares in the variation due to age, after taking out the sum of squares for the linear regression of log body weight on age, indicates that from 1 to 8 weeks of age the
<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>D.F.</th>
<th>Mean square</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>0.07096071</td>
<td>2</td>
<td>0.03548035</td>
<td>30.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age¹</td>
<td>3.23927026</td>
<td>7</td>
<td>0.46275289</td>
<td>396</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatments²</td>
<td>0.04351873</td>
<td>7</td>
<td>0.00621696</td>
<td>5.32</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Age x treatments interaction³</td>
<td>0.02327113</td>
<td>49</td>
<td>0.00047492</td>
<td>0.41</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Residual</td>
<td>0.14723785</td>
<td>126</td>
<td>0.00116855</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3.52425868</td>
<td>191</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| ¹Age                                    |                |      |             |       |       |
| Regression                              | 3.23480564     | 1    | 3.23480564  | 2768  | <0.001|
| Residual                                | 0.00446462     | 6    | 0.00074410  | 0.64  | >0.2  |

| ²Treatments                             |                |      |             |       |       |
| 100                                     | 0.00253316     | 1    | 0.00253316  | 2.17  | >0.1  |
| 010                                     | 0.01806140     | 1    | 0.01806140  | 15.46 | <0.001|
| 001                                     | 0.00006165     | 1    | 0.00006165  | 0.05  | >0.2  |
| 110                                     | 0.00157552     | 1    | 0.00157552  | 1.35  | >0.2  |
| 101                                     | 0.00561817     | 1    | 0.00561817  | 4.81  | <0.05 |
| 011                                     | 0.00326535     | 1    | 0.00326535  | 2.79  | >0.05 |
| 111                                     | 0.01240347     | 1    | 0.01240347  | 10.61 | <0.01 |

| ³Age vs treatments                      |                |      |             |       |       |
| Regression                              | 0.00906540     | 1    | 0.00906540  | 7.76  | <0.01 |
| Residual                                | 0.000049826    | 1    | 0.000049826 | 0.43  | >0.2  |
| 110                                     | 0.00057241     | 1    | 0.00057241  | 0.49  | >0.2  |
| 101                                     | 0.00522795     | 1    | 0.00522795  | 4.47  | <0.05 |
| 011                                     | 0.00001768     | 1    | 0.00001768  | 0.02  | >0.2  |
| Residual                                | 0.00035204     | 1    | 0.00035204  | 0.30  | >0.2  |

Table 4. Analysis of Variance
Log Body weights 1 to 8 weeks of age
relationship between log body weight and age did not deviate significantly from linearity (P > 0.2). Therefore it appears that nourishment was adequate during this period and the fall off in the rate of growth during the ninth week reflects the decreased amount of milk fed prior to weaning.

The item for treatments is significant (P < 0.01) indicating significant differences between the overall mean log body weights in the various treatment groups. However, unless the relationships between log body weight and age in the various treatment groups differ significantly, the significantly different means might indicate nothing more than fortuitously different birth weights in these groups. The sum of squares attributable to differences in relationships between log body weight and age is the age x treatment interaction sum of squares which does not indicate significant differences (P > 0.2).

However if the relationship between log body weight and age in each treatment group is simply a linear regression, as was the case for mean log body weight over all treatment combinations, then the 7 degrees of freedom for comparisons between linear regression coefficients in the different treatment groups might account for a large proportion of the sum of squares for interactions between log body
weight and age; if this were so significant differences between these regressions would be suggested. The sum of squares for treatments was therefore further partitioned into sums of squares for single degrees of freedom for the treatment main effects, and their interactions, on log body weight. The sum of squares for interactions between treatments and age was partitioned into sums of squares for treatment main effects, and their interactions, on the regressions of log body weight on age, and a residual sum of squares to test for deviations from these regressions. These partitions are presented in Table 4, and although some of the comparisons indicate statistically significant differences, the biological significance of these differences is doubtful. Certainly the complete analysis does not support the hypothesis that in the absence of overt infectious disease, the different levels of circulating γ-globulin produced by the various treatments influenced the rate of growth of the lambs. The regression equations relating log body weight to age for the individual treatments are given in Table 5.

Both lambs which died acutely in the early part of the experiment did not receive any serum. However the numbers involved are too few to allow any conclusions
to be drawn as to the effectiveness of the amounts of adult serum used in preventing early death.

Table 5. The regressions of log body weight on age for the period from 1 to 8 weeks of age for the individual treatments of the experiment.

Regression equation:

\[ \log \text{wt.} = \text{mean log wt.} + b (\text{age} - \text{mean age}) \]

<table>
<thead>
<tr>
<th>Treatment code</th>
<th>Regression equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>000</td>
<td>( Y = 1.1879 + 0.03117(x-x) )</td>
</tr>
<tr>
<td>001</td>
<td>( Y = 1.2076 + 0.0300(x-x) )</td>
</tr>
<tr>
<td>010</td>
<td>( Y = 1.1820 + 0.02936(x-x) )</td>
</tr>
<tr>
<td>011</td>
<td>( Y = 1.1861 + 0.02871(x-x) )</td>
</tr>
<tr>
<td>100</td>
<td>( Y = 1.2277 + 0.02612(x-x) )</td>
</tr>
<tr>
<td>101</td>
<td>( Y = 1.1937 + 0.02455(x-x) )</td>
</tr>
<tr>
<td>110</td>
<td>( Y = 1.1783 + 0.02815(x-x) )</td>
</tr>
<tr>
<td>111</td>
<td>( Y = 1.1929 + 0.02848(x-x) )</td>
</tr>
</tbody>
</table>

The effects of treatments on response to antigenic challenge

In nearly all lambs the response to primary challenge with SIV was non-specific and no meaningful antibody titres could be measured. After secondary challenge most lambs produced titratable levels of antibody but a few still gave a non-specific response which obscured the specific response. Because of this non-specific response it was not possible to analyse the results as completely as the body weight data. The mean titres at the various times following secondary challenge for all lambs giving
a measurable response are presented in Table 6. for each treatment combination. The numbers for each treatment combination are less than 4 when lambs died or gave non-specific responses. It can be seen from Table 6. that there was no obvious relationship between the treatment administered and the antibody response. Moreover there is no apparent difference between the maximal responses for colostrum-deprived lambs and colostrum-fed lambs whose responses are also given in Table 6.

Table 6. The antibody titres (log₂) appearing in the serum of lambs following a secondary challenge of SIV, as measured by a haemagglutination inhibition titration

<table>
<thead>
<tr>
<th>Treatment code</th>
<th>Days after secondary challenge</th>
<th>No. of lambs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>000</td>
<td>4.1</td>
<td>4.5</td>
</tr>
<tr>
<td>001</td>
<td>4.1</td>
<td>5.1</td>
</tr>
<tr>
<td>010</td>
<td>4.4</td>
<td>4.9</td>
</tr>
<tr>
<td>011</td>
<td>4.0</td>
<td>4.1</td>
</tr>
<tr>
<td>100</td>
<td>3.9</td>
<td>4.5</td>
</tr>
<tr>
<td>101</td>
<td>4.2</td>
<td>5.1</td>
</tr>
<tr>
<td>110</td>
<td>3.7</td>
<td>4.4</td>
</tr>
<tr>
<td>111</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Colostrum-fed lambs</td>
<td>4.9</td>
<td>4.9</td>
</tr>
</tbody>
</table>

No haemolysins were produced by any of the lambs in response to challenge by rabbit RBC. Later tests on both normal lambs and adult sheep showed that the dose given
subcutaneously was not sufficient to produce a haemolysin response in these animals. Some agglutinins were present in the serum but these could not be measured satisfactorily and may have been non-specific.

The effects of feeding regimen on body weight

The rate of growth during their first 12 weeks of three groups of lambs which were reared under different conditions are compared in Fig. 12. Lambs reared by their mothers grew faster during the first few weeks after birth but then their rate of increase in body weight declined and their mean weight at 12 weeks was approximately the same as the two groups of lambs which were reared by hand. Of the two hand-reared groups, one consisted of the colostrum-deprived lambs given the serum and saline treatments described above, and the other of the four lambs which received colostrum but were reared in the same manner as the colostrum-deprived group. The differences in body weight between all three groups were slight but in appearance the hand-reared lambs were smaller in frame and were distinctly pot-bellied, probably because they received their milk in a few large feeds each day. A larger ration of milk fed in small quantities throughout the day might have improved the quality of these lambs.
The similar rates of growth in the colostrum-fed and colostrum-deprived groups suggests that under the conditions of the experiment, in which some antibiotic treatment was given to control infectious disease, colostrum had no beneficial effects on growth.

The effects of colostrum on serum protein concentrations in lambs were evident from birth onwards. Total serum protein concentrations decreased slightly from birth to 2 weeks of age and then gradually increased. A noticeable increase in total protein levels after 4 weeks of age occurred at about the same time as endogenous synthesis ceased in the serum. Colostrum-fed lambs had a mean level of 93.5 mg/ml at 1 days of age, almost double the level in colostrum-deprived lambs. Colostrum-fed lambs, reared by their mothers, showed a higher mean serum protein level of the colostrum-fed group, but did not fall between 2 and 4 weeks so that at 4 weeks of age their serum protein levels were higher than the hand-reared lambs.

**Fig. 12** The mean body weight (lb) of 3 groups of lambs, reared under different conditions, plotted against age in weeks.
- o colostrum-deprived, hand-reared lambs.
- ● colostrum-fed, hand-reared lambs.
- x normal lambs reared by their mothers.
The similar rates of growth in the colostrum-fed and colostrum-deprived groups suggests that under the conditions of the experiment, in which some antibiotic treatment was given to control infectious disease, colostrum had no beneficial effects on growth.

**The effects of colostrum on serum protein concentrations in lambs**

In colostrum-deprived lambs, total serum protein concentrations decreased slightly from birth to 2 weeks of age and then steadily increased. A noticeable increase in total protein levels after 4 weeks of age occurred at about the same time as endogenous γ-globulin became apparent in the serum. Colostrum-fed lambs reached a mean level of 93.5 mg/ml. at 2 days of age, almost double the level in colostrum-deprived lambs and then fell progressively to 4 weeks of age at which age there was no difference between the serum protein levels of these lambs and those of the colostrum-deprived group. In normal lambs reared by their mothers, the serum protein levels increased after the ingestion of colostrum, were similar at 2 weeks of age to the other colostrum-fed group, but did not fall between 2 and 4 weeks so that at 4 weeks of age their serum protein levels were higher than the hand-reared lambs.
The serum protein levels for the 3 different groups of lambs are given in Table 7.

Table 7. The total serum protein levels (mg/ml.) of lambs reared under different conditions, expressed as mean values ± S.E. of the mean. The numbers in parentheses show the number of observations from which each mean value is calculated.

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Hand-fed lambs</th>
<th>Normal lambs reared by ewes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No colostrum</td>
<td>Fed colostrum</td>
</tr>
<tr>
<td>1</td>
<td>54.6±3.73 (7)</td>
<td>77.3±10.0 (3)</td>
</tr>
<tr>
<td>2</td>
<td>49.1±3.60 (7)</td>
<td>93.5±9.47 (4)</td>
</tr>
<tr>
<td>14</td>
<td>48.0±3.31 (6)</td>
<td>67.2±9.71 (3)</td>
</tr>
<tr>
<td>28</td>
<td>52.7±4.57 (10)</td>
<td>55.8±2.95 (3)</td>
</tr>
<tr>
<td>42</td>
<td>59.5±4.57 (3)</td>
<td>-</td>
</tr>
</tbody>
</table>

Cellulose acetate electrophoretograms of the serum from colostrum-fed and colostrum-deprived lambs showed that most of the increase in total serum protein could be accounted for by the increase in serum γ-globulin (Fig. 13). Densitometer tracings (Analytrol, Beckman) showed that after the ingestion of colostrum the levels of γ-globulin in lamb serum became higher than in adult serum. In the colostrum-deprived lamb, serum γ-globulin only reached levels detectable by this method after about 4 weeks, and even at 10 weeks the levels of circulating γ-globulin were less than in adult sheep.
Fig. 13 Cellulose acetate electrophoretograms of serum from lambs and adult sheep with densitometer tracings derived from the stained cellulose acetate membranes.
(a) Serum of lamb after the ingestion of colostrum, 2 days of age.
(b) Serum from colostrum-deprived lamb at 2 days of age.
(c) Adult sheep serum.
(d) Serum from colostrum-deprived lamb at 10 weeks of age.
The absorption of maternally γ-globulin by the small intestine of the newborn lamb has been studied previously by several workers (McCarthy, 1935; Martin and Middle, 1948; McCarthy and 1953). The intestine is able to transfer a variety of proteins to the plasma during the first 30 hours after birth but this capacity is rapidly lost and absorption ceases by 48 hours after birth (McCarthy and McEvedy, 1953;Boce and Morgan, 1962). The experiments described in this chapter were carried out in order to study the absorption of γ-globulin in Merino and Cross-bred Merino lambs and to test whether 125I-labelled γ-globulin pre-incubated with the relative protein, or not, behaved similarly in vivo to the native protein. As far as this could be determined in studies with the proteins of ovine colostrum, the results of the experiments in Merino lambs are recorded.
CHAPTER 4.

STUDIES OF γ-GLOBULIN METABOLISM IN THE NEWBORN LAMB

The absorption of maternal γ-globulin by the small intestine of the lamb has been studied previously by several workers. It has been shown that intact antibodies appear in the plasma between 1 and 4 hours after the ingestion of colostrum (Mason et al., 1930) and there is a concurrent increase in the globulin fraction of the plasma (Earle, 1935; Charlwood and Thomson, 1948; McCarthy and McDougall, 1953). The intestine is able to transfer a variety of proteins to the plasma during the first 30 hours after birth but this capacity is rapidly lost and absorption ceases by 48 hours after birth (McCarthy and McDougall, 1953; Lecce and Morgan, 1962).

The experiments described in this chapter were carried out in order to study the absorption of γ-globulin in Merino and Cross-bred Merino lambs and to test whether ¹²⁵I-labelled γ-globulin prepared as described earlier behaved similarly in vivo to the native protein, in so far as this could be determined in studies with the intact newborn animal.

The proteins of ovine colostrum whey

After the ingestion of colostrum by the newborn lamb, the casein is coagulated to form a curd in the abomasum and
the whey which is liberated moves fairly rapidly down the small intestine, and it is from this fluid that γ-globulin is absorbed. The level of γ-globulin in ovine colostrum whey is high, reported values being as high as 150 mg/ml in colostrum obtained immediately after parturition (McCarthy and McDougall, 1953). In one sample analysed in the course of the work reported in this thesis, the amount of protein precipitated by 18% Na₂SO₄ was 91 mg/ml. or 55% of the total whey protein.

The colostrum γ-globulin was characterised by agar gel immunoelectrophoresis of colostrum whey using antiserum to adult sheep serum (Fig. 14). Three precipitin arcs corresponding to three classes of γ-globulin, IgM, fast IgG and slow IgG, were present in the whey. The fast IgG was the predominant class, and IgM appeared to be present in higher concentration than in adult serum. Results suggesting a selective mechanism in the mammary gland of the ewe favouring the concentration of fast IgG over slow IgG in ovine colostrum have previously been reported (Mackenzie and Lascelles, 1968).

**Estimation of the rate of absorption of γ-globulin by the intestine**

Adult sheep γ-globulin was purified and labelled with ¹²⁵I as described previously. Portions of the labelled
protein were added to either colostrum or a sterile solution of \( \gamma \)-globulin in saline. These were then fed to eight lambs at various ages as shown in Table 8. Blood samples were taken every 2-4 hours during the 12 hours after feeding. Subsequent blood samples were taken at regular intervals during the experiment. The levels of radioactivity present in the serum are shown plotted against time after feeding.

\( ^{125}I \)-labelled \( \gamma \)-globulin was seen from this figure that the levels of radioactivity in the serum peaked between 2 and 5 hours after feeding, and tended to a maximum 6-10 hours after feeding.

In all three treatments, the serum was bound to protein.

**Table 8.** The schedule according to which lambs were given \( ^{125}I \)-labelled \( \gamma \)-globulin including the estimate of the maximum percentage of the dose present in the circulation, assuming a plasma volume of 40 ml./kg.

<table>
<thead>
<tr>
<th>Lamb No.</th>
<th>Weight (kg)</th>
<th>100ml colostrum</th>
<th>97ml colostrum</th>
<th>100ml colostrum</th>
<th>106ml colostrum</th>
<th>100ml colostrum</th>
<th>106ml colostrum</th>
<th>88ml ( \gamma )-globulin</th>
<th>34ml ( \gamma )-globulin</th>
<th>15ml ( \gamma )-globulin</th>
<th>Intrapertioneally</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.93</td>
<td>2</td>
<td>2</td>
<td>100ml colostrum</td>
<td>12.3</td>
<td>97ml colostrum</td>
<td>18.4</td>
<td>100ml colostrum</td>
<td>19.7</td>
<td>106ml colostrum</td>
<td>19.7</td>
</tr>
<tr>
<td>4</td>
<td>3.27</td>
<td>2</td>
<td>2</td>
<td>97ml colostrum</td>
<td>14.6</td>
<td>100ml colostrum</td>
<td>19.4</td>
<td>100ml colostrum</td>
<td>10.9</td>
<td>106ml colostrum</td>
<td>18.7</td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>6</td>
<td>2</td>
<td>97ml colostrum</td>
<td>14.6</td>
<td>100ml colostrum</td>
<td>10.9</td>
<td>100ml colostrum</td>
<td>10.9</td>
<td>106ml colostrum</td>
<td>18.7</td>
</tr>
<tr>
<td>8</td>
<td>2.5</td>
<td>17</td>
<td>2</td>
<td>100ml colostrum</td>
<td>12.3</td>
<td>97ml colostrum</td>
<td>18.4</td>
<td>100ml colostrum</td>
<td>19.7</td>
<td>106ml colostrum</td>
<td>19.7</td>
</tr>
<tr>
<td>2</td>
<td>2.48</td>
<td>29</td>
<td>2</td>
<td>100ml colostrum</td>
<td>12.3</td>
<td>97ml colostrum</td>
<td>18.4</td>
<td>100ml colostrum</td>
<td>19.7</td>
<td>106ml colostrum</td>
<td>19.7</td>
</tr>
<tr>
<td>5</td>
<td>2.77</td>
<td>0.5</td>
<td>2</td>
<td>88ml ( \gamma )-globulin</td>
<td>10.9</td>
<td>34ml ( \gamma )-globulin</td>
<td>7.9</td>
<td>15ml ( \gamma )-globulin</td>
<td>7.9</td>
<td>Intrapertioneally</td>
<td>77.4</td>
</tr>
<tr>
<td>7</td>
<td>2.73</td>
<td>5</td>
<td>2</td>
<td>88ml ( \gamma )-globulin</td>
<td>10.9</td>
<td>34ml ( \gamma )-globulin</td>
<td>7.9</td>
<td>15ml ( \gamma )-globulin</td>
<td>7.9</td>
<td>Intrapertioneally</td>
<td>77.4</td>
</tr>
<tr>
<td>3</td>
<td>2.93</td>
<td>2.5</td>
<td>2</td>
<td>88ml ( \gamma )-globulin</td>
<td>10.9</td>
<td>34ml ( \gamma )-globulin</td>
<td>7.9</td>
<td>15ml ( \gamma )-globulin</td>
<td>7.9</td>
<td>Intrapertioneally</td>
<td>77.4</td>
</tr>
</tbody>
</table>

**Fig. 14** Protein profiles of ovine colostrum whey and adult sheep serum obtained by agar gel immunoelectrophoresis.
protein were added to either colostrum or a sterile solution of γ-globulin in saline. These were then fed to eight lambs at various ages as shown in Table 8. Blood samples were taken every 2-4 hours during the 12 hours after feeding. Subsequent blood samples were taken at regular intervals during the next three weeks. The levels of radioactivity present in the serum are shown plotted against time after feeding $^{125}$I-labelled γ-globulin in Fig. 15. It can be seen from this figure that the levels of radioactivity in the serum increased rapidly between 2 and 5 hours after feeding, and tended to a maximum 6-10 hours after feeding. In all the lambs nearly all the radioactivity in the serum was bound to protein.

Table 8. The schedule according to which lambs were given $^{125}$I-labelled γ-globulin including the estimate of the maximum percentage of the dose present in the circulation, assuming a plasma volume of 40 ml./kg.

<table>
<thead>
<tr>
<th>Lamb No.</th>
<th>Body Weight (kg)</th>
<th>Age at feeding (hr)</th>
<th>Solution fed</th>
<th>Maximum % of dose present in circulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.93</td>
<td>2</td>
<td>100ml. colostrum</td>
<td>13.2</td>
</tr>
<tr>
<td>4</td>
<td>3.27</td>
<td>2</td>
<td>97ml. colostrum</td>
<td>14.4</td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>8</td>
<td>100ml. colostrum</td>
<td>14.7</td>
</tr>
<tr>
<td>8</td>
<td>2.5</td>
<td>17</td>
<td>100ml. colostrum</td>
<td>8.1</td>
</tr>
<tr>
<td>2</td>
<td>2.48</td>
<td>29</td>
<td>100ml. colostrum</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>2.77</td>
<td>0.5</td>
<td>68ml. γ-globulin</td>
<td>10.0</td>
</tr>
<tr>
<td>7</td>
<td>2.73</td>
<td>5</td>
<td>34ml. γ-globulin</td>
<td>7.9</td>
</tr>
<tr>
<td>3</td>
<td>2.93</td>
<td>2.5</td>
<td>15ml. γ-globulin</td>
<td>77.4</td>
</tr>
</tbody>
</table>
One lamb was fed a solution of γ-globulin, and serum samples taken at regular intervals after feeding were examined by immunoelectrophoresis (Fig. 15). There was only a trace of γ-globulin in the serum 3 hours after feeding, but after 4 hours both the fast and slow IgG precipitin arcs are clearly visible in the protein profile, and by 12 hours the concentrations of both fast and slow IgG arcs are at a maximum level. The appearance and disappearance of the γ-globulin on the agar gels is not specified for the protein. Only a small amount of IgM can be seen in the sample of serum collected 21 hours after the step. The γ-globulin and it is possible that this γ-globulin is derived from the circulation more rapidly than fast IgG.

Estimates of the amount of γ-globulin transferred to the circulation

In the infant, the amount of protein which is transferred to the circulation is not measured. Estimates of the amount of absorbed prolein present in the circulation at some time after feeding have been made in the piglet (Olsson, 1959; Pierce and Smith, 1967a) the calf (Bangham et al., 1958) and the lamb (Olsson et al.,

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Fig. 15 The levels of radioactivity in the serum of 5 of the 8 lambs given 125I-γ-globulin plotted against the time after feeding in hours.
One lamb was fed a solution of $\gamma$-globulin, and serum samples taken at regular intervals after feeding were examined by immunoelectrophoresis (Fig. 16). There was only a trace of $\gamma$-globulin in the serum 2 hours after feeding, but after 4 hours both the fast and slow IgG precipitin arcs are clearly visible in the protein profile, and by 12 hours the concentrations of both fast and slow IgG appear to be at a maximum level. The appearance of both classes of $\gamma$-globulin in the serum of the lambs shows that the absorption process is not specific for either protein. Only a small amount of slow IgG can be seen in the sample of serum collected 71 hours after the feed of $\gamma$-globulin and it is possible that this $\gamma$-globulin is removed from the circulation more rapidly than fast IgG.

**Estimates of the amount of $\gamma$-globulin transferred to the circulation**

In the intact animal the actual amount of protein which is transported by the intestine cannot be measured. Estimates of the amount of absorbed protein present in the circulation at some time after feeding have been made in the piglet (Olsson, 1959; Pierce and Smith, 1967a) the calf (Bangham et al., 1958) and the lamb (Mason et al.,
Fig. 16 The serum protein profiles obtained by agar gel immunoelectrophoresis of serum from a lamb fed labelled γ-globulin. Blood samples were taken before feeding, and 2, 4, 6, 12, 24, and 71 hours after the protein was fed.
Hours after feeding +

0

2

4

6

12

24

71
1930) using measured or assumed values for the plasma volume of the animals. These estimates, which are all likely to be less than the actual amount absorbed, were 5-17% of the amount fed for the piglet, 8-16% for the calf and 1% for the lamb. Locke, Segre and Myers (1964) used an alternative method in which the mean titre of the serum in piglets 48 hours after feeding a given dose of antibody was compared with the titre reached after the injection of an identical dose intraperitoneally. They claimed that 20-25% of the dose fed appeared in the circulation in 48 hours.

In the studies described here the quantities of γ-globulin absorbed by lambs were estimated from the peak levels of absorbed γ-globulin in the circulation, in a similar manner to those published previously. A plasma volume of 40 ml./kg or 4% of body weight was assumed. The percentage of the radioactivity which appeared in the sera of 7 lambs after colostrum or γ-globulin was fed (Table 8.) changed from about 14% at 2 hours of age to about 2% at 29 hours of age. There was a similar proportion of the dose absorbed at 2 hours and 8 hours, suggesting that there was no significant decrease in absorptive capacity during the first 8 hours after birth. The absorption of γ-globulin from a solution in saline, 7.9% and 10% of the
dose for the 2 lambs given this solution, appeared to be less than for colostrum. These percentages would be increased by about 1/5 if they were expressed relative to the maximum amount of an intraperitoneal dose which was present in the plasma following injection (77% for lamb 3).

The amount of γ-globulin in the serum of the lambs during the 24 hours after feeding the labelled γ-globulin was estimated by measuring the protein precipitable by 18% Na₂SO₄ and these values are shown plotted against time in Fig. 17. Since most of the lambs which had been fed labelled colostrum were returned to their mothers, the increase in their serum γ-globulin was a result of absorption from the initial feed and from the colostrum they ingested later from their mothers. The final levels reached would be dependent upon the amount of colostrum ingested, as well as upon the capacity of the intestine to absorb protein. One lamb (No. 5) which was fed only milk after the initial feed of γ-globulin, did not show any increase in serum γ-globulin beyond the 6th hour after that feed. In another lamb (No. 4) the absorption of γ-globulin resulted in a rise of 42 mg/ml. in the total protein in the serum, and a similar rise in the protein
precipitated by 18% Na₂SO₄. The total amount of γ-globulin transported by the intestine of this lamb was of the order of 7 g, almost equivalent to the total circulating plasma protein of the lamb at birth. Three of the other lambs absorbed about 2 g of γ-globulin.

Table 1. Estimations of total protein concentration (mg/ml.) in the serum of a normal lamb during the first 30 days of life, and the amount of γ-globulin present in the serum estimated from densitometer tracings of electrophorograms of the serum.

<table>
<thead>
<tr>
<th>Age (day)</th>
<th>Total Protein (mg/ml.)</th>
<th>γ-Globulin (mg/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.5</td>
<td>7.1</td>
</tr>
<tr>
<td>2</td>
<td>12.0</td>
<td>7.8</td>
</tr>
<tr>
<td>3</td>
<td>13.5</td>
<td>8.3</td>
</tr>
<tr>
<td>4</td>
<td>15.0</td>
<td>9.0</td>
</tr>
<tr>
<td>5</td>
<td>16.5</td>
<td>9.5</td>
</tr>
<tr>
<td>6</td>
<td>18.0</td>
<td>10.5</td>
</tr>
<tr>
<td>7</td>
<td>19.5</td>
<td>11.2</td>
</tr>
<tr>
<td>8</td>
<td>20.0</td>
<td>11.6</td>
</tr>
<tr>
<td>9</td>
<td>21.5</td>
<td>12.0</td>
</tr>
<tr>
<td>10</td>
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</tr>
<tr>
<td>30</td>
<td>53.0</td>
<td>22.5</td>
</tr>
</tbody>
</table>

Fig. 17 The amount of protein precipitable by 18% Na₂SO₄ present in the serum of lambs plotted against the time in hours after an initial feed of colostrum or labelled γ-globulin.
precipitated by 18% Na₂SO₄. The total amount of γ-globulin transported by the intestine of this lamb was of the order of 5 g, almost equivalent to the total circulating plasma protein of the lamb at birth. Three of the other lambs absorbed about 2 g of γ-globulin.

The total serum protein estimations for one normal lamb left with its mother (Table 9) showed that a rise of 60 mg/ml. occurred during the first 24 hours after birth.

Table 9. Estimations of total protein and γ-globulin concentration (mg/ml.) in the serum of a normal lamb during the first 9 days of life, and the percentage of γ-globulin present in the serum estimated from densitometer tracings of electrophoretograms of the serum

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Total protein (mg/ml.)</th>
<th>γ-Globulin (%)</th>
<th>γ-Globulin (mg/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>105.5</td>
<td>68</td>
<td>71.7</td>
</tr>
<tr>
<td>3</td>
<td>96</td>
<td>66</td>
<td>63.4</td>
</tr>
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<td>59.1</td>
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<tr>
<td>9</td>
<td>86.5</td>
<td>52</td>
<td>45.0</td>
</tr>
</tbody>
</table>

Cellulose acetate electrophoresis of the serum samples taken during the first 8 days of life showed that most of the increase in protein concentration was due to γ-globulin (Fig. 18). Densitometer tracings of the stained cellulose acetate membranes were used to estimate the percentage of
Fig. 18  Cellulose acetate electrophoretograms of serum from a lamb suckled by its mother. Samples of serum were taken daily for the first 8 days after birth. The densitometer tracings of the stained membranes for the 2, 5 and 8 day serum samples show that the level of $\gamma$-globulin in the serum decreases during this period.
the total protein present as γ-globulin, and the estimates are given in Table 9. These results suggested that about 20 g of γ-globulin could be absorbed by the small intestine and transferred to the circulation during the first 2 days of life. The concentration of γ-globulin decreased by 9 mg/ml. from the second to the third day. It was not until 5 days that the level was more normal. The parts of the curve represent the contribution of albumin from various plasma pools to the plasma protein concentration and are measured. In the growth curve, the plasma volume could not be measured when the radioactive label in the globulin was too high, but the plasma volume could be measured when the label present in the circulation from previous determinations would interfere with subsequent
the total protein present as γ-globulin, and the estimates are given in Table 9. These results suggested that about 8-10 g of γ-globulin could be absorbed by the small intestine and transferred to the circulation during the first 24 hours of life. The concentration of γ-globulin decreased by 9 mg/ml. from the second to the third day but thereafter the decrease was more gradual.

The rate of disappearance of absorbed γ-globulin from the circulation

The rate of turnover, or the rate of catabolism of any plasma protein in the body, can only be estimated accurately in a steady state system in which the size and rate of turnover of the various protein pools remain constant, and can be measured. In the rapidly growing lamb the plasma volume and the total plasma protein pool in the body are increasing, and if a true circulating half-life for labelled γ-globulin was to be estimated, the plasma volume would have to be measured whenever the radioactivity in the circulation was measured. This presents difficulties since if the plasma volume is estimated repeatedly with T-1824 or radioactively labelled albumin, the label present in the circulation from previous determinations would interfere with subsequent
determinations. Even if the true circulating half-life was measured, it would be difficult to relate this to the metabolic half-life, and hence the rate of catabolism. Neither can the specific activity of the $\gamma$-globulin in the plasma be used in turnover studies in young lambs, because during the period of the experiment the concentration of autologous $\gamma$-globulin in the circulation increases, thereby reducing the specific activity of the absorbed protein more rapidly than would occur as a result of loss from the circulation.

In these studies only the level of radioactivity in the serum (cpm/ml.) was used to measure the disappearance of $^{125}\text{I}$ activity from the circulation, and the resultant "half-life" is an underestimate of the true circulating half-life of the homologous $\gamma$-globulin since any increase in plasma volume results in an increase in the apparent rate of disappearance of the protein, i.e., a shorter "half-life". This effect of growth is evident from Table 10, where the lambs which showed the greatest change in body weight during the experiment gave the lowest values for the apparent "half-life", calculated from the regression of radioactivity in the serum on time (Fig. 19). The mean "half-life" of the $^{125}\text{I}-\gamma$-globulin (289 hr)
Time course for the level of radioactive protein in the serum of 5 lambs following the neonatal absorption of $^{125}$I-$\gamma$-globulin from the gut or the peritoneal cavity. The level of radioactivity, on a log scale, is plotted against the time in hours after feeding the labelled protein, on a linear scale. The regression line for each lamb was fitted by the method of least squares, and the 'half-life' calculated from the regression coefficient.
estimated in this way compares reasonably on a body weight basis with the values obtained for other species (Dixon et al., 1952; Cohen et al., 1956) which suggests that the protein is behaving similarly in vivo to the native protein. If the protein had been altered during the isolation and labelling procedures, then the rate of catabolism would be increased, since animals are able to recognise denatured proteins and preferentially destroy them (McFarlane, 1956).

Table 10. Body weights of five lambs at birth and 21 days of age and the regressions calculated from the rate of disappearance of \( ^{125} \text{I} \) radioactivity from their serum following a feed of labelled \( \gamma \)-globulin

Regressions are of the form:

\[
\log \text{cpm/ml.} \ (Y) = a + b \ (\text{age} \ (x) \ \text{in hours})
\]

<table>
<thead>
<tr>
<th>Lamb No.</th>
<th>Weight (kg)</th>
<th>Constants for regression equation</th>
<th>&quot;Half-life&quot; calculated from regression line (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
<td>21 days</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.93</td>
<td>4.32</td>
<td>3.6577, -0.0009</td>
</tr>
<tr>
<td>2</td>
<td>2.48</td>
<td>3.40</td>
<td>2.8174, -0.0007</td>
</tr>
<tr>
<td>3</td>
<td>2.93</td>
<td>6.40</td>
<td>3.4429, -0.0012</td>
</tr>
<tr>
<td>4</td>
<td>3.27</td>
<td>8.80</td>
<td>3.5648, -0.0013</td>
</tr>
<tr>
<td>5</td>
<td>2.77</td>
<td>5.00</td>
<td>3.8209, -0.0015</td>
</tr>
</tbody>
</table>

Mean 289
CHAPTER 5.

STUDIES ON THE ABSORPTION OF PROTEIN BY THE INTESTINE OF THE FOETAL LAMB

The data presented in the previous chapter, and the results of other workers, show that it is difficult to assess accurately the kinetics of absorption of γ-globulin in the newborn animal, since the circulating plasma volume and the rate at which newly absorbed γ-globulin leaves the circulation to equilibrate with extravascular pools, cannot be measured precisely in these animals. During the first day of life, the metabolism of the newborn animal is becoming adjusted to independent existence, and in this changing system the rate of any one process, such as γ-globulin absorption, can only be estimated very approximately. In order to understand this process better, more information about the kinetics of protein absorption in vivo is required, and this would necessitate a more direct measurement of absorption. Cannulation of the lymphatic vessels and collection of lymph draining from the gut might give some useful information, but this has been done only in anaesthetized calves (Balfour and Comline, 1962), and the effects of anaesthesia on the process are unknown. Even if time were available to allow
the animals to recover from the anaesthetic, the acute effects of surgery might still compound with the stresses of being born and lead to experimental results which have little relevance to the normal physiological situation. In the experiments described in this chapter, the absorption of protein from the gut of the foetal lamb has been studied, to see to what extent the absorption process and its rapid cessation are consequences of birth.

**Characteristics of thoracic duct lymph in foetal lambs**

**The rate of flow of lymph**

The rate of flow of lymph from the thoracic duct of 18 foetuses varied from 7.6 ml./hr to 42.5 ml./hr, but in the majority the rate was 10-15 ml./hr. In any one foetus, flow was remarkably constant from one hour to the next. The overall mean rate of flow for successive 12-hour periods is shown in Table 11, and despite the wide range of flow rates in individual foetuses, there is little change in the overall mean flow rate during the first 60 hours of lymph flow.

**Protein concentration**

Protein estimations on lymph samples showed that although there was a decrease in protein concentration in some animals, particularly those with a high flow
rate, other foetuses were able to maintain a steady concentration of protein in their lymph, and presumably in their blood plasma, for several days. The mean protein output per hour is relatively constant for 60 hours following surgery (Table 11.), but since the protein output of some of the foetuses was increased by infused protein absorbed from the intestine, the overall mean output conceals the fact that in several foetuses the hourly output of protein did decrease by about 25% during this period.

Table 11. The characteristics of thoracic duct lymph flow in foetal lambs during the 60 hours after thoracic duct fistulae were established in the foetuses. The values for mean flow rate (ml./hr), protein output (mg/hr), and lymphoid cell output (cells/hr) were calculated from the hourly lymph samples collected from 18 foetal lambs over successive 12-hour periods. The number of foetuses used for each estimate is given in parentheses.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Mean flow rate (ml./hr)</th>
<th>Mean protein output (mg/hr)</th>
<th>Lymphoid cell output (cells/hr x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-12</td>
<td>12.3 ±1.5 (18)</td>
<td>275 ±32 (17)</td>
<td>111 ±10 (16)</td>
</tr>
<tr>
<td>13-24</td>
<td>13.5 ±1.3 (18)</td>
<td>270 ±26 (17)</td>
<td>78 ±8 (16)</td>
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<tr>
<td>25-36</td>
<td>14.0 ±2.0 (18)</td>
<td>272 ±26 (17)</td>
<td>60 ±7 (16)</td>
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<tr>
<td>37-48</td>
<td>14.0 ±2.5 (15)</td>
<td>273 ±22 (14)</td>
<td>55 ±6 (14)</td>
</tr>
<tr>
<td>49-60</td>
<td>14.2 ±2.9 (13)</td>
<td>265 ±24 (12)</td>
<td>49 ±5 (10)</td>
</tr>
</tbody>
</table>
The output of lymphoid cells

The cells present in thoracic duct lymph were almost entirely medium lymphocytes, with relatively few mitotic figures, large cells, or eosinophils. No change in the type of cells appearing in the lymph occurred during drainage, although the numbers of cells declined considerably during prolonged drainage. The cell count was low while the ewe and foetus were recovering from anaesthesia, but after the ewe stood up, the cells increased to about 15-20 x 10^6 cells/ml. Then the cell content of the lymph declined fairly rapidly for several hours, to about 8 x 10^6 cells/ml., but thereafter the fall was more gradual.

Survival of lambs following thoracic duct drainage

The fate of the foetal lambs used depended both on the experiment performed, and the age of the foetus at surgery. Of a total of 24 foetuses which flowed for periods ranging from 8 hours to 6 days, 9 were delivered under anaesthesia and killed subsequently for histological studies or for radioactivity measurements, 3 were dead when delivered by the ewe, and 12 survived birth. In 5 out of these 12 lambs, the cannula pulled out during delivery and the pleural cavity became filled with lymph,
causing death within 24 hours. Seven of the lambs were born with fistulae intact, one of them being shown in Fig. 20, and 3 of these were fed labelled γ-globulin soon after birth, to measure the absorption of radioactive protein postnatally. Of those in which the cannula was tied off up to 24 hours after birth, only two survived more than a week. The others apparently failed to develop adequate connections between the lymphatic vessels and the circulation, and died within 7 days with gross congestion of the lymphatics draining the viscera.

Since a number of foetuses survived the stresses of birth after prolonged lymphatic drainage, and the flow rate and the protein content of lymph did not change drastically during the first 60 hours after surgery, it would seem that the foetal lamb preparation can be considered reasonably physiological during this period, or at least comparable with other animals with lymphatic fistulae.

**Relationships between plasma and lymph protein in the foetal lamb**

The concentration of protein in lymph relative to that in blood plasma reflects, in part, the permeability of blood capillaries to protein in the tissues in which
Fig. 20 A lamb born with an intact thoracic duct fistula, photographed a few hours after birth. The fistula was established in utero two days previously, and lymph had been collected continuously for 45 hours before birth.
the lymph is formed. Whilst it is relatively simple to collect lymph from the unanaesthetized foetus, it is difficult to collect blood samples at the same time. The mean concentration of protein in the thoracic duct lymph of 18 foetuses during the 12 hours after surgery was $22.2 \pm 1.05 \text{ mg/ml.}$ The mean concentration of protein in the plasma of 4 foetuses at surgery was $48.4 \pm 6.39 \text{ mg/ml.}$, whilst the mean plasma protein concentration measured in 10 newborn lambs immediately after birth was $47.9 \pm 1.09 \text{ mg/ml.}$ which is not significantly different from that measured in foetuses at surgery. The ratio of the mean protein concentration of foetal thoracic duct lymph to the mean plasma protein concentration in foetuses and newborn lambs was 0.45. This value is within the range of values measured for lymph draining from various regions of the adult sheep (Lascelles and Morris, 1961; Cowie, Lascelles and Wallace, 1964; Morris and Sass, 1966) and there is therefore no reason to suspect that capillary permeability to proteins in the foetus in late gestation is greatly different from that in adult sheep.

The rate of equilibration of plasma albumin with the albumin in lymph was measured in one foetus (F7/69) in which both the right jugular vein and the thoracic
duct were cannulated. Evans Blue dye (T-1824; 15 mg) dissolved in 1 ml. of physiological saline was injected into the jugular vein at 5 and 44 hours after surgery. Unfortunately blood samples could only be obtained during the two hours after the second injection, due to persistent clotting of blood in the cannula. The time course of the specific activity of albumin in the lymph after each injection of T-1824 is shown in Fig. 21, where it can be seen that it was similar for both injections. The amount of dye collected in the lymph during the ten hours after each injection was 1.63 mg and 1.76 mg for the first and second injection respectively. These results suggest that the rate of leakage of albumin from the circulation did not change significantly during the first 48 hours of lymph collection.

The specific activity of albumin in the lymph was equal to that in the serum 2 hours after the second injection (Fig. 21), indicating that plasma albumin equilibrated rapidly with extravascular albumin in the tissues drained by the thoracic duct. The ratio of the protein concentration in lymph to that in plasma rose from 0.53 at 24 hours to 0.60 at 44 hours, with a decrease in total protein in the serum from 53.5 mg/ml. to 44 mg/ml. in the same period.
Fig. 21 The time course for the specific activity of T-1824 labelled albumin in the thoracic duct lymph of a foetal lamb (F7/69) following the first (x) and second (o) injections of the dye. Also shown is the specific activity of the albumin in the plasma for the 2 hours following the second injection (●).
Absorption of protein from the foetal intestine

Absorption of $^{131}\text{I}$-human serum albumin

In a preliminary experiment to show whether or not the intestine of the foetal lamb was capable of absorbing intact protein, approximately 40 µc of $^{131}\text{I}$-HSA in sterile sheep serum was infused into the duodenum of a foetal lamb (F2/67) at a constant rate of 2 ml./hr for 5 hours. Radioactive iodine was detected in the lymph within 2 hours of the commencement of the infusion and reached a peak level seven hours after the beginning of the infusion. The radioactivity in the lymph then declined rapidly and was no longer detectable 33 hours after the infusion ended. The foetus was then given a similar infusion of $^{131}\text{I}$-HSA in sterile sheep serum for 10 hours. The radioactivity in the lymph resulting from this infusion was separated into albumin-bound and non-albumin-bound radioactivity, using the method of Debro, Tarver and Korner (1957). The time course for the levels of albumin-bound and total radioactivity are shown in Fig. 22. The rate of absorption of albumin-bound radioactivity reached a peak of about 18% of the rate at which it had been infused soon after the infusion ended, and showed no sign of reaching a plateau during the infusion. From this preliminary experiment it
appeared that the foetal intestine was able to absorb protein, and transfer it to the lymphatic system.

Absorption of $^{125}\text{I}$-γ-globulin in saline

(i). The time course of absorption

In the first experiment using γ-globulin, 16 ml. of solution of γ-globulin in saline containing $^{125}\text{I}$-labelled globulin was infused into the duodenum of a foetal lamb (F2/67) for 18 hours at a constant rate of 1 ml./hr (equivalent to about 150 μg of protein infused/hr). The radioactivity in a 1 ml. portion of hourly lymph collections was measured and the ratio of protein:non-protein radioactivity was estimated. Although the radioactivity in the infused solution in the scintillator, up to the total radioactivity in the lymph was soluble, indicating possible breakdown of the labelled protein. The maximum rate of absorption of intact protein, estimated from the protein-bound radioactivity, 105 min. after infusion and this was 25 (Fig. 31).

The rate of flow of lymph from the thoracic duct of this foetus was 3-4 times higher than that measured subsequently in any other foetus. There was no noticeable

Fig. 22 The time course for the total radioactivity (o) and the albumin-bound radioactivity (x) in the thoracic duct lymph of a foetal lamb (F2/67) during and after the infusion of $^{131}\text{I}$-human serum albumin.
appeared that the foetal intestine was able to absorb protein, and transfer it to the lymphatic system.

Absorption of $^{125}$I-$\gamma$-globulin in saline

(i) The time course of absorption

In the first experiment using $\gamma$-globulin, 18 ml. of a solution of $\gamma$-globulin in saline containing $^{125}$I-labelled $\gamma$-globulin was infused into the duodenum of a foetal lamb (F3/67) for 18 hours at a constant rate of 1 ml./hr (equivalent to about 150 mg protein infused/hr). The radioactivity in 1 ml. portions of hourly lymph collections was measured and the ratio of protein:non-protein radioactivity was estimated. Although less than 2% of the radioactivity in the infused solution was soluble in the scintillator, up to 63% of the total radioactivity in the lymph was soluble, indicating possible breakdown of the labelled protein. The maximum rate of absorption of intact protein, estimated from the protein-bound radioactivity in the lymph, was 14% of the rate of infusion and this was reached only at the end of the infusion (Fig. 23).

The rate of flow of lymph from the thoracic duct of this foetus was 3-4 times higher than that measured subsequently in any other foetus. There was no noticeable
The transport of protein-bound radioactivity (o) in the thoracic duct lymph of a foetal lamb (F3/67), infused intraduodenally for 18 hours at a rate of 1 ml/hr with a solution of 125I-γ-globulin in saline (equivalent to about 150 mg of protein infused per hour). The rate of transport of labelled protein in the lymph, expressed as a percentage of the rate of infusion, is shown plotted against time. The volume of lymph produced each hour (x) is also shown plotted against time.
increase in the flow rate as a result of the infusion (Fig. 23).

Similar experiments were carried out with two more foetuses (F2/68, F3/68) to confirm that the foetal intestine can absorb intact γ-globulin from a solution of the protein in saline. The two infusions were made as similar as possible, with the same amount of \(^{125}\text{I-}\)γ-globulin added to each infusion. The γ-globulin was infused continuously into the intestines of both foetuses for 20 hours at a rate of 4 ml./hr (4 times the rate used in the preceding experiment). The infused solution contained 157 mg of γ-globulin per millilitre, so the total amount of protein infused was greater than 12 grams, equivalent to the amount of γ-globulin in about 100 ml. of colostrum.

The time course of the rates of absorption of radioactivity into the lymph is shown in Fig. 24. Both foetuses reached a maximum rate of absorption of about 5% of the rate of infusion. However, F2/68 reached a maximum in only 15 hours compared with 20 hours for F3/68. The curve for F2/68 shows a plateau region from 15 to 20 hours, suggesting that some form of steady state had been reached, and the rate of absorption was maximal
The transport of protein-bound radioactivity in the thoracic duct lymph of two foetal lambs [F2/68 (o), F3/68 (x)], infused intraduodenally for 20 hours at a rate of 4 ml/hr with a solution of 125I-γ-globulin in saline (equivalent to about 630 mg of protein infused per hour). The rate of transport of labelled protein in the lymph, expressed as a percentage of the rate of infusion, is shown plotted against time. The volume of lymph produced each hour for each foetus [F2/68 (o), F3/68 (x)] is shown plotted against time.
for the conditions of the experiment. The level of non-protein-bound radioactivity in F2/68 (17%) was approximately half that in F3/68 (30%). This might have been related to differences in the maturity of the two foetuses since F3/68 was considerably larger, and from its crown-rump length was estimated to be 135 days post-conception, compared with 123 days for the smaller animal. About 83% of the radioactivity in the lymph of F2/68 was precipitable by 18% Na₂SO₄, which is in good agreement with the estimate of protein-bound radioactivity given above.

(ii) Analysis of foetal lymph during absorption of ¹²⁵I-γ-globulin

In order to confirm that the protein-bound radioactivity present in the thoracic duct lymph was associated with intact γ-globulin, samples from one of the above foetuses (F3/68) collected throughout the infusion, were examined by agar gel immunoelectrophoresis. The precipitin arc corresponding to fast IgG was first evident 6 hours after the infusion began (Fig. 25) and significant amounts of radioactivity appeared in the lymph at about the same time. The concentration of fast IgG appeared to increase steadily up to the end of the infusion. Slow IgG was detectable after 8-10 hours and appeared to reach a maximum concentration after about 20 hours.
Fig. 25  Agar gel immunoelectrophoretograms obtained from the thoracic duct lymph of a foetal lamb (F3/68) which was infused intraduodenally for 20 hours at a rate of 4.5 ml./hr with a solution of 125I-γ-globulin dissolved in physiological saline. The precipitin arc corresponding to fast IgG appears after 6 hours infusion and increases in intensity with increased time of infusion. The slow IgG arc only appears after 8-10 hours.
<table>
<thead>
<tr>
<th>Hours of infusion</th>
<th>+</th>
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<th></th>
<th></th>
<th>-</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>10</td>
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</tbody>
</table>
Fig. 26  Gel filtration of foetal lymph obtained during the intraduodenal infusion of γ-globulin. The Sephadex G-200 column was pumped at a constant rate of 5 ml./hr, and the optical density at 280 mµ of the effluent, on an arbitrary scale, is shown plotted against time. Three fractions (1, 2 and 3) were taken as indicated by the broken lines, and fractions 2 and 3 were characterized by agar gel immunoelectrophoresis, the results of which are shown.
Three samples of lymph from 12/20, taken 15, 16, and 17 hours after the beginning of the infusion, were pulsed to give 22 ml. of labelled lymph. This was applied to a Sephadex G-200 column (3.2 cm. I.D., 70 cm. length) equilibrated with 0.02 M Tris buffer pH 8.4, and eluted with the same buffer. The sample and eluting buffer were driven upward through the column at a constant rate.

The effluent from the column was monitored continuously at 280 mp if three peaks obtained were separated and each fraction concentrated to a volume of 10 ml. (Diaflo apparatus, Amicon). The radioactivity in 1 ml of these fractions was measured, and 65% of the radioactivity eluted to the column was recovered in fractions 2, 3, and 3 (23a). No radioactivity was found in fraction 1.

The solutions from fractions 2 and 3 were subjected to agar gel immunoelectrophoresis and it was shown that fraction 2, which contained a protein proportionally of the radioactivity recovered, was predominantly fast SDS while fraction 3 contained albumin, and several proteins moving in the β-globulin region (Fig. 36).

(iii) Fate of protein not absorbed into the lymph

The two foetuses used in the previous experiments...
Three samples of lymph from F2/68, taken 15, 16 and 17 hours after the beginning of the infusion, were bulked to give 22 ml. of labelled lymph. This was applied to a Sephadex G-200 column (3.2 cm. I.D. x 1 m length) equilibrated with 0.02 M Tris buffer pH 8.4, and eluted with the same buffer. The sample and eluting buffer were driven upwards through the column at a constant rate of 5 ml./hr. The effluent from the column was monitored continuously at 280 mÅ (Fig. 26), and the three peaks obtained were separated and each fraction concentrated to a volume of 10 ml. (DiaFlo apparatus, Amincon). The radioactivity in 1 ml. portions of these fractions was measured, and 68% of the radioactivity applied to the column was recovered in fractions 2 (45%) and 3 (23%). No radioactivity was found in fraction 1.

The solutions from fractions 2 and 3 were subjected to agar gel immunoelectrophoresis which showed that fraction 2, which contained a large proportion of the radioactivity recovered, was predominantly fast IgG, while fraction 3 contained albumin, and several proteins moving in the β-globulin region (Fig. 26).

(iii) Fate of protein not absorbed into the lymph

The two foetuses used in the previous experiments
were killed at the end of the experiments and samples of the foetal fluids and maternal serum were taken for radioassay. The amniotic and allantoic fluids were homogenized, made to a known volume, and the radioactivity in 1 ml samples was measured. The amount of radioactivity found in the fluids was about 3% of the total infused for F2/68, and about 10% for F3/68, most of it as non-protein-bound radioactivity. A further 12% of the infused radioactivity was recovered in various organs of F3/68. The gut and its contents accounted for 3% of the total radioactivity infused, the plasma 3%, and the thyroid 6%, and in each case most of the radioactivity was non-protein-bound. Therefore including the output in the lymph, only about 25-30% of the total infused radioactivity was recovered. The activity in the maternal serum was negligible, so it is unlikely that any intact protein, which may have been present in the foetal circulation, was lost across the placenta.

Other possible routes of absorption

The results presented above suggested that some of the \( \gamma \)-globulin infused was absorbed intact by the foetal intestine and transported in the thoracic duct lymph. However, it was not known whether the thoracic duct was
the sole route of transport, or whether some of the
γ-globulin present in the thoracic duct lymph had leaked
from the blood after being absorbed by another route.
Therefore a solution of $^{125}$I-γ-globulin in saline was
infused intravenously for 20 hours at the same rate of
4 ml./hr into one foetal lamb (F4/68). In this lamb
the thoracic duct was cannulated in two directions; one
cannula was used for intravenous infusion and one for
the collection of thoracic duct lymph. With the exception
of a period of about four hours in the middle of the
infusion, the radioactivity present in the lymph increased
rapidly throughout the infusion, reaching a level of 17%
of the amount of radioactivity infused per hour (Fig. 27).
Thirty hours after the infusion had ended the output was
still high, equivalent to over 14% of the infusion rate.
In all samples the level of non-protein radioactivity was
less than 2% of the total.

This result was very different from those obtained
in the previous experiments with respect to the rate of
increase in the radioactivity in the lymph, the rate of
decrease in the radioactivity in the lymph after the
infusion ended, and the amount of non-protein-bound
radioactivity present in the lymph. This evidence does
not support the hypothesis that the labelled protein appearing in the lymph during infusion into the intestine came from the circulation and it is concluded that the protein absorbed by the intestinal epithelium is transferred directly to the lymphatics in a manner similar to that in the newborn animal.

In this experiment, the amount of protein in the fibrin-free lymph which was insoluble in 18% Na₂SO₄ was measured throughout the experiment. The variation of the amount of protein absorbed obtained from the infusion of the lymph was also shown plotted against time. This content supports the hypothesis that the protein absorbed by the intestinal epithelium is transferred directly to the lymphatics in a manner similar to that in the newborn animal.

Factors influencing the absorption of protein from the foetal intestine

Fig. 27 The transport of protein-bound radioactivity (●) in the thoracic duct lymph of a foetal lamb (F4/68) infused intravenously for 20 hours at a rate of 4 ml/hr with a solution of 125I-γ-globulin in saline. The rate of transport of labelled protein in the lymph, expressed as cpm/hr x 10⁻⁴, is shown plotted against time. The concentration in the lymph of protein precipitable by 18% Na₂SO₄ (x), is also shown plotted against time.
not support the hypothesis that the labelled protein appearing in the lymph during infusion into the intestine comes from the circulation, and it is concluded that the protein absorbed by the intestinal epithelium is transferred directly to the lymphatics in a manner similar to that in the newborn animal.

In this experiment, the amount of protein in the fibrin-free lymph which was insoluble in 18% $\text{Na}_2\text{SO}_4$ was measured throughout the infusion. The estimates of the amount of protein absorbed obtained by this method compare favourably with the radioactivity measurements (Fig. 27). In later experiments, precipitation by 18% $\text{Na}_2\text{SO}_4$ was used routinely to estimate the amount of protein absorbed, and to relate this to the measurements of radioactivity in the lymph.

Factors influencing the absorption of protein from the foetal intestine

The effects of electrolytes on absorption

The time taken for the rate of absorption of $\gamma$-globulin from the gut of the foetal lamb to become maximal in the preceding experiments was certainly longer than in newborn lambs. Also, the maximum rate of absorption of the infused protein into the lymph was only about 5% of the rate of
infusion, less than the minimal estimates of absorption of γ-globulin from colostrum by newborn lambs, given in the preceding chapter. To test whether different electrolytes would increase the amount absorbed, γ-globulin dissolved in the solution found to be 'optimal' for absorption in the anaesthetized calf (Balfour and Comline, 1962), was infused intraduodenally into a number of foetuses. The infusion of this solution resulted in both a more rapid appearance of radioactivity in the lymph, and a higher maximum rate of absorption, than in the foetuses infused with the same protein dissolved in saline (Fig. 28). A peak level of absorption was reached after only 7 hours infusion, with a mean of about 11% of the radioactivity infused. Individual levels varied from 7 to 20%, and this variation between foetuses was possibly due to differences in age. The amount of non-protein-bound radioactivity in the lymph was lower than for previous infusions, and varied from 2-5% of the total radioactivity. The protein profiles obtained by agar gel immunoelectrophoresis confirmed that there was a difference in the rate of appearance of γ-globulin in the lymph (Fig. 29).
Fig. 28  The effect of electrolytes and colostrum whey on the rate of absorption of $^{125}\text{I-} \gamma$-globulin by the foetal intestine. The rate of transport of labelled protein in the lymph, expressed as a percentage of the rate of infusion, is shown plotted against time.

- $^{125}\text{I-} \gamma$-globulin in physiological saline (mean of 3 infusions)
- $^{125}\text{I-} \gamma$-globulin in an 'optimal' electrolyte solution (mean of 3 infusions)
- $^{125}\text{I-} \gamma$-globulin in colostrum whey (mean of 2 infusions).
Agar gel immunoelectrophoretograms obtained from the thoracic duct lymph of a foetal lamb which was infused intraduodenally for 20 hours at a rate of 4.5 ml./hr with a solution of $^{125}\text{I}-\gamma$-globulin dissolved in an 'optimal' electrolyte solution. The precipitin arc corresponding to fast IgG appears in the lymph in less than 4 hours, and slow IgG is detectable after 6-8 hours of the infusion. The $\gamma$-globulins are present in the lymph in detectable concentrations after a much shorter time than when the protein is dissolved in physiological saline (cf. Fig. 25).
The effects of prolonged infusion of $\gamma$-globulin on absorption.

24 hours of infusion of foetal lamb was infused with $\gamma$-globulin to determine whether a high protein load or the continued absorption of protein could initiate the cessation of absorption. The solution of $\gamma$-globulin was infused at a rate of 3 ml/hr (1.4 g of $\gamma$-globulin/hr). The rate of absorption of radioactivity in the lymph reached a plateau level of about 7% of the radioactivity infused per hour after 8 hours (Fig. 3). Thereafter the rate of absorption fluctuated between 7 and 10% of the rate of infusion, but there was no sign of any decrease in the rate of absorption over the 24-hour period. The protein in the lymph precipitable by 1.8% $\text{Na}_2\text{SO}_4$ (Fig. 3) showed that the radioactivity measurements reflected the absorption of the protein. The animal was given peritoneal adrenocorticotropic hormone (ACTH) subcutaneously as part of a later experiment, also showed no decrease in the rate of absorption per hour in the presence (Fig. 35). Therefore it is unlikely that the absorption
The effects of prolonged infusion of $\gamma$-globulin on absorption

One foetal lamb was infused with $\gamma$-globulin continuously for a prolonged period to determine whether a high protein load or the continued absorption of protein could initiate the cessation of absorption. The solution of $\gamma$-globulin was infused at a rate of 9 ml./hr (1.4 g of $\gamma$-globulin/hr) for 33 hours. The rate of absorption of radioactivity in the lymph reached a plateau level of about 7% of the radioactivity infused per hour after 8 hours (Fig. 30). This was equivalent to the absorption of 100 mg protein/hr. Later, the rate of absorption fluctuated between 7 and 10% of the rate of infusion, but there was no sign of any decrease in the rate of absorption during the 33 hours of the infusion. The estimation of the protein in the lymph precipitable by 18% Na$_2$SO$_4$ (Fig. 30) showed that the radioactivity measurements reflected the amount of protein absorbed.

Another foetus, which was given porcine adrenocorticotrophic hormone (ACTH) subcutaneously as part of a later experiment, also showed no decrease in the rate of absorption during a 48 hour infusion (see Fig. 35). Therefore it is unlikely that the absorption
The transport of protein-bound radioactivity (●) in the thoracic duct lymph of a foetal lamb (F21/68) infused intraduodenally for 33 hours at a rate of 9 ml./hr with a solution of 125I-γ-globulin in an 'optimal' electrolyte solution (equivalent to about 1.4 g protein infused per hour). The rate of transport of labelled protein in the lymph, expressed as a percentage of the rate of infusion, is shown plotted against time. The amount of protein precipitable by 18% Na₂SO₄ (x), transported in the lymph each hour, is also shown plotted against time.
of protein for long periods is sufficient to initiate the cessation of absorption.

The effects of products of protein hydrolysis

Smith and Pierce, (1967) showed that the presence of L-leucine and L-methionine inhibited the uptake of protein by the intestinal epithelium of the newborn piglet in vitro. Therefore, in order to test whether the products of protein digestion might inhibit the absorption of protein by the intestinal epithelium in vivo, the effect of adding amino acids to the infusion solution was measured in 3 foetal lambs. In the first, a solution of $^{125}\text{I-\gamma-globulin}$ was infused at 4.5 ml./hr for 4 hours (700 mg protein/hr) into the duodenum of a foetal lamb (F23/68), and then the infusion was continued for a further 6 hours with the same solution to which L-leucine (10mM) had been added. The rate at which the radioactive protein transported in the lymph increased, decreased coincident with the addition of L-leucine to the infused solution (Fig. 31), and the maximum percentage of the infused radioactivity in the lymph (8%) was not reached until 2 hours after the infusion ended, i.e. 12 hr after the infusion commenced. This result suggests that L-leucine at a concentration of 10 mM might reduce the absorption of protein to some extent.
The effect of a protein hydrolysate ("Protogast", Burroughs Wellcome, 8% protein hydrolysate) on absorption was measured in 3 foetuses each given 2 infusions as shown in Table 12.

Fig. 31

The effect of L-leucine on the absorption of protein. The transport of protein-bound radioactivity (●) in the thoracic duct lymph of a foetal lamb (F23/68) infused intraduodenally for 10 hours at a rate of 4.5 ml./hr with a solution of 125I-γ-globulin in an 'optimal' electrolyte solution. L-leucine (10 mM) was added to the infused solution for the last 6 hours of the infusion (+). The rate of transport of labelled protein in the lymph, expressed as a percentage of the infusion, is shown plotted against time.
The effect of a protein hydrolysate ("Protogest", Burroughs Wellcome, 8% protein hydrolysate) on absorption was measured in 2 foetuses each given 2 infusions as shown in Table 12.

Table 12.

<table>
<thead>
<tr>
<th>Foetal lamb</th>
<th>Infusion</th>
<th>Solution infused</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1/69</td>
<td>1</td>
<td>γ-globulin solution (50 ml.) + Electrolyte solution (25 ml.)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>γ-globulin solution (50 ml.) + &quot;Protogest&quot; (25 ml.)</td>
</tr>
<tr>
<td>F2/69</td>
<td>1</td>
<td>Colostrum whey (50 ml.) + &quot;Protogest&quot; (25 ml.)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Colostrum whey (50 ml.) + Electrolyte solution (25 ml.)</td>
</tr>
</tbody>
</table>

The two infusions were given for 6 hours each at an infusion rate of 12 ml./hr, the second beginning 18 hours after the first infusion ended. The amount of radioactivity present in the lymph of both foetuses throughout the experiment is shown in Fig. 32, and there does not appear to have been any inhibition of protein absorption in either foetus under the conditions of the experiment. The percentage of the infused radioactivity which was absorbed was lower than in most other foetuses, but these were
Fig. 32 The effect of a protein hydrolysate on the absorption of γ-globulin by the foetal lamb. The transport of protein-bound radioactivity in the thoracic duct lymph of 2 foetal lambs [F1/69 (x), F2/69 (o)], given two successive infusions, 18 hours apart, at a rate of 12 ml./hr, of different solutions containing 125I-γ-globulin, according to Table 12. The rate of transport of labelled protein in the lymph, expressed as a percentage of the rate of infusion, is shown plotted against time.
infused with smaller volumes of more concentrated solutions of γ-globulin per hour. The rate of infusion of fluid, and the concentration of protein in it, might be more important than products of protein digestion in determining the rate at which protein is absorbed by the foetal intestine.

Absorption of \(^{125}\)I-γ-globulin in colostrum whey

In order to compare the rate of absorption of serum γ-globulin from the 'optimal' electrolyte solution with the rate of absorption of γ-globulin from colostrum whey, one foetus (F24/68) was given 2 separate infusions of colostrum whey 63 hours apart, for 20 hours each. The whey contained 140 mg/ml. of protein and was infused at a rate of 4.5 ml./hr, equivalent to 630 mg protein infused per hour. The radioactivity appearing in the lymph during both infusions followed a similar time course for the first 10 hours of each infusion, and this time course was similar to those obtained when γ-globulin, dissolved in the 'optimal' electrolyte solution, was infused into other foetuses in previous experiments (Fig. 28). Although the rate of absorption of γ-globulin dissolved in the electrolyte solution reached a maximum within 10 hours infusion, the rate of absorption of the γ-globulin in colostrum whey continued to increase throughout the
Fig. 33 The transport of protein-bound radioactivity (●) in the thoracic duct lymph of a foetal lamb (F24/68) given 2 successive intraduodenal infusions at a rate of 4.5 ml/hr of colostrum whey containing 125I-γ-globulin (equivalent to 630 mg protein infused per hour). The rate of transport of labelled protein in the lymph, expressed as a percentage of the rate of infusion, is shown plotted against time. The amount of protein in the lymph precipitable by 18% Na₂SO₄ (x), expressed as a percentage of the rate of infusion, is also shown plotted against time.
first infusion, from 15% of the rate of infusion at 10 hours to 22% at 20 hours (Fig. 33). The amount of protein precipitable by 18% Na$_2$SO$_4$ increased in a similar manner to the radioactivity in the lymph. During the second 10 hours of the second infusion, L-leucine (100 mM) was added to the colostrum whey infused, and the rate of absorption continued to increase for about 1 hour, and then decreased from 17% at 11 hours to 14-15% at 20 hours. It is possible that this decrease in the rate of absorption was due to the L-leucine.

The similar rate of absorption in the first 10 hours of each infusion suggests that factors in colostrum whey are unlikely to initiate the cessation of absorption.

The effects of steroid hormones on protein absorption

Halliday (1959) showed that comparatively large doses of cortisone acetate and deoxycorticosterone acetate (DOCA) caused newborn rats to cease absorbing intact protein at a younger age than control animals. This work suggested that steroid hormones might initiate the cessation of protein absorption. In order to investigate whether or not corticosteroids affected protein absorption in the foetal lamb, 50 mg DOCA in 5 ml. of oil was infused over a 2 hour period into the duodenum of a foetal lamb (Fl8/69),
and a solution of $^{125}$I-$\gamma$-globulin was infused 19 hours later. The amount of protein-bound radioactivity, and the amount of protein precipitable by 18% Na$_2$SO$_4$, present in the lymph during the infusion is shown by Fig. 34. The amount of protein absorbed was less than for other foetuses given similar infusions and the results resembled those obtained with infusions of $^{125}$I-$\gamma$-globulin dissolved in saline. It is possible that absorption of protein was delayed by the DOCA infusion and it could not be determined whether or not this was possible. If DOCA persisted because the lambs were born 20 hours after the infusion of the steroid, the third cannulae pulled out during delivery. DOCA in this way was born in the way the steroid was administered, before any protein was infused.

![Graph](image)

**Fig. 34** The transport of total radioactivity (•) and protein-bound radioactivity (o) in the thoracic duct lymph of a foetal lamb (FL8/69), infused intraduodenally with 50 mg DOCA, followed 4 hours later by an infusion of $^{125}$I-$\gamma$-globulin in an 'optimal' electrolyte solution for 19 hours at a rate of 4.5 ml./hr (equivalent to about 700 mg protein infused per hour). The rates of transport of labelled protein and total radioactivity in the lymph, expressed as a percentage of the rate of infusion, are shown plotted against time. The amount of protein in the lymph precipitable by 18% Na$_2$SO$_4$ (x), expressed as a percentage of the rate of infusion, is also shown plotted against time.
and a solution of $^{125}\text{I-}\gamma\text{-globulin}$ was infused 2 hours later. The amount of protein-bound radioactivity, and the amount of protein precipitable by 18% Na$_2$SO$_4$, present in the lymph during the infusion are shown in Fig. 34. The amount of protein absorbed was less than for other foetuses given a similar infusion and the results resembled those obtained with infusions of $^{125}\text{I-}\gamma\text{-globulin}$ dissolved in saline. It is possible that absorption of protein was delayed by the DOCA infusion. It could not be determined whether or not this possible effect of DOCA persisted because the lamb was born 24 hours after the infusion of the steroid, and both cannulae pulled out during delivery. Another foetus which was given 50 mg DOCA in the same way was born 20 hours after the steroid was administered, before any protein was infused.

In two other experiments, a preparation of porcine ACTH in gelatin (C.S.L.) was given subcutaneously. One lamb was born within 7 hours of being given 1 unit of ACTH. Another (F11/69), which was infused subcutaneously with ACTH at a rate of 1 unit/day for 2 days, was born after the end of this infusion, after 70 hours of lymph collection. This foetus was infused with $^{125}\text{I-}\gamma\text{-globulin}$ at a rate of 4.5 ml./hr for 48 hours: The infusion was
Fig. 35 The transport of protein-bound radioactivity in the thoracic duct lymph of a foetal lamb (F11/69), infused intraduodenally for 48 hours at a rate of 4.5 ml./hr with 125I-γ-globulin in an 'optimal' electrolyte solution (equivalent to about 700 mg of protein infused per hour). The rate of transport of labelled protein in the lymph, expressed as a percentage of the rate of infusion, is shown plotted against time. The amount of protein in the lymph precipitable by 18% Na₂SO₄ (o), expressed as a percentage of the rate of infusion, is also shown plotted against time. The drop in the rate of transport between 6 and 10 hours was caused by the ewe lying down and obstructing lymph flow in the cannula. From 12 hours (arrow), porcine ACTH was infused subcutaneously at a rate of 1 unit per day.
begun 12 hours before the infusion of ACTH, and continued throughout most of the infusion of ACTH. The γ-globulin was absorbed at a rate of 16-23% of the rate of infusion until the end of the infusion, and there was no evidence for a decrease in the rate of absorption as a result of the ACTH given (Fig. 35).

**Postnatal absorption of $^{125}$I-γ-globulin by lambs born with thoracic duct fistulae**

Three of the lambs which were born with intact thoracic duct fistulae after varying periods of lymph drainage were used to study the absorption of $^{125}$I-γ-globulin by the intestine after birth. Two of the lambs, F20/68 and F23/68, had each been infused intraduodenally for 9-10 hours in utero with a solution of $^{125}$I-γ-globulin, each lamb receiving about 7 g of protein. During these infusions, the rate of absorption of protein-bound radioactivity in the thoracic duct lymph of each foetus reached a level of about 7% of the rate of infusion. The absorption of radioactive γ-globulin from a single feed of 100 ml. of colostrum, fed orally 1-2 hours after birth, did not appear to be reduced by the absorption of γ-globulin in utero (Fig. 36). About 20% of the radioactivity in the colostrum fed to F20/68 was recovered
The transport of protein-bound radioactivity in the thoracic duct lymph of 2 lambs [F20/68 (a), F23/68 (b)] after a single feed of 100 ml. of colostrum containing 125I-γ-globulin given 1-2 hours after birth. The radioactivity in the lymph (x), expressed as cpm/hr, is shown plotted against time. Both lambs had absorbed γ-globulin prior to birth from an intraduodenal infusion of 125I-γ-globulin.
as protein-bound radioactivity during the 7 hours after feeding, and about 28% of the amount fed to F23/68 was recovered in the 10 hours after feeding. Nearly all the radioactivity present in the lymph was protein-bound (>90%). From Fig. 36 there does not appear to be much delay between feeding and the appearance of absorbed protein in the lymph, so that the coagulation of the colostrum casein does not trap the colostrum whey in the abomasum for any significant period. The radioactivity in the lymph reaches a maximum level between 4 and 7 hours after feeding and then the level appears to decline rapidly.

In the third lamb given colostrum containing $^{125}\text{I}$-γ-globulin (F9/69), only about 3% of the radioactivity fed was recovered in the thoracic duct lymph. This lamb had not been infused previously, but had been given 1 unit of porcine ACTH subcutaneously 7 hours before the lamb was born and 11 hours before the labelled colostrum was fed. It appears that the ACTH may have reduced the absorptive capacity of the intestine of this lamb.

Summary of results

Foetal lambs with thoracic duct fistulae appear to remain physiologically normal for at least 48 hours after
surgery, and viable lambs have been born after lymph has been drained from their thoracic ducts for several days whilst in utero. The identification of the labelled proteins in thoracic duct lymph subsequent to their infusion into the gut, has shown that intact protein can be absorbed from the lumen of the foetal intestine.

The rate of absorption of γ-globulin from the foetal intestine has been shown to be influenced by several factors. When γ-globulin dissolved in physiological saline is infused, the rate of absorption appears to be lower than when the protein is infused dissolved in either a suitable electrolyte solution containing potassium and phosphate, or colostrum whey. The addition of L-leucine to the infused solution appears to reduce slightly the rate of absorption, but the rate of absorption is not affected by large amounts of protein hydrolysate. The administration of DOCA to the foetal lamb, as well as apparently hastening its delivery, also appears to delay protein absorption and decrease the rate of absorption. However, the rate of absorption is not affected appreciably by the infusion of large amounts of protein, or the prolonged absorption of protein. Nor does the infusion of γ-globulin into the intestine in utero influence postnatal
absorption, since lambs which had absorbed large amounts of γ-globulin in utero and were born with intact thoracic duct fistulae, rapidly absorbed γ-globulin from an oral dose of colostrum, given soon after birth.

The absorption of γ-globulin in the fetal lamb

In an early experiment, a fetal lamb which had been given a continuous intraduodenal infusion of a solution of γ-globulin at 4.5 ml/hr for 11 hours was killed, and successive sections of portions of the small intestine were

exposed with fluorescent antibodies to sheep γ-globulin, and examined under ultraviolet light. Fluorescent material
CHAPTER 6.
MORPHOLOGICAL STUDIES RELATING TO \( \gamma \)-GLOBULIN
ABSORPTION IN THE LAMB

The results presented in the previous chapter have shown that the intestine of the foetal lamb can transport \( \gamma \)-globulin in considerable quantities. In order to investigate whether this absorption is analogous to that which occurs in the newborn lamb after the ingestion of colostrum, some morphological aspects of the absorption of protein in utero, and following birth, were studied. Some of the changes which take place in the intestine after birth were also examined to see whether the cessation of protein absorption could be related to a morphological change in the intestine.

Absorption of \( \gamma \)-globulin in the foetal lamb

The demonstration of \( \gamma \)-globulin in the intestinal epithelium

In an early experiment, a foetal lamb which had been given a continuous intraduodenal infusion of a solution of \( \gamma \)-globulin at 4.5 ml./hr for 11 hours was killed, and frozen sections of portions of the small intestine were incubated with fluorescent antibodies to sheep \( \gamma \)-globulin, and examined under ultraviolet light. Fluorescent material
Immunofluorescent studies of foetal intestinal epithelium during the absorption of $\gamma$-globulin. Paraffin sections of intestine, fixed in 95% alcohol, from a foetal lamb (F7/68) which had been infused with $^{125}$I-$\gamma$-globulin.

(a) A section which had been incubated with fluorescein-conjugated antibodies to chicken $\gamma$-globulin, showing that the only cells which fluoresced under U-V light were eosinophilic cells scattered in the tissue.
Magnification: 130 x.

(b) A section from the same lamb which had been incubated with fluorescein-conjugated antibodies to sheep $\gamma$-globulin, showing the areas on the margins of the villi which fluoresced under U-V light.
Magnification: 130 x.

(c) A higher power photomicrograph of the same section, showing that most of the fluorescence appears to be concentrated in vacuoles.
Magnification: 600 x.
could be seen in the intestinal villi, but the tissue structure was preserved poorly, and representative sections from different regions of the intestine were difficult to obtain. Therefore in subsequent experiments, the method of Sainte-Marie (1962), using alcohol fixation and embedding in paraffin, was adopted for fluorescent antibody localization. The intestinal epithelium of a foetus which had been infused with \( \gamma \)-globulin for 11 hours contained vacuoles, the contents of which bound fluorescent antibodies to sheep \( \gamma \)-globulin. These were similar to those seen in the newborn piglet which had been fed fluorescent antibodies (Payne and Marsh, 1962b). There was no similar fluorescence in sections from control animals, or in sections which had been incubated with fluorescent antibodies to chicken \( \gamma \)-globulin, showing that the test was specific for sheep \( \gamma \)-globulin (Fig. 37). The distribution of the vacuoles along the gut suggested that the central portion of the small intestine was responsible for most of the absorption of \( \gamma \)-globulin.

In order to study the vacuoles in more detail and to obtain a better picture of the position of the vacuoles in the tissue, serial sections from the same foetus were either incubated with fluorescent antibodies, or stained
Fig. 38 A comparison of serial paraffin sections taken from the intestine of a foetal lamb (F7/68), which had been infused intraduodenally with $^{125}\text{I}-\gamma$-globulin, and then segments of the intestine fixed in 95% alcohol. The sections were treated by two methods to identify the $\gamma$-globulin in the tissue.

(a) A section which had been incubated with fluorescein-conjugated antibodies to sheep $\gamma$-globulin, photographed under U-V light, showing the localization of fluorescence in the margins of some of the villi. Magnification: 130 x.

(b) A similar section which had been stained with haematoxylin-eosin, showing densely-staining vacuoles in the areas which contained fluorescent material in (a) above. Magnification: 130 x.

(c) A higher power photomicrograph of the inset from (b), showing more clearly the densely-staining vacuoles (v) present in the epithelial cells. Magnification: 330 x.
with two histological stains, haematoxylin-eosin or Mallory's stain (Fig. 38). Not only did the stained sections show the histology of the tissue more clearly, but the vacuoles which bound fluorescent antibodies were found to be stained orange-red with haematoxylin-eosin and green with Mallory's stain, making them easily identifiable in the sections. The intestinal epithelium from control foetuses which had not been infused with γ-globulin did not contain any such vacuoles, and the cytoplasm of the cells took up very little stain. These observations showed that stained paraffin sections could be used to demonstrate the presence of γ-globulin in the intestinal epithelium of foetal lambs which had been infused with γ-globulin, and in later studies this method was used routinely.

**Distribution of γ-globulin in the gut of foetal lambs during and after the intraduodenal infusion of $^{125}$I-γ-globulin**

Foetuses in which only duodenal fistulae had been established were infused with $^{125}$I-γ-globulin solution at a rate of 4.5 ml./hr for 2, 4, 6 and 11 hours. The animals were killed immediately afterwards, and portions of the intestine were taken at 1 m intervals along the
gut and fixed and stained as described above. Four other foetuses, each of which had been infused intraduodenally for 10 hours with $^{125}_I\gamma$-globulin were killed at 8, 14, 38 and 60 hours after the end of the infusion, and segments of intestine were fixed and stained for histological examination.

Segments of intestine, 5 cm in length, were taken at 30 cm intervals along the gut from all the foetuses infused as described above, and the radioactivity in the segments was measured.

(i) **Morphological assessment of the kinetics of accumulation of $\gamma$-globulin in the epithelium**

Densely-staining vacuoles could be demonstrated in the gut after 4 hours infusion with $\gamma$-globulin. These vacuoles increased in number and size with increasing time of infusion, but were usually most numerous in the mid-portion of the gut (Fig. 39, Table 13). These results show that even when the protein had been infused for relatively short periods of time, some of it had reached the distal region of the small intestine. The vacuoles are initially located in the apical region of the epithelial cells. As the vacuoles increase in number and size the nuclei are displaced basally. At later times, some vacuoles can be
Fig. 39 Photomicrographs of sections of intestine from a foetal lamb which had been infused for 11 hours with $^{125}$I-γ-globulin, showing that the concentration of densely-staining vacuoles (v) in the epithelium increases as the distance from the pylorus increases. The tissue was fixed in 95% alcohol, and stained with haematoxylin-eosin.
(a) 1 metre distal to the pylorus.
(b) 2 metres distal to the pylorus.
(c) 3 metres distal to the pylorus.
(d) 4 metres distal to the pylorus.
Magnification: 330 x.
Photomicrographs of paraffin sections of the intestines of 4 foetal lambs, taken from the same region of the intestine (3 m from the pylorus) at different times after 10-11 hour infusions. The concentration of the densely-staining vacuoles (v) in the epithelium can be seen to decrease with increasing time after the infusion. The tissue was fixed in 95% alcohol and stained with haematoxylin-eosin.
(a) Immediately after an 11 hour infusion.
(b) 8 hours after a 10 hour infusion.
(c) 14 hours after a 10 hour infusion.
(d) 38 hours after a 10 hour infusion.
Magnification: 330 x.
seen basal to the nuclei, which are displaced back towards the apex of the cells. Most of the densely-staining vacuoles had disappeared from the epithelium by 14 hours after the end of the infusion of γ-globulin, and none were present after 38 hours (Fig. 40, Table 13). These results give some idea of the rate at which protein is released from those cells which contain large amounts of absorbed protein in these vacuoles.

Table 13. The relative numbers of vacuoles present in sections of the intestines of foetal lambs taken after different periods of infusion, and at different times after a 10 hour infusion. The results are scored as no vacuoles present (−), or present in increasing numbers (+, ++, etc.)

<table>
<thead>
<tr>
<th>Segment</th>
<th>Period of infusion (hours)</th>
<th>Hours after 10 hour infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 4 6 11</td>
<td>8 14 38 60</td>
</tr>
<tr>
<td>1</td>
<td>− − ++ −</td>
<td>+ − − −</td>
</tr>
<tr>
<td>2</td>
<td>− − + ++</td>
<td>+++ + − −</td>
</tr>
<tr>
<td>3</td>
<td>− + − +++</td>
<td>+++ ++ − −</td>
</tr>
<tr>
<td>4</td>
<td>− + + ++++</td>
<td>+ − − −</td>
</tr>
<tr>
<td>5</td>
<td>− − − −</td>
<td>− − − −</td>
</tr>
</tbody>
</table>

(ii) Distribution of radioactivity in the gut

As the time of infusion increased, the amount of radioactivity present in segments of tissue taken from the mid-portion of the small intestine increased (Fig. 41).
Fig. 41 The amount of radioactivity in 5 cm segments of the small intestines of foetal lambs after 2 (o), 4 (+), 6 (●), and 11 (x) hours intraduodenal infusion with $^{125}$I-γ-globulin.
The radioactivity at either end of the intestine was much lower than in the central region: most of the radioactivity had been removed from the gut within 16 hours of the end of the 10-hour infusion, and by 38 hours there was no detectable radioactivity in the tissues (Fig. 42). These findings corroborate the histological observations and other evidence to support the proposal that the polypeptides present in the epithelium contain γ-globulin. 

The structure of the intestinal mucosa during the absorption of γ-globulin by foetal and newborn lambs has been described in several species. Carless (1984) showed that the newborn rat can absorb a variety of proteins and colloids, these substances could be identified in vacuoles in the granules of the epithelial cells, apparently taken into the cell by phagocytosis. Recently, Waring and Grunenfeld (1988) described in rat, the central portion of the epithelial cells filled by a giant vacuole which contains some degenerated lamellae in a poorly staining matrix. In the intestine, Day et al. (1983) have shown that γ-globulin absorption of 125I-γ-globulin intraduodenally.
The radioactivity at either end of the intestine was much less than in the central region. Most of the radioactivity had been removed from the gut within 14 hours of the end of a 10 hour infusion, and by 38 hours there was no detectable radioactivity in the tissue (Fig. 42). These findings corroborated the histological observations and added further evidence to support the proposal that the vacuoles present in the epithelium contain $\gamma$-globulin.

The fine structure of the intestinal mucosa during the absorption of $\gamma$-globulin by foetal and newborn lambs

Changes in the ultrastructure of the intestinal epithelium during $\gamma$-globulin absorption have been described in several species. Clark (1959) showed that when newborn rats and mice absorbed a variety of proteins and colloids, these substances could be identified in vacuoles in the cytoplasm of the epithelial cells, apparently taken into the cells by pinocytosis. Recently, Wissig and Graney (1968) demonstrated that in young rats absorbing $\gamma$-globulin, the central portion of the epithelial cells is filled by a giant vacuole which contains some homogeneous dense granules in a poorly staining matrix. In the piglet, Staley et al. (1969) have examined the intestinal mucosa during the absorption of $\gamma$-globulin and have described large
densely-staining vacuoles within the epithelial cells. Vacuoles containing granular material which stained less intensely were also present. In the work described below, the changes in the epithelial cells of the intestine during the absorption of \( \gamma \)-globulin by foetal and newborn lambs were studied by electron microscopy.

Intestinal segments from foetal lambs used in some of the experiments described previously, and from lambs at varying times after birth, were fixed in 1\% OsO_4, or 1.25\% glutaraldehyde, and resin sections were examined by light microscopy (ca. 1\( \mu \) sections) and in the electron microscope.

In the foetal lambs absorbing \( \gamma \)-globulin, large protein-filled vacuoles were present in the cytoplasm of the epithelial cells, their size and number varying with both the time of infusion of \( \gamma \)-globulin and the region of the gut examined. In 1\( \mu \) sections stained with Mallory's stain large, very densely staining vacuoles were present in the epithelium, in most cases situated above the nucleus (Fig. 43). Sections which had been stained with uranyl acetate and lead citrate were examined in the electron microscope, and the vacuoles stained with a uniformly electron opaque background, against which could be seen more dense inclusions, particularly near the periphery of
Fig. 43  Photomicrographs of resin sections (ca. 1µ) of intestine from a foetal lamb (F7/68), 3 metres from the pylorus, showing the large numbers of densely-staining vacuoles (v) in the epithelium. The tissue was fixed in 1.25% Glutaraldehyde, and the sections were stained with Mallory's stain. Magnification: (a) 330 x  (b) 1320 x.
The vacuoles were membrane-bound, though in some vacuoles the membrane appeared to be discontinuous.

The vacuoles were often rich and well-distended, similar to those seen in some collenchyma. In other vacuoles, a few vacuoles were displaced by the nuclei, as can be seen in the figure. The vacuoles indicated by the arrows were particularly prominent, suggesting a high concentration of glycogen granules in the cytoplasm which
the vacuoles (Fig. 44). The vacuoles were membrane-bound, though in some vacuoles the membrane appeared to be discontinuous (Fig. 44).

The intestinal epithelium of newborn lambs which had been given colostrum contained large numbers of vacuoles similar to those seen in the foetal lamb (Fig. 45). In some cells, the vacuoles practically filled the cytoplasm (Fig. 46). Many more vacuoles could be seen basal to the nuclei than was the case in the foetus, and when the vacuoles were in this position the nuclei were displaced apically, and in some cases could be seen almost at the apices of the cells.

Several features of the intestinal epithelial cells of the foetal and newborn lamb are worthy of comment. The apical region of the cells is characterized by a complex network of tubules (Figs. 47,48,49,50). These can be seen in control lambs as well as in those given γ-globulin or colostrum, but are virtually absent from the epithelial cells of a 4 day old lamb (Fig. 51). Invaginations of the cell membrane at the base of the microvilli are common, particularly during protein absorption (Fig. 48), and suggest that there is active pinocytosis. There is a high concentration of glycogen granules in the cytoplasm which
Fig. 44 A large protein-filled vacuole (v1) present in the apical region of an epithelial cell in the intestine of a foetal lamb, following a 10 hour infusion with a solution of γ-globulin. A smaller vacuole (v2) can be seen on the right. The vacuoles appear to be contained within a membrane which is discontinuous in some areas. Interdigitations (t) of the cell membranes of 2 adjoining cells are present. Fixation in 1% OsO₄ and staining with uranyl acetate and lead citrate. 8,800 x.
Fig. 45 A large protein-filled vacuole ($v_1$) in the apical region of an epithelial cell in the intestine of a newborn lamb during the absorption of protein from colostrum. Pinocytotic activity of the cell is suggested by the large number of vacuoles ($v_2$) and tubules (t) between the protein-filled vacuole and the microvilli (mv). Fixation in 1% OsO$_4$ and staining with uranyl acetate and lead citrate. 7,200 x.
Fig. 46 The basal region of three epithelial cells in the intestine of a lamb on the day of birth, following the ingestion of colostrum. The large, protein-filled vacuoles (v) are close to the base of the cells, with groups of mitochondria (m) between the vacuoles and the cell membrane. A small blood vessel (bv) can be seen in close proximity to the epithelial cells. Fixation in 1% OsO₄ and staining with uranyl acetate and lead citrate. 8,000 x.
Fig. 47 The apical region of an epithelial cell in the intestine of a foetal lamb. Many tubular and vesicular profiles are present (p), and an invagination of the plasma membrane (i) at the base of the microvilli (mv) can be seen. Fixation in 1% OsO₄ and staining with uranyl acetate and lead citrate. 20,000 x.
Fig. 48  The apical region of an epithelial cell in the intestine of a lamb on the day of birth during the absorption of protein from colostrum. A deep invagination of the plasma membrane (i) at the base of the microvilli (mv) is present, and many tubular profiles (p) can be seen. Fixation in 1% OsO₄ and staining with uranyl acetate and lead citrate. 38,000 x.
Fig. 49  A longitudinal section of the apical region of two epithelial cells in the intestine of a newborn lamb after the ingestion of colostrum. As well as large protein-filled vacuoles ($v_1$), numerous vacuoles containing little stained material ($v_2$) are present in the cells. These vacuoles suggest very active pinocytotic activity during the absorption of protein. Fixation in 1% OsO$_4$ and staining with uranyl acetate and lead citrate. 8,000 x.
Fig. 50  A transverse section of the apical regions of three epithelial cells in the intestine of a newborn lamb after the ingestion of colostrum. The complex network of vesicles and tubules filling the cytoplasm suggests intense pinocytotic activity. A grazing section of a protein-filled vacuole (v₁) can be seen in the photograph. Several desmosomes (d) are present at the junctions of the cell membranes. Fixation in 1% OsO₄ and staining with uranyl acetate and lead citrate. 12,000 x.
Fig. 51  The apical region of an epithelial cell in the intestine of a lamb 4 days after birth. There is no evidence for pinocytosis in this cell. The rootlets (r) of the microvilli (mv) can be seen in the terminal web (w) which contains only a few vesicles (v). Fixation in 1% OsO₄ and staining with uranyl acetate. 27,000 x.
disappear within a few days of birth. Desmosomes, tight junctions and interdigitations of the cell membranes of adjoining cells are present, and these features suggest that there is little opportunity for protein migration between cells, or for release of protein through the sides of the cells.

Changes in the intestine of the lamb after birth

Smith (1925) observed that in the foetal calf the intestinal epithelium contained large poorly-staining vacuoles, and these disappeared from the epithelium within 3 days after birth. Other workers have reported a change in the character of the cells of the epithelium after birth, particularly at about the time when the absorption of protein ceases in the different species studied (Clark, 1959; Staley et al., 1969). In view of these findings the changes which occurred in the intestine of lambs during the early postnatal period were studied.

Growth of the small intestine after birth

The intestine of the lamb after birth appears grossly different from that of the foetal lamb. Besides being distended with ingesta, the intestine appears relatively hyperaemic compared with that of the foetus, and gut motility is more obvious. For this reason, the increase
in size of the intestine was studied in a series of lambs up to 5 weeks of age.

The length of the small intestine was measured after it had been freed of mesentery. Due to the mobile nature of the tissue, these measurements are subject to considerable error, but the figures suggest that there is little change during the first week, though there is a considerable increase thereafter (Table 14.). The wet and dry weights of segments of duodenum, jejunum and ileum were also measured, but there was no consistent change in the dry weight to wet weight ratio up to 5 weeks of age (Table 14.).

Table 14. Changes in the intestine of lambs during the 5 weeks after birth. The length of the small intestine (cm), and the dry weight:wet weight ratio (%) for the duodenum, jejunum and ileum are given together with the body weight (kg).

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Mean body weight (kg)</th>
<th>Length of intestine (cm)</th>
<th>Dry weight:Wet weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Duodenum</td>
</tr>
<tr>
<td>1</td>
<td>3.95</td>
<td>824</td>
<td>16.39</td>
</tr>
<tr>
<td>2</td>
<td>4.13</td>
<td>979</td>
<td>19.19</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>873</td>
<td>18.14</td>
</tr>
<tr>
<td>4</td>
<td>4.05</td>
<td>983</td>
<td>18.75</td>
</tr>
<tr>
<td>5</td>
<td>4.55</td>
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<td>7</td>
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<td>21</td>
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<tr>
<td>28</td>
<td>10.9</td>
<td>1490</td>
<td>17.46</td>
</tr>
<tr>
<td>35</td>
<td>12.7</td>
<td>1720</td>
<td>18.96</td>
</tr>
</tbody>
</table>
Most of the increase in the volume of the gut during the week after birth appears to be due to distension with ingested material rather than to an increase in the amount of tissue.

Morphological changes in the epithelium of the small intestine

The epithelial cells of the intestine of the foetal lamb showed different staining characteristics from those of lambs 3 or more days after birth when they were fixed and treated in the same manner (Fig. 52). Accordingly, intestines from lambs between birth and three days of age were examined to see if the difference between the intestinal epithelial cells of foetal lambs and lambs at three days of age was due to progressive changes during this period. Whereas in the foetal lambs, and in lambs at birth, the epithelial cells fixed in 95% alcohol showed very little staining of the cytoplasm, in 2-3 day old lambs an increasing proportion of the intestinal villi appeared to be covered by epithelial cells in which the apical region could be stained quite intensely. From Fig. 53, it is apparent that most of the villi are covered by a different type of epithelium by 3 days after birth. These observations suggested that, during the first 2-3
Fig. 52 Sections of small intestine from a foetal lamb (a) and a lamb 3 days after birth (b). In both cases, the gut was fixed in 95% alcohol and stained with haematoxylin-eosin. In the foetal epithelium (a), a large proportion of the cytoplasm of the cells is unstained and appears to contain a single large vacuole. At 3 days after birth, the apical region of the epithelium is quite densely stained and the cells appear to be more columnar, and uniform in size.
Magnification: 330 x.
Fig. 53  Sections of the intestines of 3 lambs, taken on the day of birth, and on the 2nd and 3rd days after birth. The tissue was fixed in 95% alcohol, and stained with haematoxylin-eosin.

(a) A section showing large densely-staining vacuoles (v) in the intestinal epithelium of a lamb on the day of birth, following the ingestion of colostrum. The vacuoles are present over the whole length of the villi. Magnification: 130 x.

(b) A section taken from a lamb on the second day after birth, showing that only the upper portions of the villi contain densely-staining vacuoles (v). The basal portion of the villi is covered with a more mature type of cell, taking up more stain than the cells of foetal and day-old lambs. Magnification: 130 x.

(c) A section taken from a lamb on the third day after birth, showing that the whole length of the villi is covered with a mature type of epithelium, with basal nuclei and well-stained cytoplasm. Magnification: 130 x.
days after birth, the immature type of epithelial cells matured, or were replaced by a mature type of cell as a result of migration of cells from the crypts of Lieberkühn.

In order to investigate further the apparent change in the intestinal epithelium of the lamb after birth, 4 lambs were each fed 200 ml. of fresh colostrum at different times after birth (4, 15, 23 and 32 hours). Four hours after the colostrum was fed, the lambs were killed and portions of the small intestine were fixed in Zenker's formol, and paraffin sections of the tissue were examined after staining with Dominici's stain. In the lamb fed 4 hours after birth, intestinal epithelial cells containing densely-staining vacuoles could be seen along the entire length of the intestinal villi. In the lambs fed at longer intervals after birth there appeared to be a region near the bases of the villi in which the epithelial cells did not contain any vacuoles, but resembled the more densely-staining, mature type of cell characteristic of older lambs. This region appeared to be more extensive at 19 and 27 hours than at 8 hours after birth (Fig. 54). In the lamb examined at 36 hours, there were still some cells containing protein-filled vacuoles, covering a considerable length of the villi. In general, these observations were
Sections of the intestines of two lambs, taken on the first and second day after birth and fixed in Zenker's formol.

(a) A section from a lamb 8 hours after birth, and 4 hours after being fed colostrum, showing the large densely-staining vacuoles (v) present in the epithelial cells along the whole length of the villi. The section was stained with haematoxylin-eosin. Magnification: 330 x.

(b) A section from a lamb 27 hours after birth, and 4 hours after being fed colostrum, showing the change in the type of epithelial cells (arrows), apparently migrating up from the crypts. The section was stained with haematoxylin-eosin. Magnification: 330 x.

(c) A section from the same lamb as in (b), but stained with Dominici's stain. The transition in the epithelium can be seen (arrows) with densely-staining vacuoles (v) only present in the upper portions of the villi. Magnification: 330 x.
consistent with a rapid replacement of the epithelium characteristic of foetal and newborn lambs which is capable of absorbing intact protein, by a mature type of epithelium proliferating from the crypts. There appears to be some variation between lambs, and a larger number of animals in a similar series would be needed to investigate this phenomenon further.

Estimation of the rate of renewal of the intestinal epithelium before and after birth

Portions of the intestines of both foetal and newborn lambs were incubated in vivo with $^3$H-thymidine as described previously. The radioactivity disappeared from the lumen quite rapidly in both the foetal and newborn lambs (Fig. 55), most of the activity being taken up by the tissue during the first 30 minutes after the injection of $^3$H-thymidine. At different times after the incubation the tissues were examined by autoradiography. In both foetal and newborn lambs labelled epithelial cells could be found only in the crypts immediately after the incubations. The rate at which the epithelial cells migrated from the crypts up the villi definitely increased after birth. After 24 hours, labelled cells in the postnatal lamb could be seen well beyond the mouths of the crypts, but in the foetus the labelled cells were still within the crypts (Fig. 56).
Fig. 55 The time course for the disappearance of $^3$H-thymidine radioactivity from the lumen of isolated segments of the intestines of foetal (○, ●) and newborn (x) lambs. The radioactivity in a sample of the lumen fluid, expressed as a percentage of the radioactivity in a similar sample taken 2 minutes after the injection of $^3$H-thymidine, is shown plotted against the time after the injection.
Fig. 56  Autoradiographic comparisons of the intestines of foetal and newborn lambs. The intestine was incubated with $^3$H-thymidine in vivo, and 24 hours later the animals were killed and the intestine fixed in Zenker's formol. Paraffin sections were then subjected to autoradiography.
(a) A section from a foetal lamb 24 hours after incubation with $^3$H-thymidine.
(b) A section from a newborn lamb 24 hours after incubation with $^3$H-thymidine.
By 72 hours, the labelled cells in the young lamb had traversed about half the length of the villi and practically no labelled cells remained in the crypts. These results suggested that the epithelium of the newborn lamb would be almost completely replaced in about the first 72 hours after birth. The pattern of labelled cells seen at 36 and 48 hours after the incubation with \( ^3 \)H-thymidine in the foetus suggested that the cells are sedentary, and in the foetus there was little or no migration of the labelled cells up the sides of the villi.

**Comparison of the mitotic activity in the crypts of foetal and newborn lambs**

The mitotic activity in the crypts of foetal and newborn lambs' intestines was estimated by counting the numbers of mitotic figures visible in 5 \( \mu \) sections of the crypts of the small intestine used for autoradiography. An analysis of the results of these counts (Table 15), shows that the rate of cell division was significantly higher in the newborn lambs (\( P < .001 \)), but the mechanism by which the suggested increased rate of division is initiated is not known. It is interesting to note that in the intestine of the foetal lamb there is considerable mitotic activity, despite the apparent lack of migration of cells from the crypts.
Table 15. Estimation of the mitotic activity in the crypts of the intestines of foetal and newborn lambs. Duplicate determinations of the mean number of mitotic figures per crypt are tabulated. These were calculated from the number of mitotic figures counted in at least 100 crypt sections for each duplicate.

Mean number of mitotic figures per crypt

<table>
<thead>
<tr>
<th>Foetal lambs</th>
<th>Newborn lambs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.56</td>
<td>2.79</td>
</tr>
<tr>
<td>1.02</td>
<td>3.34</td>
</tr>
<tr>
<td>1.21</td>
<td>2.07</td>
</tr>
<tr>
<td>1.37</td>
<td>1.78</td>
</tr>
<tr>
<td>1.54</td>
<td>3.11</td>
</tr>
</tbody>
</table>

Analysis of Variance

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>D.F.</th>
<th>Mean square</th>
<th>V.R.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duplicates</td>
<td>0.1217</td>
<td>1</td>
<td>0.1217</td>
<td>0.3002</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Foetuses vs. lambs</td>
<td>9.6605</td>
<td>1</td>
<td>9.6605</td>
<td>23.8316</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>6.8912</td>
<td>17</td>
<td>0.4054</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16.6734</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 7.

DISCUSSION

Since Emrlich (1892) first studied the transfer of antibodies from mother to offspring in mice, a considerable body of data has been accumulated by numerous workers concerning the way in which the offspring of many species are immunized passively against infectious diseases which they are likely to encounter during the early postnatal period. The routes by which antibodies are transferred, the time at which this transfer takes place, and the time during which antibodies can be transferred, have all been investigated, and the findings published for the different species studied have already been discussed in Chapter 1 of this thesis. Despite all this work on young animals, there are still many unanswered questions concerning the mechanism by which the transfer of antibodies takes place, and why this transfer ceases when it does. The experiments described in this thesis, in which several aspects of \( \gamma \)-globulin metabolism in the lamb have been studied, were carried out to try and answer some of these questions, and the significance of the results and their relationship to the findings of other workers are discussed below.
The importance of maternal antibodies in the development of the newborn lamb

Earlier workers have shown that the calf, the piglet, and the lamb are born with very little circulating antibody, and during the first day of life large quantities of antibody are absorbed from the colostrum ingested soon after birth. The economic value of the calf and piglet, and the economic advantages to be gained by weaning these animals from their mothers as soon as possible, have stimulated considerable interest in their successful artificial rearing. The importance of colostrum to the calf has been stressed since the early work of Orcutt and Howe (1922) and Smith and Little (1922). The latter workers raised the question of whether or not colostrum has a secondary role, besides that of providing antibodies for passive immunity, but they concluded that calves deprived of colostrum could grow normally if they survived early infections. Hansen and Phillips (1947) were able to increase the rate of survival of colostrum-deprived calves by the use of antibiotics, but the rate of growth of these calves was lower than that of normal calves, due to either inadequate nutrition or some other factor. More recently, Staub and Boguth (1956) and Lecce and Morgan (1962) have
shown that, in the piglet, colostrum promotes normal plasma protein synthesis, since colostrum-deprived piglets produced antibodies later than control piglets, and showed delayed maturation of the plasma protein profile.

The successful rearing of lambs by hand has received less attention than that of calves or piglets, because under usual husbandry conditions, little benefit derives from being able to wean lambs from their mothers at an early age. The absorption of antibodies from colostrum by lambs has been demonstrated (Mason et al., 1930; Earle, 1935; McCarthy and McDougall, 1953), and the importance of these antibodies in the protection of the newborn lamb from some specific diseases has been confirmed (Chauveau, 1888; Mason et al., 1930; Oxer, 1936). However, no reports of studies on the survival of colostrum-deprived lambs have been found in the literature.

The results of the experiments described in Chapter 3. of this thesis showed that colostrum-deprived lambs grew as well as colostrum-fed lambs reared on the same diet, suggesting that the normal growth of lambs does not depend on them ingesting colostrum. These findings are contrary to those of Penning (P.D. Penning, 1969, personal communication) who found that lambs of British breeds of
sheep reared by hand in large numbers in Berkshire, U.K., require colostrum as an essential factor for survival. The rate of survival of the colostrum-deprived lambs used in the experiments described in this thesis may have been influenced favourably by the generally dry weather conditions, and the antibiotics supplied during the first week after birth.

No effects could be detected of the various treatments with adult serum given to the lambs, or of colostrum fed immediately after birth, on the lambs' rate of growth, the appearance of autologous \(\gamma\)-globulin in their circulation, or their antibody response to swine influenza virus. Since the immunoelectrophoretic profiles demonstrated that \(\gamma\)-globulin precipitin arcs did appear in the serum of lambs after treatment with 50 ml. of adult serum, there were significant quantities of \(\gamma\)-globulin present in the circulation, though these levels were far below those resulting from the feeding of colostrum. However, the quantities of adult serum which would be required to duplicate the effects of colostrum in this respect would be completely impracticable for routine use. The results do not support the hypothesis that colostrum has a secondary role in stimulating the normal development of
the lamb, and any differences between hand-fed and normal lambs are likely to be due to levels of food intake. If colostrum-deprived lambs are to be hand-reared successfully, it would appear more practicable to prevent early infections by some means other than the provision of homologous antibodies, and to ensure that the lambs receive adequate nourishment.

Studies of the absorption of γ-globulin by the intestine of the newborn lamb

Most of the studies of antibody absorption by the intestine have been carried out on intact newborn animals. These animals have been fed a known dose of antibodies and the serum titre reached within a certain time has been used to measure the amount of antibody transferred. Alternatively, labelled γ-globulin has been fed, and the peak levels of labelled protein reached in the circulation have been used to estimate the extent to which the protein is absorbed. Similar studies were carried out on lambs to obtain as much information as possible about the absorption of γ-globulin in this situation, and the results are described in Chapter 4. of this thesis.

The intestine of the lamb was able to transfer at least 14% of the γ-globulin fed in colostrum to the
circulation during the first 8 hours after birth. The capacity for absorption was lost almost entirely by 29 hours after birth, which confirms the findings of McCarthy and McDougall (1953) who detected some absorption at 29 hours, but none at 48 hours after birth. The period of permeability of the intestine is therefore similar to that in the calf, where there is little absorption after 24-30 hours (Smith, Reed and Erwin, 1964). The percentage of the \( \gamma \)-globulin fed which is absorbed is also similar to that measured in the calf (Bangham et al., 1958) and in the piglet (Olsson, 1959; Pierce and Smith, 1967a). A greater proportion of \( ^{125}I \)-labelled \( \gamma \)-globulin was absorbed when it was fed dissolved in colostrum than when it was fed dissolved in physiological saline. This difference in the rate of absorption of protein will be discussed more fully later when the results of studies with the foetal intestine are considered.

The levels of serum globulin in newborn lambs were found to rise by 19-35 mg/ml. after the ingestion of colostrum. These increases are higher than those reported by McCarthy and McDougall (1953) (9-12 mg/ml.) and lower in general than those reported by Earle (1935) (30-35 mg/ml.). From the increase in serum globulin levels, it was estimated
that from 2-5 g of γ-globulin was transported to the circulation from the intestine in several lambs, and that in one lamb about 10 g was transported. Since the maximum levels of serum globulin were reached about 10 hours after the ingestion of colostrum, protein must have been transported to the circulation at mean rates of 0.2-1.0 g per hour in individual lambs. The peak rates of absorption were certainly much higher than these mean figures.

The mechanism by which γ-globulin is absorbed by the intestine of the lamb does not appear to be very selective. The 3 classes of γ-globulin present in colostrum whey can be detected in the plasma after the ingestion of colostrum, and both fast IgG and slow IgG are absorbed from a solution of serum γ-globulin. The relative concentrations of these immune globulins in the plasma reflect those in the original solution fed, as judged visually from the immunoelectrophoretograms. Other workers have reported that lambs will absorb other large molecules besides γ-globulin, such as ovalbumin and PVP (Lecce and Morgan, 1962). In this respect, the lamb is similar to the calf which can absorb all the proteins in colostrum whey (Pierce, 1961b), and the piglet which can absorb dextrans, PVP, and a variety
of proteins not found in colostrum (Lecce, Matrone and Morgan, 1961).

The rate of γ-globulin absorption in the lamb was estimated previously by Mason et al. (1930). Lamb dysentery antitoxin was detectable in the serum one and a half hours after feeding, and the antibody titre rose rapidly to a maximum at 12 hours. In some animals, the antibodies could not be detected until 4 hours after feeding. The figures presented here for the rate of absorption of I-labelled homologous γ-globulin are similar to those of Mason et al. (1930) for the antitoxin. There is a delay initially in the appearance of radioactivity in the plasma after feeding 125 I-γ-globulin, which would be expected since there are possible delaying pools in both the gut contents and in the gut wall.

A mean circulating half-life of absorbed γ-globulin of at least 12 days was estimated from the rate at which the concentration of absorbed 125 I-γ-globulin in the plasma decreased. The error in the method of estimation is considerable, because of the changes in the plasma volume and the total plasma protein pool during the first few weeks after birth. However, the result does suggest that the labelled protein preparation behaved similarly in vivo
to the colostrum $\gamma$-globulin, since the rate of disappearance of the latter from the circulation appeared to follow a similar time course. Assuming a half-life of 12 days, the circulating maternal $\gamma$-globulin would have decreased to about 6% of the original level by 7 weeks after birth, or from about 30 mg/ml. to only 2 mg/ml. This represents about 4% of the total serum protein, and since endogenous $\gamma$-globulin becomes apparent in the serum after 4 weeks, the differences in the serum proteins between lambs fed colostrum and those deprived of colostrum would have practically disappeared by 7 weeks of age. McCarthy and McDougall (1953) reported that the difference between the serum globulin levels of colostrum-fed and colostrum-deprived lambs had disappeared by 5-6 weeks of age, which agrees with the results presented in this thesis and suggests that an estimate of 12 days for the circulating half-life is reasonable. This estimate of the circulating half-life is longer than would be expected from a comparison with figures for other species on a body weight basis (Dixon et al., 1952; Cohen et al., 1956; Dich and Nielsen, 1964), but these other estimates were made from experiments with adult animals, in which conditions approached a steady state. Since the changes in blood
volume resulting from the growth of the lambs would cause the apparent half-life to decrease, the relatively long circulating half-life measured in the lambs suggests that there is a slower rate of catabolism of $\gamma$-globulin in these young animals than in adults.

Other systems used to study the absorption of $\gamma$-globulin by the intestine

The studies described using the intact lamb show that although a certain amount of information concerning the absorption of $\gamma$-globulin by the intestine can be obtained, the actual process by which the protein is absorbed cannot be studied directly. The short time during which the intestine is permeable to intact protein, the complexity of the system, and the lack of direct measurement of absorption, all combine to limit the usefulness of information which can be obtained from such experiments. A different experimental model is required if the process is to be studied in more detail, and if an explanation for the cessation of absorption is to be found.

One of the preparations developed to study the absorption of $\gamma$-globulin more directly in the newborn animal was the anaesthetized calf with a thoracic duct fistula (Comline, Roberts and Titchen, 1951a). Colostrum
was infused directly into the duodenum, and the amount of protein appearing in the thoracic duct lymph was measured. These workers established that practically all the absorbed protein entered the blood by way of the intestinal lymphatic vessels and the thoracic duct, and the rate of absorption reached a peak after 2-3 hours infusion with colostrum. This preparation, which has been used more recently by Balfour and Comline (1962), suffers from the disadvantages that the animals are anaesthetized, and the experiments are acute in nature, lasting up to a maximum of only about 5 hours.

Isolated everted sacs of intestine from newborn animals, similar to those described by Wilson and Wiseman (1954), have been used to study the absorption of \( \gamma \)-globulin \textit{in vitro} in the rat (Bamford, 1966) and the piglet (Pierce and Smith, 1967b; Smith and Pierce, 1967). The sacs of everted intestine were incubated in solutions containing \( \gamma \)-globulin, and the absorption of protein was estimated from the amount of protein recovered from the fluid inside the sacs. This method has provided useful information concerning the influence of different factors on the absorption of protein, but these findings need to be corroborated by \textit{in vivo} studies.
In order to avoid some of the disadvantages of the preparations used previously for the direct measurement of \( \gamma \)-globulin absorption in the intestine of newborn animals, the process was studied in foetal lambs in which chronic indwelling thoracic duct and duodenal fistulae had been established. The foetal lamb has been used previously by several groups of workers for a variety of studies involving surgical manipulations in utero. The development of immunological competence in the foetal lamb has been studied by means of the rejection of skin grafts (Schinckel and Ferguson, 1953), antibody response to antigenic challenge at various stages of gestation (Silverstein, Uhr, Kraner and Lukes, 1963) and following thymectomy in utero (Silverstein, Parshall and Prendergast, 1968). Acute studies of sugar and acetate metabolism (Alexander, Britton and Nixon, 1967) and acid-base relationships (Assali, Manson, Holm and Ross, 1963), and chronic studies of changes in blood haemoglobin and oxygen concentrations (Meschia, Cotter, Breathnach and Barron, 1965), have also been carried out in foetal lambs. The only measurements of lymph flow in the foetal lamb to my knowledge have been made in acute preparations in which lymph flow from the right lymph duct and the thoracic duct was measured (Boston, Humphreys, Reynolds and Strang, 1965;
Humphreys, Normand, Reynolds and Strang, 1967). In these studies, which were concerned with the clearance of fluid from the lung during ventilation, the foetal lamb was anaesthetized throughout the experiments, and lymph was only collected for 2-4 hours.

The foetal lambs used in the experiments described in Chapter 5 of this thesis healed very rapidly after surgery, and when born 1 or 2 days post-operatively showed no ill-effects from the quite complex manipulations involved. Several lambs were born with their fistulae intact, despite the expectation that the cannulae would be pulled from the foetus during delivery, because they were necessarily very long, and were anchored at several places exterior to the foetuses. The risk of the cannulae being pulled out of the foetuses could be virtually eliminated by delivering the lambs by Caesarean section.

The observations on flow rate and protein content of thoracic duct lymph, and the subsequent birth and survival of some of the foetal lambs used for experiments in utero, show that the foetus is able to tolerate the loss of lymph for a considerable time, at least 2-3 days, without severe effects. Therefore the foetal lamb with
thoracic duct and duodenal fistulae appears to remain physiologically normal for sufficient time to allow prolonged studies of \( \gamma \)-globulin absorption to be carried out in the unanaesthetized animal, under conditions which approach a steady state.

It is possible that these and various other surgical preparations of foetal lambs might be useful for studying changes in the metabolism of the foetus which occur at or near birth. In view of my experiences, it might be suggested that such surgical preparations required for studies in the newborn lamb could be established in utero, and sufficient time allowed for recovery from surgery before the lambs are delivered by Caesarean section. These lambs would then be available for experiments immediately after birth, unanaesthetized and without having been subjected to the stress of surgery soon after birth.

**Quantitative aspects of \( \gamma \)-globulin absorption by the intestine of the foetal lamb**

A considerable amount of the work reported in Chapter 5 was carried out to establish that the foetal lamb can absorb protein through the intestinal epithelium in a manner similar to that which occurs in the intestine of the newborn lamb. The appearance in the thoracic duct
lymph of labelled protein which is antigenically similar to the \(\gamma\)-globulin infused, and the association of \(^{125}\)I radioactivity in the lymph with the appearance of intact \(\gamma\)-globulin, provide evidence that the foetal intestine is able to absorb intact protein. At the same time it was shown that only a small proportion, about 10-15\%, of the protein infused into the intestine reached the thoracic duct lymph. The fate of the remaining protein has not been determined conclusively, but the amount of non-protein-bound \(^{125}\)I radioactivity found in the lymph suggests that there is active proteolytic digestion in the foetus even at this stage of development. Pierce and Smith (1967a) reported similar observations in the newborn piglet in which about 10\% of the \(\gamma\)-globulin fed was absorbed and the other 90\% appeared to be digested.

Later experiments with the foetal lamb preparation were concerned mainly with studying the effects of different factors on the rate of absorption of protein by the intestine. Balfour and Comline (1962) reported that \(\gamma\)-globulin infused into the intestine of the calf was absorbed more slowly when given as a solution in saline than when given in an electrolyte solution similar to colostrum whey. Similar results were obtained with the
foetal lamb. Not only was there a more gradual rise in the amount of radioactivity in the lymph during the infusion of $^{125}$I-γ-globulin dissolved in saline, but the maximum rate of absorption was lower than in foetuses infused with a solution of $^{125}$I-γ-globulin dissolved in an electrolyte solution similar to that used by Balfour and Comline (1962). This effect could be explained by either low Na$^+$ concentration or high K$^+$ concentration enhancing the absorption process, or by the uptake of protein being dependent on the presence of phosphate. The enhancement of absorption in the calf by glucose-6-phosphate (Balfour and Comline, 1962) does suggest some form of energy-dependent transfer mechanism.

The maximum rate at which protein was absorbed was higher when colostrum whey was infused into the foetal intestine, and this suggests that some factor or factors present in the whey might have had an effect on the amount of protein absorbed. Possibly there is a trypsin inhibitor in ovine colostrum whey, similar to that described in both bovine and porcine colostrum (Laskowski and Laskowski, 1951; Laskowski, Kassell and Hagerty, 1957), which is responsible for the increased amount of protein absorbed from colostrum whey.

Smith and Pierce (1967) showed that the amino acids L-leucine and L-methionine interfered with protein
absorption in *vitro*, and the results of experiments described here do suggest that high concentrations of L-leucine might reduce absorption by the foetal lamb. However, a protein hydrolysate did not reduce the rate of absorption of γ-globulin *in utero*, and it was shown that there is active digestion of protein in the foetus at the same time as intact protein is being absorbed.

A significant finding concerning γ-globulin absorption in the foetal lamb was that prolonged infusion of protein at a constant rate, for up to 48 hours, did not result in any decrease in the rate of absorption. This is very different from the situation in the newborn lamb where absorption has practically ceased by 36 hours after birth. The load of protein given during the prolonged infusions varied from 30-45 g, equivalent to 300-400 ml. of colostrum whey, and was greater than the amount of γ-globulin normally ingested by newborn lambs.

**A comparison of the rates of protein absorption in foetal and newborn lambs**

The amount of protein absorbed by the gut, and transported in the thoracic duct lymph during the prolonged infusions of protein into the duodenum of the foetal lamb, was about 3.5 g (about 100 mg protein per hour). This
amount is equivalent to the amount of \(\gamma\)-globulin absorbed by a normal lamb after birth. Since there was no evidence for a cessation of absorption during these prolonged infusions, it is possible that the foetal lamb could absorb much more \(\gamma\)-globulin. The rate of protein absorption in foetal lambs is considerably less than that in the newborn lamb after the ingestion of colostrum, when the intestine can transfer at least from 2-10 g of \(\gamma\)-globulin to the circulation in less than 10 hours, i.e., 0.2-1.0 g/hr. However, the situation in the foetal lamb is different from the lamb given a single large feed of colostrum, and the slow rate of infusion of the protein may limit the rate of absorption. The rate of transfer is greater than that reported for \textit{in vitro} systems, based on the amount of protein transferred per gram of wet tissue per hour. Pierce and Smith (1967b) found a maximum rate of transfer \textit{in vitro} of about 50 \(\mu\)g protein/g wet tissue/hr, which they estimated to be about one fiftieth of that in the newborn piglet. A higher rate of about 2 mg/g wet tissue/hr was obtained when sacs of intestine were taken from piglets 2 hours after a feed of colostrum, in which case, possibly, only the release of previously absorbed protein from the epithelium was being measured \textit{in vitro}. From the results obtained with
the foetal lamb, and using estimates for the weight of the intestine based on data obtained from newborn lambs, about 2.5 mg γ-globulin/g wet tissue/hr is transported by the middle portion of the small intestine of the foetal lamb. The lower rate of absorption of protein in the foetal lamb than in the newborn lamb may also be influenced by the blood supply to the tissue. It is likely that the foetal intestine which is not actively absorbing nutrients for the maintenance of the animal is relatively poorly supplied with blood, and consequently with oxygen and metabolites necessary for the process of protein absorption. An inadequate supply of these factors to the epithelium of everted sacs of intestine may also explain the low rate of absorption in vitro.

**Morphological comparisons of the intestines of foetal and newborn lambs during γ-globulin absorption**

The morphological comparisons between the intestines of foetal and newborn lambs provide further evidence that in both situations the absorption of protein takes place by the same process. Similar large densely-staining vacuoles appear in the epithelium of the foetal intestine following infusion with serum γ-globulin and in the newborn lamb's intestine after the ingestion of colostrum. The contents of these vacuoles have been identified as
γ-globulin by fluorescent antibody localization. The histological appearance and the staining properties of the vacuoles are identical in both situations. That the process in the foetus is an active one is shown by the uneven distribution of the protein-filled vacuoles along the gut. The concentration of vacuoles, and the level of $^{125}\text{I}$ radioactivity, in the epithelium are highest in the middle region of the gut which agrees with the findings of Pierce and Smith (1967b) and Bamford (1966) using in vitro systems, and with other observations in the rat (Clark, 1959). The same region of the intestine is important in the active transfer of amino acids in the adult animal (Wiseman, 1967), but the relationship between protein and amino acid transfer, if any, is not clear.

Possible mechanisms for the absorption of γ-globulin

The transfer of protein from the intestinal lumen to the lymph can be considered in three successive stages. First, the uptake of protein from the intestinal lumen appears to involve pinocytosis. It is not certain that the presence of protein stimulates the formation of pinocytotic vacuoles, since the apical region of normal foetal epithelial cells contains many tubules and vacuoles in the absence of exogenous γ-globulin. Whether the uptake of protein is initiated by the hydrostatic pressure of the
gut contents, the presence of an ionic gradient across the intestinal epithelium, or an active energy-dependent transfer of protein attached to specific receptor sites, is not clear. Secondly, the concentration of absorbed protein into a single vacuole and its subsequent displacement to the base of the cell can be observed histologically, but the mechanisms involved are not understood. Possibly an ionic gradient, coupled with a nett transfer of fluid across the cell, contributes to the movement of the protein-filled vacuole. Thirdly, the release of the absorbed protein from the cell may involve either a release of all the contents of the vacuoles by rupture of the plasma membrane, or a slow release of the protein by dissolution and dispersal in the cell fluids.

The apparent enhancement of the overall process by phosphate suggests that a considerable amount of energy might be required for protein transfer, and the groups of mitochondria at the apex and base of the cells may supply some of the energy required. Phosphate could also enhance the rate of synthesis of phospholipids necessary for the increased formation of membranes which must take place during pinocytosis.
Possible mechanisms involved in the cessation of \(\gamma\)-globulin absorption

It appears that neither the presence of protein in the gut for long periods, nor a large amount of protein transferred by the intestine, initiates the cessation of protein absorption \textit{in utero}. Therefore some change associated with birth, or occurring after birth, during the adjustment to independent existence, must be responsible for the cessation of absorption of intact protein by the intestine of the newborn lamb. The changes occurring in the circulation after birth, with increased blood flow to the intestine and a reduced portal vein pressure due to the absence of the placental circulation, could play an important role in initiating the cessation of absorption. The onset of digestive activity has also been suggested as an important factor in decreasing the amount of protein absorbed (Hill, 1956), as have the products of protein digestion (Smith and Pierce, 1967). However, the fact that digestion appeared to be taking place to a considerable extent even in the foetal lamb, and the fact that a protein hydrolysate did not reduce absorption of protein by the foetal lamb, would tend to discount the onset of digestion alone as being responsible for the cessation of absorption
of intact protein, and supports the results of Deutsch and Smith (1957) and Steck (1962).

Another factor which could be involved in the cessation of absorption is the secretion of the adrenal cortex, since in response to the stresses of birth, increased production of corticosteroid hormones could be expected. Previous work by Halliday (1959) implicated cortisone acetate and DOCA in the cessation of absorption of protein by the intestine of the young rat, and Clark (1959) observed that the cells of the intestinal epithelium of the young rat appeared more mature histologically 48-72 hours after the administration of cortisone acetate. In the sheep, steroids secreted by the foetal adrenal have been shown to play an important part in the onset of parturition (reviewed by Holm, 1967). Secretion of cortisol, corticosterone, and aldosterone by the foetal adrenal was demonstrated by Chester Jones, Jarrett, Vinson and Potter (1964), and Alexander, Britton, James, Nixon, Parker, Wintour and Wright (1968). There was an increase in both the rate of secretion and the weight of the adrenals of normal foetal lambs towards term (Alexander et al., 1968), and the levels of secretion were generally higher in lambs after birth than in foetuses before birth. Bilateral
adrenalectomy of the foetus (van Rensburg, 1967; Drost and Holm, 1968), or electrocoagulation of the hypophysis of the foetus (Liggins and Kennedy, 1968), caused prolonged gestation in pregnant ewes, whereas the administration of ACTH or corticosteroids to foetal lambs caused their premature delivery (van Rensburg, 1967; Halliday and Buttle, 1968; Liggins, 1968). The findings reported in this thesis support those of other workers since DOCA and possibly ACTH appeared to cause premature birth of the foetuses. Because of this complication, it was difficult to assess the effects of corticosteroids on \( \gamma \)-globulin absorption in utero, but the results of one experiment did suggest that a large dose of DOCA had an effect on protein absorption within a few hours of its administration.

The morphological changes in the epithelium of the gut after birth, observed in these studies and reported by Smith (1925) and Staley et al. (1969), and the autoradiographic evidence for a more rapid proliferation of cells from the crypts after birth, suggest that a different type of epithelium replaces the foetal type of cell during the first 3 days after birth. Since antibody absorption has been shown to decrease rapidly during the first 2 days after birth, and there is no evidence for
absorbed protein being present in the cells which migrate from the crypts after birth, this change in the epithelium could be the cause of the cessation of absorption. It appears that the ability to absorb intact protein is primarily a property of the foetal type of cell, and only when the epithelium is not being rapidly replaced, as in the foetal lamb, can absorption continue indefinitely. Since the foetal cells are not being replaced to any great extent, they are probably 'old' cells. It is interesting to speculate whether the digestive cells which appear after birth, and which are characteristic of older animals, might eventually change to the type of cell characteristic of the foetus, if the rate of replacement of the epithelium were decreased in the older animal. In order to do this, the factors governing the life span of intestinal epithelial cells and their rate of migration up the intestinal villi need to be understood better. These might include increased abrasion of the epithelium with consequent loss of cells from the apices of the villi, due to the presence of ingesta in the gut or increased gut motility, or circulating levels of corticosteroids.
SUMMARY

1. It has been shown that lambs can be reared successfully without colostrum, and the normal growth and immunological development of the lambs is maintained in the absence of maternal antibodies. A relatively small dose of homologous antibody, given in sterile sheep serum, did not appear to influence the survival, growth rate, or development of colostrum-deprived lambs. Under the conditions of the experiment, in which antibiotics were administered to the lambs during the first 5 days after birth, there was no beneficial effect of colostrum on subsequent development.

2. In a series of newborn lambs, certain aspects of $\gamma$-globulin absorption by the intestine were studied, and the rate of absorption, and the amount of protein transported to the circulation, were estimated as accurately as possible in the intact lamb.

3. The absorption of $\gamma$-globulin by the intestine of the newborn lamb was shown to be non-selective; the 3 classes of $\gamma$-globulin present in colostrum whey, IgM, fast IgG and slow IgG, were transported to the circulation after the ingestion of colostrum.

4. The rate of disappearance of absorbed $\gamma$-globulin from the circulation of newborn lambs was measured, and from the
values obtained for the apparent half-life of the labelled protein, the 125I-γ-globulin prepared as described in Chapter 2. of this thesis appeared to behave similarly in vivo to the native protein.

5. A preparation using the foetal lamb as an experimental animal for the study of γ-globulin absorption was developed. Chronic indwelling fistulae were established in the thoracic duct and duodenum, which permitted a direct assessment of the amount of protein transported in the thoracic duct lymph following the infusion of protein into the duodenum.

6. A series of experiments using the foetal lamb preparation showed that protein was absorbed by the foetal intestine in a manner similar to that which occurs in the newborn lamb. The effects of various factors on the rate of absorption of protein were studied. The rate of absorption of protein was influenced by the vehicle in which the protein was dissolved. Absorption was enhanced by the presence of potassium and phosphate. L-leucine reduced slightly the amount of protein absorbed, but a protein hydrolysate did not reduce the rate of absorption. No cessation of absorption occurred in utero.

7. The changes in the epithelium during the absorption of protein were studied by both light and electron
microscopy. These studies showed that the absorption of protein by the foetal intestine was analogous to that in the newborn lamb. Large, densely-staining vacuoles were observed in the epithelium after the infusion of γ-globulin, and these vacuoles were similar to those found in the lamb after the ingestion of colostrum.

8. Morphological observations and autoradiographic studies suggested that immediately after birth, the epithelium of the intestine of the lamb is renewed at a greater rate than in the foetal lamb. The type of cell proliferating from the crypts after birth appears to be different from that characteristic of the foetus. These cells are not able to absorb intact γ-globulin. This change in the epithelium is proposed as an explanation of the cessation of absorption in the newborn lamb.
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The lambs used in Chapter 3. were obtained with the co-operation of the CSIRO Division of Plant Industry. Figure 1. is reproduced with the permission of the Academic Press.

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