STUDIES ON MAMMARY GLAND LYMPH IN RELATION TO THE SECRETION AND RESOLUTION OF MILK

A thesis submitted for the degree of
Doctor of Philosophy

by

A.K. LASCELLES

Department of Experimental Pathology,
John Curtin School of Medical Research.

Australian National University,
Canberra.

G.37745

STATEMENT

The experimental work presented in Chapters 3 and 4 of this thesis was carried out jointly with Dr. B. Morris and forms the basis of two publications:

1. "Surgical Techniques for the Collection of Lymph from Unanaesthetized Sheep". Quart. J. exp. Physiol., 46, 199, 1961.
2. "The Flow and Composition of Lymph from the Mammary Gland in Merino Sheep". Quart. J. exp. Physiol., 46, 206, 1961.

The experimental work presented in Chapters 5 and 6 was carried out entirely by myself. This work forms the basis of three publications:

1. "Removal of Milk Constituents from the Mammary Gland of the Ewe". Nature, 191, 1404, 1961.
2. "The Absorption of Albumin and Casein from the Mammary Gland of the Merino Ewe". Quart. J. exp. Physiol., 47, No. 1, 1962.
3. "The Function of the Mammary Lymphatics in Staphylococcal and Streptococcal Mastitis in the Sheep". In preparation.

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CONTENTS

PREFACE

"The histological units of our organs express the phenomena of life and if the functions of these units show no variations under the influence of the outer atmosphere it is because they are immersed in an organic environment which does not change with variations in the cosmic environment. The living body, especially in the higher animals, never falls into chemico-physical indifference to the cosmic environment; it has ceaseless motions, an organic evolution apparently spontaneous and constant and though this evolution requires outer circumstances for its manifestation, it is nevertheless independent in its advance and modality." - Claude Bernard.

The "milieu intérieur" of the organism was described by Claude Bernard as "the circulating organic liquid, the lymph or plasma which surrounds and bathes all the tissue elements". It is reasonable to propose, and there is good evidence to support the proposition, that the tissue fluid of the body is not homogeneous in composition. The special metabolic activities of an organ may alter the composition and the volume of tissue fluid which forms in that organ. Again, peculiarities in anatomy and function may also impose conditions which will lead to changes in

the composition of the tissue fluid.

The mammary gland provides an example of an organ with specialized metabolic activities and with a specialized anatomy and function. There is a periodicity in the gland's activities which is related to pregnancy and parturition. During lactation the gland consists of a large mass of secretory tissue richly supplied with blood. Large amounts of protein fat and carbohydrate are continually passing out of the secretory acini into the milk cistern from whence they are removed by the suckling young. In the dry state, the gland has lost most of its secretory elements and there remains an atrophied mass of tissue with a relatively poor blood supply.

On the one hand, these changes in the physiology of the gland might be expected to lead to alterations in the local production and the composition of the tissue fluid and lymph. On the other hand, the nature of the gland's activities and its structure exposes the organ to risks of infection and trauma that might also be expected to influence the character of the tissue fluid. This thesis records the results of some experiments which were carried out to study the changes which may occur in the tissue fluid and lymph from the mammary gland in response to the secretion and to the resolution of milk constituents. The effects that concurrent pathology in the gland has on

the flow and composition of lymph has also been studied in animals with experimentally induced bacterial mastitis.

CHAPTER 1.

INTRODUCTION

In recently published text-books on lymphatics (cf. Yoffey and Courtice, 1956; Rusznyák, Földi and Szabó, 1960) no mention is made of mammary lymph or lymphatics. Moreover, Linzell (1959) emphasizes in a review article "Physiology of the Mammary Glands", that there are no published figures on the flow and composition of mammary lymph. Nothing has been published on the mechanism of removal of milk constituents from the mammary gland or on the role of the lymphatics in this process.

The interpretation of the results described in this thesis relate to the physiology of tissue fluid and lymph. It has also been necessary to appreciate the variations which may occur in the disposition of the lymphatics of the mammary gland and the routes by which lymph drains from it. For these reasons, the introduction includes a review of certain aspects of the physiology of the lymphatic system and a description of the anatomy of the lymphatic system of the udder.

HISTORICAL

It seems probable that lymphatic vessels were seen in the times of Hippocrates and Aristotle and it is certain that physicians of the Alexandrian school saw and described the lacteals in humans and animals. The first comprehensive description of the lymphatic system however was given by the Italian anatomist and surgeon Gasparo Aselli who, in 1622, found the distended lacteals in the mesentery of a dog which had recently had a large meal. On pricking one of the largest of the "white cords" Aselli observed that a liquid like milk gushed out. Two of his friends arranged for the publication of his book "De Lactibus Sivi Lacteis Venis" after his death in 1626 (cf. Aselli, 1627). Although many of the ideas he expressed are now known to be incorrect, the belief that the lacteals were involved in the absorption of the products of digestion has proved to be true, at least in part. Pecquet (1651) discovered the receptaculum chyli and traced the path of the thoracic duct through the thorax to where it entered the venous system at the base of the neck. By 1700 it was realised that lymphatics were present in various areas of the body, and that the fluid within these vessels emptied into the thoracic and right lymphatic ducts.

William Hunter (1718-1783) developed the generalization by analogy with the lacteals that lymphatic vessels in all regions of the body functioned in absorbing proteinized fluid, bacteria and foreign particles from the tissues (Hunter, 1784). He believed that the process of absorption was an active one, being a function of the endothelial cells of the lymphatics.

The lymphatic system of fishes, amphibia, birds and mammals including man, had been described by the latter part of the 18th century (cf. Hewson, 1846). The absence of lymphatic nodes in fishes, the presence of a few in birds and a large number in mammals was also noted.

Although the lymphatic vessels and lymph trunks of major importance had been described by the end of the 18th century, the origin of the lymph vessels or lymph capillaries within the various organs was still unknown. It was assumed at this time that lymph was derived directly from the blood by way of a series of fine tubes. However, many years later, von Recklinghausen denied that there was any direct connections between the blood and the lymph vascular systems. He suggested that fluid passed from the extravascular space to the lumen of the lymph capillaries through the open ends of the lymphatic capillaries or through other apertures which he called stomata. He also demonstrated the plate-like outlines

of the endothelial cells of the lymphatic capillaries by outlining the endothelial cells with metallic silver (cf. von Recklinghausen, 1862, 1863). Finally Sabin (1913) brought the structural issue to its present position by demonstrating the integrity of the lymphatic capillary wall.

Ludwig (1816-1895) and his pupils provided the first impetus to the modern physiology of the lymphatic system by the development of techniques for collecting lymph from various parts of the body. In Ludwig's experiments, the effects of massage and of passive movement in promoting lymph flow were clearly demonstrated. Ludwig became convinced that lymph was a filtrate from the blood. This belief, although not derived clearly from Ludwig's experimental results, was confirmed by the experiments of Starling (1894, 1894/95, 1895/96). Starling expounded his hypothesis on the factors concerned in lymph formation in a monograph published some years later (cf. Starling, 1909). The modern views on the physiology of the lymphatic system have been based on these original concepts.

FACTORS INVOLVED IN THE TRANSFER OF FLUIDS AND SOLUTES ACROSS THE CAPILLARY ENDOTHELIUM

Lymph is believed to be derived directly from the interstitial fluid and changes which occur in the formation of interstitial fluid ultimately will be reflected in

changes in the lymph. Although alterations in the metabolism of the cells may effect the composition of the lymph collected from a region of the body, it is the exchange of solutes and water through the capillary wall which are largely concerned with lymph formation. The exchange of substances between the blood and extravascular fluid occurs by diffusion and bulk flow.

Diffusion. Diffusion studies using radioactive isotopes have shown that this process, which appears to occur without energy transformation on the part of the capillary endothelial cells, leads to a rapid exchange of ions and lipid insoluble molecules across the capillary membrane. The rate of transfer is related to the size of the diffusing particles. Thus it has been calculated that in the guinea pig 140 per cent of the plasma water and 60 per cent of the plasma sodium exchange each minute with extravascular water and sodium (Flexner, Gellhorn and Merrell, 1942; Merrell, Gellhorn and Flexner, 1944). Much faster rates of exchange for similar small molecules have been calculated by Pappenheimer, Renkin and Borrero (1951) in their studies on the perfused hind limb of cats and dogs.

Bulk Flow. The exchange by bulk flow of fluid on the other hand amounts, in both directions, to about 2 per cent of the plasma flow (Pappenheimer, 1953). The rate

of nett fluid movement across the capillary wall is proportional to the difference between hydrostatic and osmotic forces acting across the capillary membrane. This concept was originally conceived by Starling (1895/96) following his experiments on the absorption of fluid from connective tissue spaces in the hind limbs of dogs. He regarded the capillary endothelium as a semi-permeable membrane which was impermeable to proteins but permeable to water and crystalloids. Filtration occurred at the arteriolar end of the capillary because "hydrodynamic" pressure was greater than the colloid osmotic pressure of the plasma proteins. However, as the "hydrodynamic" pressure at the venular end of the capillary was below the colloid osmotic pressure, absorption of protein free interstitial fluid occurred. Thus, according to the "Starling hypothesis" the direction and the rate of fluid transfer between plasma and tissue fluids are determined by three factors: (a) the hydrostatic pressures on each side of the capillary membrane; (b) the protein osmotic pressures of plasma and tissue fluids acting across the membrane; and (c) the physical properties of the capillary membrane considered as a mechanical filter.

The role of these factors in the trans-capillary flow of fluids was expressed in quantitative terms by Landis following his experiments on capillaries of the

mesentary of the frog (Landis, 1925/26, 1927a, 1927b, 1927/28). He found that the average pressure at the arteriolar end of the capillary was about 14.5 cm. water and at the venous end 10.0 cm. while the average osmotic pressure of the plasma proteins was 11.5 cm. of water. He showed that filtration occurred when the capillary pressure exceeded 11.5 cm. of H_2O and when it fell below this absorption occurred. A gradient of hydrostatic pressure along the course of the capillaries has also been demonstrated in other species, viz. in the mesentary of the rat and the guinea pig (Landis, 1930) and in the skin of man (Landis, 1929/31). This was regarded by Landis (1934) as circumstantial evidence which supported Starling's hypothesis on formation of lymph in the mammalian circulation. More recently the quantitative relations between capillary pressure, effective protein osmotic pressure and the rate of fluid exchange in the mammalian circulation were determined by Pappenheimer and Soto-Rivera (1948) using the isolated perfused hind limbs of dogs and cats. They showed that the mean pressure available for nett transfer of fluids across the capillary membrane was equal to the mean hydrostatic pressure minus the effective osmotic pressure of the plasma proteins and this principle held irrespective of the absolute values

of the osmotic and hydrostatic pressures. Thus Starling's hypothesis was established for the mammalian circulation.

Changes in capillary pressure and capillary blood flow appear to be related to the anatomy of the small circulation. The theory of Chambers and Zweifach (1946) concerning the control of the blood flow through the central channel and true capillaries is of interest in this regard but it does not appear to apply in all regions of the body. Webb and Nicoll (1944) and Nicoll and Webb (1946) for example, did not observe central channels in the capillary nets in the wing membrane of bats. However, it is true to say that changes in the diameter of the small vessels may occur only in vessels such as the arterioles and metarterioles which contain smooth muscle cells. These changes which are activated through the central nervous system, reflexly or by various local factors (Lutz and Fulton, 1958) alter the distribution of peripheral resistance between the arterioles and capillaries. As fluid exchange occurs chiefly through the wall of the blood capillaries factors which increase capillary pressure will increase the rate of formation of tissue fluid. Capillary pressure increases as a result of local heating, histamine injections and acute inflammation in the cutaneous circulation of man (Landis, 1929/31). The capillary pressure also rises after the injection of

acetylcholine (Kőniges and Ottó, 1937) or by directly stimulating the axone reflex (Landis, 1931). On the other hand, the capillary pressure decreases after severe haemorrhage or the injection of adrenalin (Landis, 1925/26) and following the local application of cold (Landis, 1929/31).

Because of the contractile nature of the arterioles, changes in capillary pressure cannot be predicted accurately by measurement of the arterial blood pressure (Bayliss and Starling, 1894). Venous pressure measurements however are known to predict accurately the level of pressure in the capillaries; capillary pressure is high in dependent parts of the body due to the hydrostatic pressure in the vertically placed veins or may be increased by direct compression of the veins (Landis, 1929/31).

"Pores". The evidence which has accumulated about the diffusion of lipid insoluble substances and ions across the endothelial membrane and about the forces involved in bulk flow has led to the "Pore Theory" of capillary permeability (Pappenheimer, 1953). This theory proposes that the passage of water and lipid insoluble substance takes place through many aqueous channels which penetrate the capillary wall. These are of a size sufficient to permit relatively unrestricted diffusion of substances of low molecular weight but which greatly restrict the free diffusion of substances of high molecular

weight such as the plasma proteins. The exchange of plasma proteins by diffusion appears to be negligible at physiological rates of filtration.

On the basis of experiments on the permeability of the blood capillaries of the hind legs of dogs and cats to lipid insoluble molecules, Pappenheimer and his colleagues postulated the existence of pores 30-45Å in radius and a population density of 1-2 x 10^9 per $cm.^2$ of capillary wall (cf. Pappenheimer, 1953). These findings are in conformity with the views of Chambers and Zweifach (1947) who proposed that the permeability of the capillary wall for lipid insoluble substances is so unselective, except for particle size, that it was more feasible to relegate their passage to some constituent of the cell wall other than the endothelial cell itself. They suggested that the material between the cells acted as a physical filter.

Electrophoretic analysis has shown that all the plasma protein fractions escape from the blood capillaries and enter the lymph (Perlmann, Glenn and Kaufman, 1943; Courtice and Morris, 1955). The albumin concentration in the lymph however is relatively higher than in plasma. It appears then that the smaller albumin molecules are filtered through the capillary wall more readily than the larger globulin molecules. This has been confirmed by

Wasserman and Mayerson (1952a) who studied the rate of exchange of labelled albumin and globulin between the plasma and the thoracic duct lymph of dogs. The transfer of foreign protein molecules of various sizes across the capillary membrane has been studied as well, and the results in general support the view that the rate of transfer of protein across the capillary wall is inversely proportional to the size of the molecule (Little and Dameron, 1943; Bott and Richards, 1941).

The rate of transfer of dextrans of various molecular weights (24,000-205,000) across the capillary endothelium has been shown to be inversely proportional to the effective diffusion diameter of the diffusing molecule (Grotte, Knutson and Bollman, 1951). Wasserman, Loeb and Mayerson (1955) working with dogs studied the distribution of dextran fractions between plasma and thoracic duct lymph. The dextran used had average molecular weights which varied from 10,600 to 412,000. They found a direct relationship between the molecular weight and the ratio of the concentration of dextran in the plasma to the concentration in the lymph. Since their calculations indicated a constant extravascular volume of distribution of dextran of molecular weight between 51,000 and 412,000 they suggested that all capillaries which leaked dextran of molecular weight of 51,000 or more

have the same proportion of small to large pores. Their results also suggested that the pores were not rigid but could increase in size if the plasma volume was increased.

The findings of Grotte (1956) on the permeability of the capillary endothelium in various regions of the body to dextran led to modification of the original theory of Pappenheimer. Grotte postulated that the capillary membrane contained a large number of pores of the size proposed originally by Pappenheimer. Grotte also postulated the existence of capillary "leaks" with a calculated radius of 116-350Å. These "leaks" would be few in number in areas drained by the cervical and leg lymphatics but numerous in the liver. Subsequently Grotte and his colleagues injected solid spherical particles of methyl-metachrylate 300-700Å in radius intravenously into dogs to determine the upper limit of permeability of the capillary endothelium in various regions of the body Grotte, Juhlin and Sandberg, 1960). The greater permeability of liver sinusoids relative to the capillaries in other regions of the body was clearly demonstrated. Juhlin (1960) also found that spheres of methyl-metachrylate up to 1,000Å in radius were transferred from plasma to bile in rabbits probably through the sinusoidal membrane into Disse's space. The findings of Courtice

(1959) that there was only very little escape of chylomicron particles of Svedberg floatation (S.f.) values greater than 400, and size up to 5,000Å in radius from the capillaries of the normal and scalded legs of the cat are in accordance with these findings.

On the basis of his experiments on the protein and lipid content of plasma and lymph from normal and scalded legs of rabbits, Courtice (1961) concluded that the permeability of capillaries to lipoprotein fractions was inversely proportional to their size. This was subsequently confirmed in similar experiments in which the lipoprotein fractions of plasma were separated by ultracentrifugation and the size of the dehydrated particles were measured electron microscopically (Courtice and Garlick, 1962). The size of these particles was found to be of the same order as that of the dextran particles used by other workers.

More recently the fine structure of the capillary endothelial membrane has been examined with the electron microscope (cf. Reynolds and Zweifach, 1959). Fundamental differences in the structure of capillaries in different regions of the body have been demonstrated (Bennet, 1958). The capillaries of muscle, skin and connective tissue which served as the experimental material for much of the work of Pappenheimer and his colleagues are of a type

which has a complete continuous basement membrane and narrow inter-endothelial cell junctions, and with endothelial cells which contain vesicles. No fenestrations or pores of the type postulated in the "Pore Theory" are demonstrable in this type of capillary (Bennet, Luft and Hampton, 1959). However intracellular fenestrations are visible running through the more attenuated portions of the capillary endothelial cells in the intestine, in endocrine glands and in the kidney. Hepatic and splenic capillaries which have no continuous basement membrane have large gaps between the endothelial cells. The available evidence suggests that vesicular transport may be significant in the transport of particles and solutes across the endothelial cell, particularly in those capillaries where there are no intracellular fenestrations or large intercellular gaps (Palade, 1953; Bennet, 1956; Moore and Ruska, 1957; Hampton, 1958). The term "cytopemphis" was suggested by Moore and Ruska to indicate vesicular transport through cells and the passage of various colloidal particles across the capillary endothelium by this mechanism has been described by Palade (1961) and Alksne (1959). On the basis of experiments in which colloidal gold and ferratin were used as tracers, Palade (1961) suggested the basement membrane was the main filtration barrier in fenestrated capillary membranes.

The ultra-structure of the lymphatic capillaries will be discussed later but it is relevant to point out at this stage that the great permeability of lymphatic capillaries is now thought to be due, at least in part, to the almost complete absence of a basement membrane.

Physiologists are aware of the possible implications of the findings of the electron-microscopists and discuss their results with these findings in view. It is interesting in this regard that Mayerson and his colleagues suggested that dextrans of molecular weights 250,000-412,000 might be transferred by "cytopemphis" as a possible alternative to transfer through a larger set of pores (Mayerson, Wolfram, Shirley and Wasserman, 1960). However, Grotte et al. (1960) did not consider that their experiments supported the view that "cytopemphis" was responsible for the transfer of methyl-metachrylate spheres across the capillary endothelium in any of the regions studied except possibly the liver.

If "cytopemphis" does play an important role in the transfer of larger macro-molecules across the capillary endothelium then an increase in the number (and size perhaps) of vesicles might be expected to occur in inflammation. This would be in accord with the increased capillary permeability to the larger lipoproteins demonstrated by Courtice (1961) in the leg after scalding.

However, Palade did not observe any change in the number and size of the endothelial vesicles of muscle venules after histamine administration but found that the intercellular space greatly increased in size. Florey (1961) found neither increase in the number or size of vesicles not any separation of the endothelial cells in the capillaries of the inflamed colon. To further confuse the picture the experiments of Alksne (1959) suggested that histamine increased the permeability of the capillaries of the skin by causing an increase in the size and number of vesicles in the endothelial cells. If the suggestion of Palade (1961) is correct that the basement membrane is the main filtration barrier, then alteration in permeability of the capillary membrane may be related to physico-chemical changes in the basement membrane, changes which may not be readily visible in electron micrographs. In any case there is little doubt that the original "Pore Theory" requires modification but any alternative theory must take into account the sieve-like properties of the capillary membrane.

FORMATION OF LYMPH

The disposition of the lymphatic capillaries in relation to the blood capillary bed was observed by Clark and Clark (1937) in transparent chambers inserted in the ears of rabbits. In some instances they found the

endothelium of the blood capillary and the lymphatic capillary virtually side by side while in others the lymphatic capillary was situated at some distance from the blood capillary. Tissue fluid is thought to be absorbed by a multitude of lymphatic capillaries situated in various positions in relation to the blood capillaries. The composition of lymph collected therefore, must be a mixture of interstitial fluid samples varying in composition from the relatively protein free fluid found at the arteriolar end of the capillary to the relatively protein rich fluid at the venular end.

Because it has been impossible to collect tissue fluid in normal animals, the relation between the protein concentration of the interstitial fluid to the lymph has been controversial (Yoffey and Courtice, 1956; Rusznyák et al., 1960). Direct evidence on this point was provided by Drinker, Field, Heim and Leigh (1934) who demonstrated a fair degree of similarity between the protein concentration of lymphoedema fluid and lymph. In view of these findings and the distribution of lymphatic capillaries around the blood capillary bed, it is now agreed by most workers that the protein concentration of lymph and an average sample of tissue fluid in any area is approximately the same.

Interstitial fluid is thought to spread through the ground substance of the connective tissue rather than

through preformed tissue spaces. There is usually a pressure gradient between the interstitial fluid and the lymph within the lymphatic capillary and when nett filtration increases this gradient increases (McMaster, 1946, 1947). It is considered that this gradient favours the continuous transfer of tissue fluid into the lymphatics. Although it seems likely that a pressure gradient is involved in the movement of tissue fluid into the lymphatic capillary, the process is undoubtedly complex and is probably related to the micro-anatomy and physico-chemical nature of interstitial substance (cf. Rusznyák et al., 1960).

In addition to removing the excess tissue fluid which forms, the lymphatics are known to absorb most, if not all, of the protein in the tissue fluid and return it to the general circulation. Evidence to this effect was presented by Lewis (1921). He injected horse serum subcutaneously into dogs and detected it in lymph and blood by serological methods. The results indicated that most of the injected protein was absorbed by way of the lymphatics. Similarly, Field and Drinker (1931a) showed that both horse serum and normal serum injected subcutaneously and intraperitoneally were absorbed rapidly by the lymphatics. More recently the almost quantitative absorption of T-1824 labelled serum albumin from the peritoneal and pleural cavities by the lymphatics was demonstrated by Courtice and his colleagues (Courtice and Simmonds, 1949;

Courtice and Steinbeck, 1950). However, there is evidence which suggests that proteins may also be transferred from tissue fluid into the blood directly when absorption of fluid is maximal as after plasmapheresis (Field and Drinker, 1931b). It appears however, that under normal conditions, the pressure relationships between blood, tissue fluid and lymph and the high degree of permeability of the lymphatic capillaries favour lymphatic absorption of the proteins from the tissue spaces.

Since the work of Starling in 1894, the capillaries of the body with the exception of those of the liver and intestines had been considered to be almost completely impermeable to protein. It was Drinker and his colleagues who first demonstrated that this was not the case and their experiments indicated that plasma proteins were continually leaking from the capillaries throughout the body (cf. Churchill, Nakazawa and Drinker, 1927; Field and Drinker, 1931a). Subsequently, the rate of protein exchange between plasma and lymph in various regions of the body was more precisely determined in experiments in which labelled plasma proteins were injected intravenously (Cope and Moore, 1944; Forker, Chaikoff and Reinhardt, 1952; Wasserman and Mayerson, 1952b; Korner, Morris and Courtice, 1954).

Thus there is a continuous filtration of fluid

and plasma protein which usually occurs at the arteriolar end of the capillaries. Estimates of the protein content of this filtrate have varied between 0.3 to 0.8 g. per cent for different areas (Landis and Gibbon, 1933; Landis, Krogh and Turner, 1932; Landis, 1934; Pappenheimer, Renkin and Borrero, 1951). The experimental evidence indicates that protein in the capillary filtrate cannot be reabsorbed directly into the blood stream as are fluids and crystalloids in the absorption process. The protein composition of the lymph is therefore considerably higher than that of the original filtrate.

An increase in the flow and protein output of lymph are known to occur under various conditions. This was demonstrated in experiments in which the capillary hydrostatic pressure was raised by intravenous transfusions of fluid (Wasserman and Mayerson, 1952c; Korner, Morris and Courtice, 1954) or by venous obstruction (Starling, 1894; Field and Drinker, 1931c; White, Field and Drinker, 1933). The protein concentration of the lymph was low under these conditions and this was probably due to a decrease in the absorption of fluid at the vemular end of the blood capillary. There is also a very rapid escape of fluid and protein from injured areas but in these circumstances the protein concentration in the lymph rises appreciably (Starling, 1894; Field and Drinker, 1931c; Cope and Moore, 1944; Courtice, 1946). The

large increase in the escape of protein through the capillary endothelium cannot be explained merely by an increase in capillary pressure and is undoubtedly associated with an increase in capillary permeability. On the other hand, a decreased flow and protein output and an increased protein content of lymph has been reported in various animals during barbiturate anaesthesia (Polderman, McCarrell and Beecher, 1943). These changes may be associated with a lowered capillary pressure in certain regions leading to decreased filtration of protein and fluid on the one hand and an increase in absorption on the other.

THE COMPOSITION OF LYMPH IN DIFFERENT REGIONS OF THE BODY

Due to variations in the forces causing capillary filtration and in the permeability of the blood capillary endothelium, the protein concentration of the lymph collected from various regions of the body differs greatly. Expressed as the percentage of the plasma protein concentration, the levels in the lymph of the dog are: liver 81, right lymph duct 69, heart 65, thoracic duct 60, intestinal duct 54, cervical duct 48 and skin 28 (Yoffey and Courtice, 1956). Lymph from all regions contains all the plasma protein and lipoprotein fractions (Page, Lewis and Plahl, 1953; Courtice and Morris, 1955). Although

the high protein concentration in liver lymph appears to be associated with a high degree of permeability of the hepatic simusoids, there is no evidence to suggest that the difference in protein content of the lymph from other regions is related to differences in permeability between the capillaries. The high protein content of lymph from the lungs for example is thought to be related to the low capillary pressure which is a characteristic of the pulmonary circulation (Drinker, 1942).

Phospholipids and cholesterol occur in the lymph from all regions of the body in lower concentrations than in plasma. These lipids are mostly associated with the globulins as lipoprotein complexes and their concentrations in lymph appear to parallel the protein concentration (Courtice and Morris, 1955). These workers also found chylomicrons in the cervical and leg lymph and showed subsequently that the number of chylomicrons in hepatic lymph was much greater (Morris and Courtice, 1956).

The electrolyte composition of plasma and lymph from various regions of the body is similar. However, in conformity with the Gibbs-Donnan equilibrium the concentration of calcium and magnesium in lymph is lower than in plasma while the concentration of chloride and bicarbonate is higher in lymph than in plasma. The concentrations of other substances of small molecular size

such as glucose, amino acids and non protein nitrogenous constituents in the lymph are on the whole the same as in the plasma. This is not surprising since it is now well established that the exchange of the smaller lipid insoluble molecules between plasma and tissue fluid occurs rapidly by diffusion.

In the fasting monogastric animal, the flow and composition of the intestinal lymph is largely governed by the transfer of fluids and solutes from the blood stream. However, during digestion and absorption, changes in the flow and composition of intestinal lymph occur as a result of the absorption of fluids and fats from the lumen of the intestine (Simmonds, 1954, 1955a, 1955b). This ingested fat is found in the lacteals in the form of chylomicrons. These chylomicrons are composed mostly of triglyceride together with small amounts of phospholipids and cholesterol and protein. The total concentration of lipid in the intestinal lymph may rise by a factor of 10 to 30 in monogastric animals during the absorption of fats (Bollman, Flock, Cain and Grindlay, 1950) and by a factor of about 2 in ruminants (Heath and Morris, 1962).

LYMPH PROPULSION

It has already been pointed out that the pressure gradient between interstitial fluid and lymph appears to

be responsible, at least in part, for the transfer of tissue fluid into the lymphatic capillary. The great permeability of the lymphatic capillary endothelium also allows the free entry of protein so that an osmotic gradient would not exist between the lymph and tissue fluid. The lymph is normally retained within the vessel because hydrostatic pressure in the interstitial fluid immediately outside the lymphatic capillary is relatively high. Lymph may move out of the vessels if the pressure inside them is increased by obstructing the main ducts (Courtice and Steinbeck, 1951).

When nett filtration increases, the pressure in the tissues rises and there is an increase in lymph flow. Small lymphatics have been shown not to collapse but to dilate when the tissue pressure is increased as in inflammation or oedema (Pullinger and Florey, 1935). The mechanism by which dilatation occurs has been attributed to the stretching of connective tissue fibres which are attached to the outside of the endothelial walls. These may hold the lymph capillaries open against even quite high pressure gradients. The mechanism thus facilitates lymph drainage under conditions where the formation of tissue fluid is increased.

The larger lymphatic trunks are known to contain smooth muscle cells and this means that they have the capacity to contract. Rhythmic contractions of a number

of lymphatic vessels (e.g. lacteals) have been observed in some species but not in others (Florey, 1927a, 1927b, 1927c; Pullinger and Florey, 1935; Smith, 1949). It was suggested that these rhythmic contractions provided an important intrinsic mechanism which contributed to the propulsion of lymph in some species. It is now generally agreed however, that in most species the transfer of lymph from one region to another is brought about by extrinsic forces such as muscular activity, respiratory movements and pulsation of blood vessels which alter tissue pressures. As the pressure in the lymph vessels increases, the lymph flows in the direction of least resistance. The provision of valves along the course of the lymphatics ensures that the direction of flow is always central.

Thus alterations in lymph flow may follow changes in the formation of lymph on the one hand or propulsion of lymph in the lymphatic vessels on the other. In the interpretation of experiments in which changes of lymph flow occur, the interplay of these factors must be borne in mind.

ANATOMY OF THE LYMPHATIC SYSTEM OF THE UDDER

Technique of Examination.

As lymphatic vessels are thin walled structures of small calibre and contain a fluid which in most regions is transparent, the smaller vessels are difficult to see.

For anatomical studies of the lymphatic system it is almost essential to inject opaque or coloured substances into the lymphatics prior to dissection. Great progress in this respect was made in the latter part of the 17th century by Nuck who introduced mercury into the main lymphatic trunks against the normal direction of lymph flow (cf. Nuck, 1692). This method has often proved difficult to carry out due to the presence of valves and many workers have filled the vessels from their origin by injecting a dye mass into the tissue spaces. Gerota (1896) made use of various pigments (e.g. prussian blue) in turpentine and sulphuric ether. This technique has been modified in various ways by later workers but in general the principle was the same.

Examination of the lymphatic system using either of these techniques is likely to be misleading because artefacts are common (cf. Rusznyák et al., 1960). More recently the lymphatic system of the kidneys of rabbits has been examined after ligation of the main efferent trunks (Kaiserling and Soostmeyer, 1939). The anatomy of the main lymphatic vessels and the lymph capillaries could be seen easily in histological sections of these preparations and artefacts were minimal. It appears that the anatomical studies on the lymphatic system of the mammary gland have been carried out after dye injection. Thus the description given in the following text must be

treated with some caution particularly with regard to the anatomy of the lymphatic capillaries and smaller ducts.

Since there appears to be nothing published on the anatomy of the lymphatic system of the udder of the sheep and goat the descriptions in the following text will refer primarily to the udder of the cow.

The Lymph Nodes.

The lymph vessels of the udder send afferent lymphatic trunks which usually pass through lymph nodes situated in the posterior and dorsal aspect of the udder. A number of workers have described the presence of at least two lymph nodes on each side of the udder. These are found in a superficial position between the ventral wall of the pelvis and the posterior part of the dorsal surface of the udder (Zietschmann, 1917; Baum, 1927; Emmerson, 1928; Sisson, 1941). El Hagri (1945a) examined the lymphatic system of 42 udders taken from slaughtered cattle. In general agreement with the earlier workers, his investigations revealed as many as four nodes on either side and the largest of these nodes, the crescentic superficial supramammary nodes of the two sides were sometimes united by a narrow isthmus. In one case the two nodes were found to form a single lymphoid mass.

One or two smaller accessory lymph nodes are found in some specimens on the lateral surface of the udder.

These nodes are situated either well forward, or posteriorly immediately below the supramammary nodes. A varying number of smaller, deep supramammary nodes are situated anterior to the superficial supramammary nodes in a superficial or deep position. El Hagri (1945a) found this group of nodes in 57 per cent of the specimens examined and in some cases these nodes were present on one side only.

The Lymph Vessels.

According to Zietschmann (1917) there is no other organ equal to the udder in possessing such a rich supply of lymphatics. These lymphatics have been subdivided in various ways by authors for convenience in description. The comprehensive system of classification proposed by El Hagri (1945b) has been adopted here, viz. (a) the lymphatics of the skin, (b) the lymphatics of the teat, (c) the lymphatics of the superficial parenchyma, and (d) the lymphatics of the deep parenchyma. This classification is based upon the area from which the main radicles are derived and does not signify completely separate drainage from these areas.

The lymphatics of the skin are derived from the lymphatic plexuses in the dermis. The smaller vessels unite to form larger trunks most of which course irregularly towards the node, although a few appear to

follow the larger blood vessels. Anastomosis is common and some of the collecting lymph vessels join either the radicles of the teats or the superficial parenchymal lymph trunks. It appears that the lymph from the skin of one side passes through the superficial supramammary node of that side, although Baum (1927) found that a certain degree of overlap of lymphatic drainage occurred in the region of the midline.

The teats are richly supplied with both blood and lymph vessels. Two or three large lymphatic trunks which are derived from radicles taking origin in all layers of the teat wall are found in each teat. The smaller lymphatics may be divided into two groups, a subcutaneous plexus and a submucosal plexus. Some of the branches of the submucosal plexus enter the deep parenchyma to join the deep parenchymal lymphatic trunks (El Hagri, 1945b). Most of the lymph from the teat appears to be carried away independently in large superficial vessels to enter the superficial supramammary nodes.

The main radicles of the lymph vessels of the superficial parenchyma originate in the superficial parenchyma of the udder but some of the lymph in these vessels comes from the teats. The lymph trunks formed by union of these radicles follow the lymphatic trunks of the teat to enter the superficial supramammary node.

The lymph from the central part of the udder is drained by a number of deep parenchymal lymph vessels which follow the branches of the mammary artery. The origin of the deep parenchymal lymphatics has been described in the cow by Regaud (1900), Tagand (1932) and in carnivores by Apostoleano (1925). There is general agreement by these authors that the lymphatic capillaries which are sometimes seen in the form of relatively large culs de sac, originate in the intralobular connective tissue. This means that the lymphatic capillaries are situated at some distance from the blood capillaries which closely invest the acini and intercalary ducts (Turner, 1952).

The final course of the lymph vessels in relation to the nodes has been a source of considerable controversy. Hess (1911) claimed that the superficial lymph vessels entered the lymph nodes at the base of the udder while the deep lymph vessels proceeded directly to the inguinal canal. On the other hand, many of the deep vessels were found to pass through the supramammary nodes by Zwart (1911). Zietschmann (1917) found that the superficial lymph vessels entered the deep supramammary nodes while Baum (1927) states that all the lymphatics of the udder pass through the superficial supramammary nodes. It is likely that the respective findings of these workers are correct

but because each made insufficient observations the enormous variation in the anatomy of the lymphatics and lymph nodes was not appreciated. El Hagri (1945b) in his more extensive series of observations noted the variations which he encountered. He confirmed the observation of Baum that all lymph vessels passed through the superficial supramammary lymph nodes but only when the deep supramammary nodes were absent. In cases where both superficial and deep supramammary nodes were present the following variations were found to occur, viz. (a) the lymph reaching each lymph node, left by efferent vessels which united with each other to form the main efferent lymph trunk or trunks of its own side; (b) the lymph reaching one of the lymph nodes of the deep group was carried away by an efferent lymph vessel which became an afferent to another node of the same group and in this way finally passed through the superficial supramammary lymph node; (c) the lymph left the superficial supramammary lymph node to enter the deep lymph nodes. This means that the flow of lymph was found to follow a course opposite to that described under (b).

The Lymph Drainage of the Udder as a Whole.

In about 77 per cent of specimens examined El Hagri found that lymph from either side of the organ left the lymph nodes of its own side independently of the other by way of 1-2 lymph trunks which accompanied the external

pudendal vessels to enter the inguinal canal. In the other 23 per cent of specimens lymph from either side or from one side only joined the lymph drainage of the other side and this was returned in a variety of ways. In two specimens the entire lymphatic drainage of the udder was effected by the lymphatics of one side. In any case lymph from the udder passes through internal iliac lymph nodes and then enters the lumbar trunks.

Histology of the Lymphatic Capillaries.

It is well known that the lymphatics are capable of absorbing particles many times the size of the largest protein molecules (Yoffey and Courtice (1956). The way in which these particles pass through the lymphatic capillaries has been controversial. There has been no satisfactory explanation for the great difference in permeability of blood and lymphatic endothelial membranes. A greater understanding of these problems has resulted from studies of the electron microscopic structure of lymphatic capillaries in various regions such as the intestine, diaphragm and skin (Palay and Karlin, 1959a, 1959b; French, Florey and Morris, 1960; Casely-Smith and Florey, 1961). It was considered that endothelial cells were more loosely bound together and adjacent cells might temporarily separate to allow the passage of macromolecules and particles. It was thought that the almost complete absence of basement membrane facilitates the separation of

these cells. It is significant in this regard that French et al. (1960) described the presence of red cells between adjacent endothelial cells of the lymphatics of the diaphragm during their absorption from the peritoneal cavity. Also Palay and Karlin (1959b) conclusively demonstrated that chylomicron particles could enter the interspace between lymphatic endothelial cells in the central lacteal of an intestinal villus.

Although the significance of vesicles in the transfer of particulate matter has not yet been fully assessed, the evidence presented supports the view that the great permeability of the lymphatic endothelium is associated with the separation of the endothelial cell junctions and also perhaps with the almost complete absence of a basement membrane.

CHAPTER 2.

MATERIALS AND METHODS

Experimental Animals. Merino ewes in good condition were used for the collection of mammary lymph. Most of the ewes were in early lactation (within 4 weeks of parturition) although some experiments were carried out on ewes at a later stage of lactation and also on some ewes in the completely dry state. The sheep were housed indoors and fed lucerne chaff or hay, oaten chaff and grain oats ad libitum. They also had access to water and a mineral lick.

Rabbits were used for the preparation of antisera against proteins in ewe's milk. The rabbits were 6 months old hutch bred laboratory animals which were fed on a standard pellet diet (17 per cent protein). Green clover was given daily and water was supplied ad libitum.

Biological Solutions. Normal saline contained 0.85 per cent sodium chloride weight for volume in distilled water. Calcium-magnesium saline (French, 1952) contained 8.5 g. sodium chloride, 0.0275 g. anhydrous calcium chloride, 0.192 g. magnesium chloride, 0.052 g. sodium borate, 1.203 g. boric acid made up to 1 litre with distilled water.

Anticoagulents. Heparin was used in the powdered form (Pularin, Evans Medical Supplies, 110-120 international units per mg.) as the anticoagulent for blood and lymph samples on which biochemical analyses were carried out. For other blood and lymph samples the cruder preparation (purified dried extract of lung, Boots, 63.8 international units per mg.) was used.

Albumin. Bovine albumin in crystalline form was obtained from Armour Ltd., London. This product is prepared by ethanol fractionation (Cohn fraction 5) and it is homogeneous on electrophoresis and is not precipitated by sodium sulphate solution.

Radioactive Iodinated Human Serum Albumin (^{131}I H.S.A.) This was obtained from the Radiochemical Centre, Amersham. This product was indistinguishable from unlabelled human albumin on electrophoresis and contained an average of 1 atom of iodine per molecule of albumin. The amount of radioactivity present in free form was less than 1 per cent of total activity. The specific activity of this preparation was very high so that only tracer amounts of albumin were used in the experiments.

Radioactive Iodine (^{131}I). This was obtained from the Radiochemical Centre, Amersham, as carrier free iodide in isotonic solution containing phosphate buffer (pH 7). The specific activity of iodine was greater than 10 curies per mg. of iodine.

T-1824. This was an Eastman Kodak (U.S.A.) product. The dye used was from a batch previously used by Courtice (1943) and had been tested for protein binding by Ogston (1943). This batch contained a minimum of red impurity.

GENERAL METHODS

Anaesthesia. Pentothal sodium (Abbott) was given intravenously to the ewes to induce anaesthesia and a No. 9 Magill's tube was passed into the trachea. Surgical anaesthesia was maintained with cyclopropane administered in closed circuit. The ewes usually recovered rapidly from the anaesthesia and were able to stand 1-3 hours after the completion of the operation.

Lymph Flow Rate and Sampling. Rates of flow were obtained by measuring the volume collected each hour over 12-24 hour periods. Observations on flow rates were also made over short periods of time (2-4 minutes) when the lambs were suckling or when the udder was being massaged.

Lymph samples for biochemical analysis were collected in centrifuge tubes containing powdered heparin. During the collection, the centrifuge tube was placed in a large plastic container which was filled with cracked ice. Each lymph sample was then centrifuged at 2,000 r.p.m. for 10 minutes. The supernatant was decanted into a test tube, stoppered and kept at $-4^{\circ}C$ until analysed.

Blood Samples. Blood was obtained from the ewes

directly from the jugular vein. When frequent blood sampling was necessary, a transflex catheter (external diameter 2.5 mm.) was passed through a 10 gauge bleeding needle into the jugular vein. The needle was removed leaving the cannula in situ. A nylon stilette (diameter 1.2 mm.) was placed inside the catheter along its entire length and removed only when collections were made. Blood samples were obtained from the catheter with a syringe and transferred to a centrifuge tube containing powdered heparin. Plasma was separated by centrifugation at 2,000 r.p.m. for 10 minutes and stored frozen at -4°C. In rabbits, blood samples were taken from the marginal ear vein, allowed to clot and the serum separated and stored at -4°C.

Intravenous Injections. In sheep, intravenous injections were made into the jugular vein either directly or by way of the transflex catheter. Intravenous injections in rabbits were made into the marginal ear vein.

DETAILED METHODS

Biochemical Analyses.

Volatile Fatty Acids (V.F.A.) were determined by a modification of the method of Annison (1954). To 1.5 ml. of plasma or lymph were added 12.5 ml. of distilled water and 1 ml. of freshly prepared 25 per cent metaphosphoric acid. The precipitated proteins were removed by

centrifugation and the supernatant containing the V.F.A. was decanted into a clean test tube. The pH of 5 ml. of the supernatant was adjusted between 2.8 and 3.0 and transferred to a Markham still. The steam was bubbled through at a fast rate and two 50 ml. samples of the distillate were collected, the second sample being used as the titration blank. Care was taken during the course of the distillation to ensure that there was an adequate flow of water through the condenser so that all the steam had condensed by the time it had reached the collecting vessel. After collection of the distillate the steam was turned off so that the remnants of the sample were sucked out of the sample container and the rinsing process was repeated 3 times with distilled water. Duplicate distillations on each plasma and lymph sample were carried out in this way. Each 50 ml. of distillate was transferred to an EEL titration vessel and 0.2 ml. of a phenolphthalein solution (0.04 per cent in 95 per cent ethanol) was added. Carbon dioxide was removed by bubbling a stream of nitrogen through the solution for 5 minutes. The titration vessel was then closed with a close fitting perspex stopper through which was drilled a hole sufficiently large to accommodate a plastic tube (Intramedic P.E. 10) which delivered the sodium hydroxide solution into the titration vessel. A "604" filter was used and the change in O.D. of the solution was followed on the spot galvanometer.

Sodium hydroxide (0.01N) was slowly added by means of an "Agla" micrometer syringe (Burroughs Wellcome & Co.) and during the titration the solutions were mixed with a magnetic stirrer. The titration assembly is depicted in Fig. 1. An empirically chosen optical density was used as an end point for all titrations and sodium hydroxide solution was added until this optical density was reached. The acidity attributable to V.F.A. was obtained by subtracting the titration reading of the blank from the reading of the first distillate.

The distillation procedure appeared to be quite satisfactory. Recovery of added acetic acid varied between 97-100 per cent. However, the accuracy of duplicate titrations of the amounts of V.F.A. in the equivalent of 0.5 ml. of sheep plasma and lymph agreed only within ±10 per cent.

Free Fatty Acids were determined by a modification of the method of Dole (1956). The free fatty acids were extracted by shaking 1 ml. of plasma or lymph with 5 ml. of extraction mixture (redistilled isopropyl alcohol 40 parts, redistilled heptane 10 parts and 1 N. sulphuric acid, 1 part). After standing for 10 minutes, 2 ml. of heptane and 3 ml. of water were added and the phases separated out without centrifugation. One ml. of the heptane phase containing the free fatty acids was transferred to a specially designed titration vessel (see Fig. 2), together

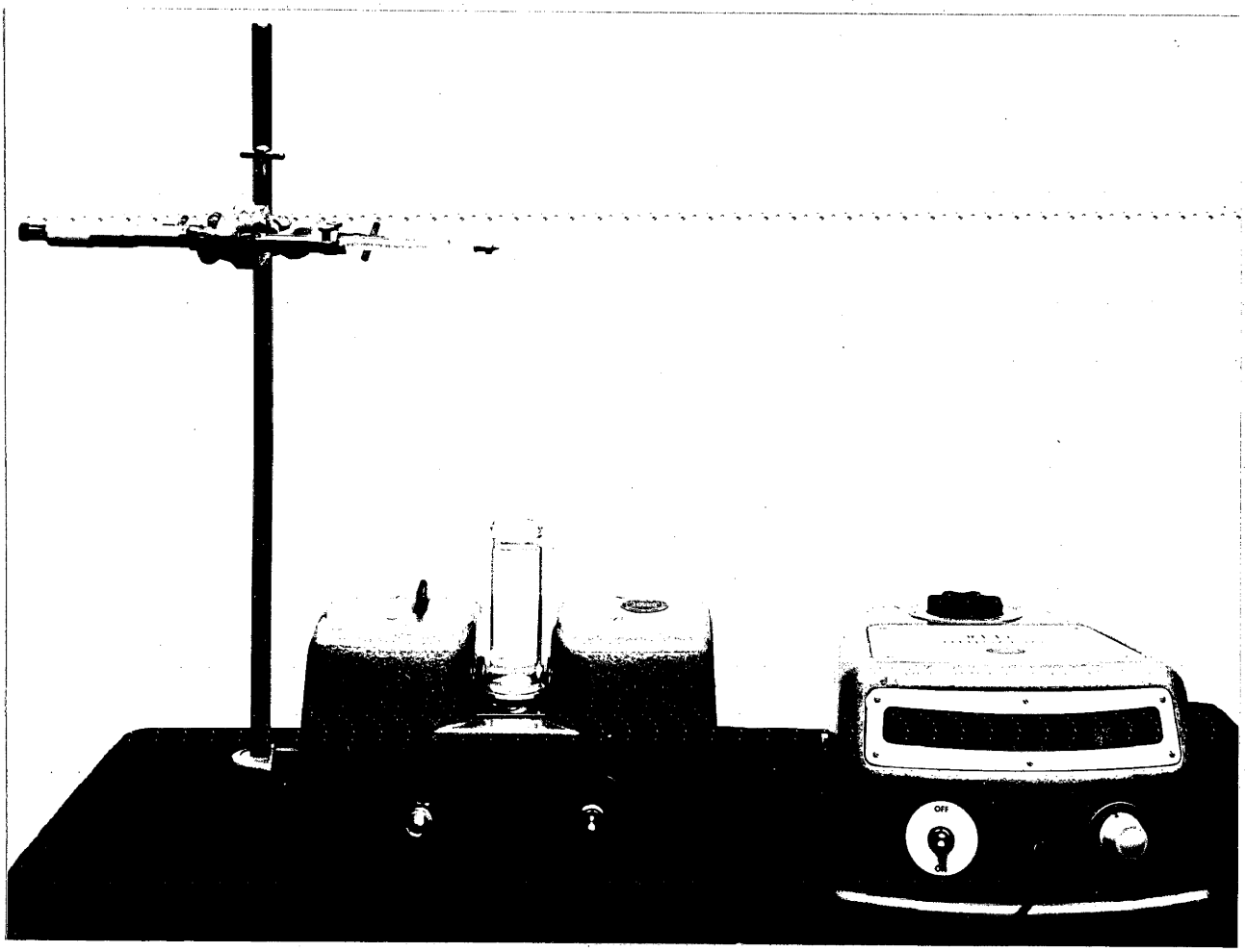

Figure 1.

Showing EEL titration assembly and "Agla" micrometer syringe which were used for the titration of V.F.A.

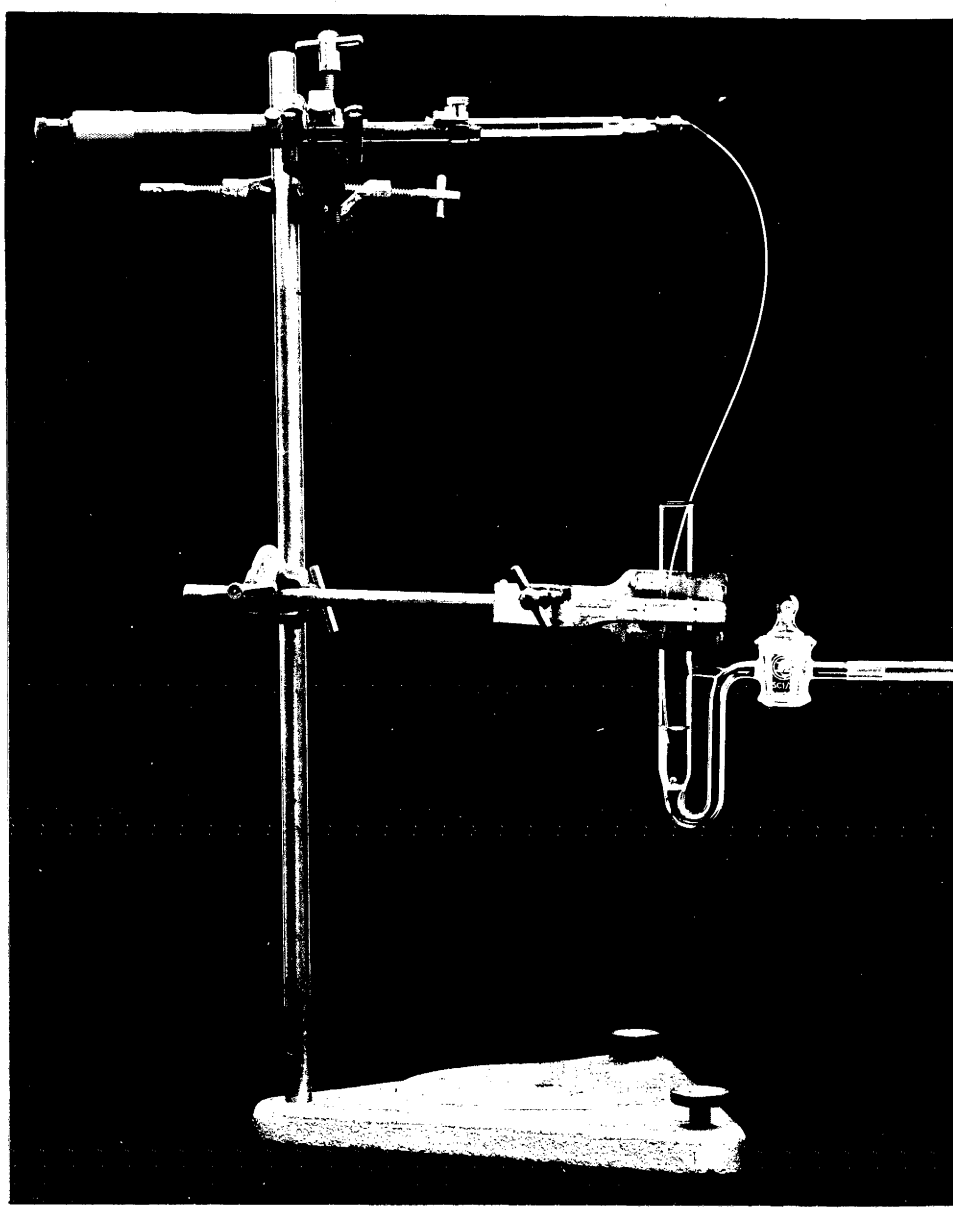

Figure 2.

Showing the apparatus for titration of free fatty acids. Nitrogen is bubbled through the contents of the titration vessel by way of a small hole at the bottom of the vessel.

with 1 ml. of the indicator solution (0.01 per cent thymol blue in 90 per cent ethanol). A stream of nitrogen was bubbled through the solutions in the titration vessel to eliminate carbon dioxide and to mix the two phases. This was continued during the course of the titration. Titration was carried out by means of an "Agla" micrometer syringe using 0.01 N. sodium hydroxide solution which was introduced into the contents of the titration vessels through a fine polythene tube (Intramedic P.E. 10) until the first appearance of a yellow-green colour. At the beginning and end of each series of estimations titrations were carried out on blanks (1 ml. heptane plus 1 ml. indicator solution) and on a standard palmitic acid solution in heptane. The blank titrations were usually negligible and were not taken into account.

The extraction procedure described was found to be efficient and recoveries of added radioactive palmitic acid which had been added to plasma varied between 99-101 per cent. The accuracy of the titration procedure was also highly satisfactory and duplicate titrations agreed within ±2.5 per cent.

Total Esterified Fatty Acids (T.E.F.A.) were measured by the method of Stern and Shapiro (1953). Samples of plasma and lymph (0.2 ml.) were extracted with 10 ml. of boiling 3:1 ethanol-ether mixture. The precipitated protein was removed by filtration through No. 43 Whatman

filter papers and 3 ml. of the filtrate was taken and reacted with 0.5 ml. hydroxylamine hydrochloride solution (0.5 M. solution in water) and 0.5 ml. of sodium hydroxide solution (3.5 N.). The mixture was allowed to stand for 20 minutes at room temperature. The solutions were then acidified with 0.6 ml. of hydrochloric acid solution (1 volume of concentrated hydrochloric acid to 2 volumes of water) and then 0.5 ml. 0.37 M. ferric chloride in 0.1 N. hydrochloric acid was added. The colour intensity developed under these conditions was proportional to the concentration of fatty acid esters in the ethanol-ether extract. Solutions containing 0.5, 1.0, 2.0 and 4.0 uEq. of triacitin were used to obtain a standard curve (see Fig. 3). The colour developed was measured in a Beckman Spectrophotometer at 525 mu.

Phospholipids and Inorganic Phosphate were determined on 0.2 ml. samples of plasma and lymph by the method of Zilversmit and Davis (1950). Phospholipids were precipitated together with the proteins by the addition of 3 ml. of trichloracetic acid (10 per cent) to a graduated test tube containing a 0.2 ml. sample and 3 ml. of water. After standing 1-2 minutes, the mixture was centrifuged and the supernatant containing the inorganic phosphate was transferred to another tube and 0.8 ml. perchloric acid (60 per cent A.R.) was added. Digestion of the precipitate was accomplished by adding 1 ml. 60 per cent perchloric acid

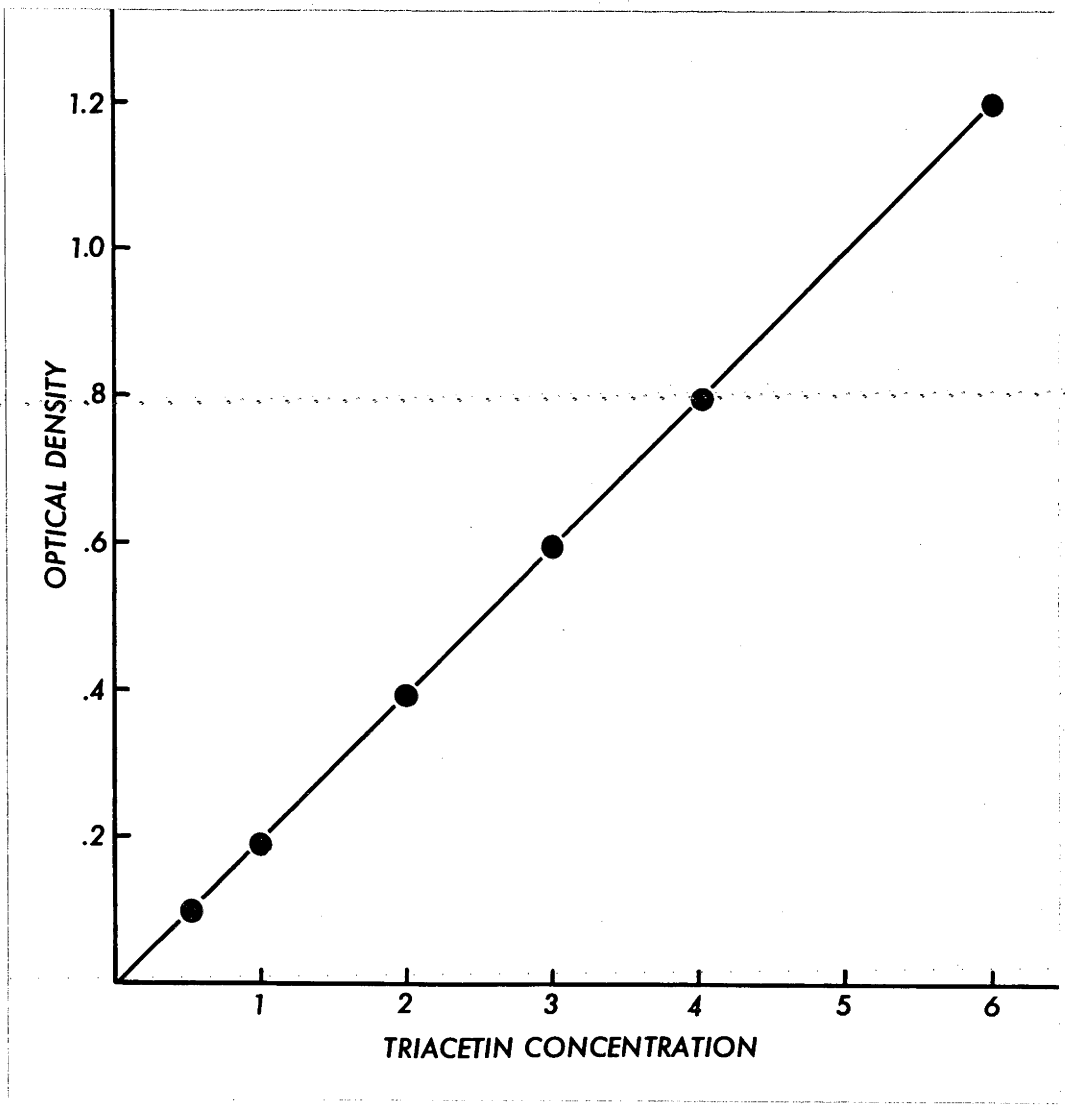

Figure 3.

The calibration line for the estimation of total esterified fatty acids.

Wave length 525 mu.
Regression coefficient = 5
Calculation: Optical Density of unknown X $\frac{5 \times 50}{3}$ = concentration of T.E.F.A. in uEq/ml.

and boiling until the solution was clear. After cooling, 5-6 ml. of water was added followed by 1 ml. 4 per cent ammonium molybdate solution. Colour development was achieved by the addition of 1 ml. of 2/5 dilution of reducing solution (0.5 g. 1:2:4 amino naphthol sulphonic, 30 g. sodium bisulphite and 6.0 g. sodium sulphite made up to 250 ml. with water). The colour was allowed to develop for 20 minutes and optical densities were read at 810 mu. The supernatant containing the inorganic phosphate was treated in the same way as the phospholipids after these were digested. A standard curve (see Fig. 4) was constructed from dilutions of a standard phosphorus solution containing 1 ml. phosphorus per ml. (4.391 g. potassium dihydrogen phosphate per litre).

Cholesterol Estimations. The method of Pearson, Stern and McGavack (1953) was used. The Lieberman-Burchardt colour is developed without prior extraction of the cholesterol. To 0.1 ml. of plasma or lymph was added 0.1 ml. glacial acetic, 0.5 ml. p-toluenesulphonic acid and 1.5 ml. acetic anhydride. After cooling, 0.2 ml. concentrated sulphuric acid was added and the mixture shaken vigorously. The optical density of the resultant colour was measured at 550 mu. A standard curve was derived from standard amounts of cholesterol in acetic acid (Fig. 5).

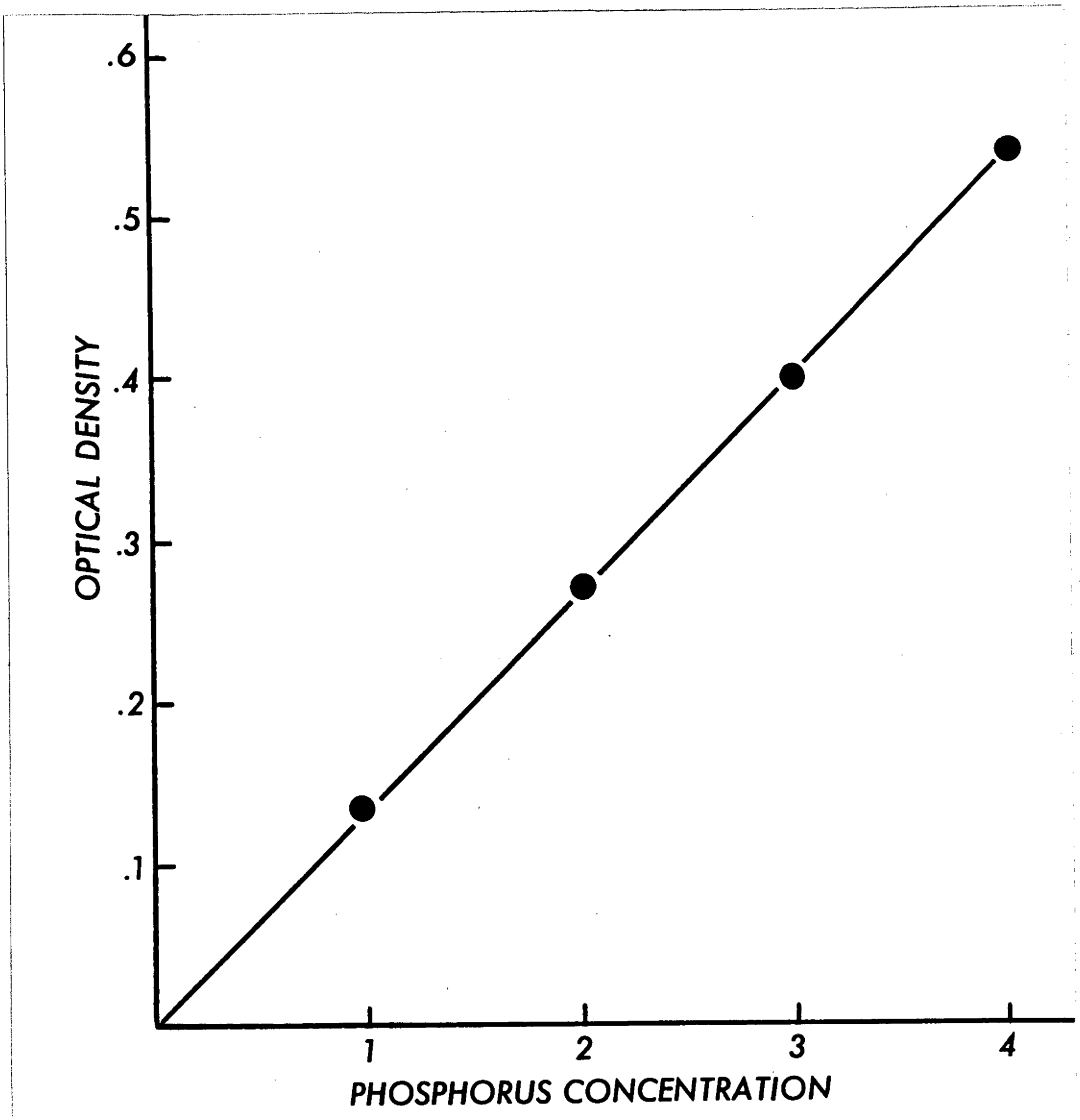

Figure 4.

The calibration line for the estimation of phospholipid phosphorus.

Wave length 810 mu.
Regression coefficient = 7.4
Calculation: Optical Density of unknown X 7.4 X 5 = concentration of phospholipid phosphorus in mg per cent.

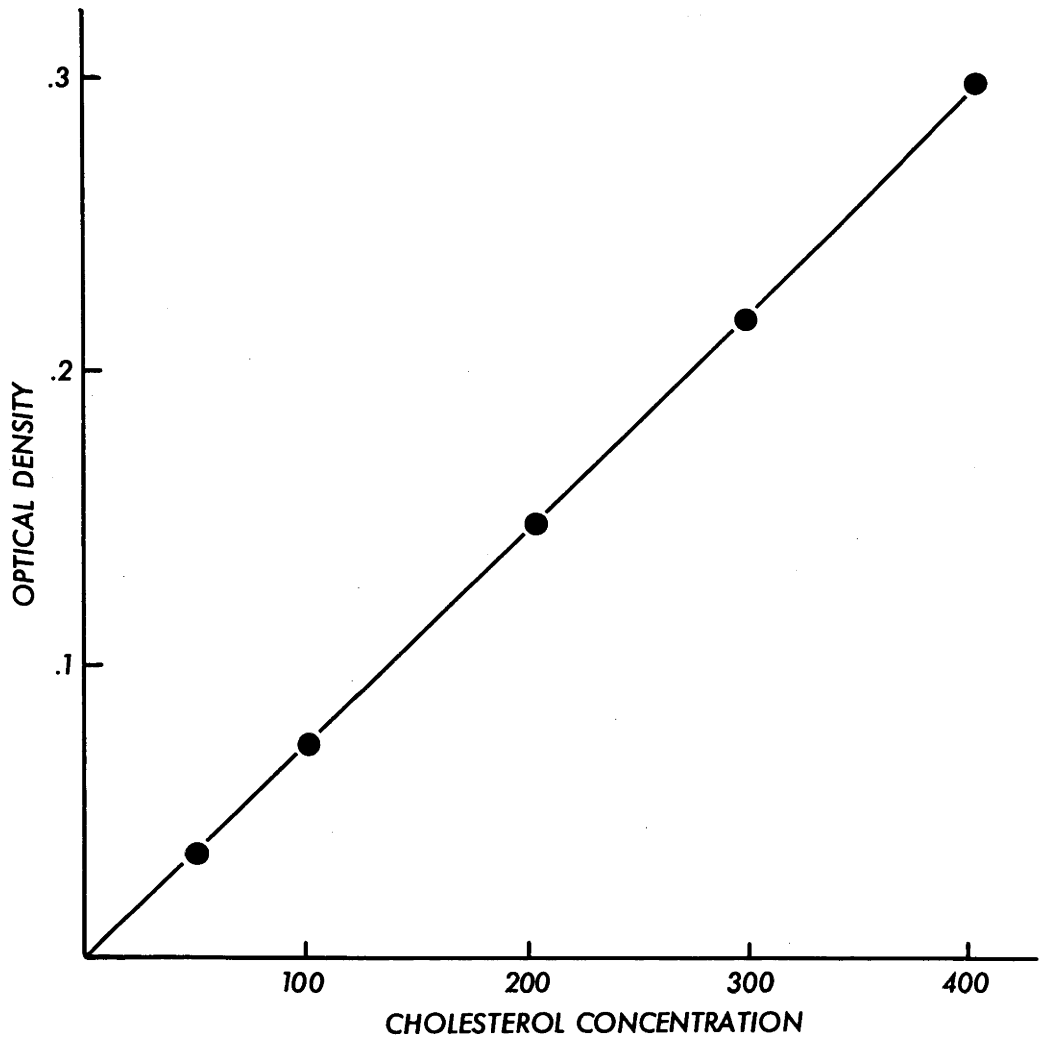

Figure 5.

The calibration line for the estimation of cholesterol.

Wave length 550 mu.
Regression coefficient = 1,350.
Calculation: Optical Density of unknown X 1,350 = concentration of cholesterol in mg per cent.

Protein Determinations. Determinations of the total protein and albumin concentrations of plasma and lymph were carried out using the method of Gornall, Bardawill and David (1949). Globulin concentrations were obtained by difference. To 0.5 ml. of the sample of plasma or lymph was added 9.5 ml. of 22.6 per cent sodium sulphate solution and mixed. For the determination of total protein 2 ml. of the mixture were transferred to another test tube to which was added 8 ml. of Biuret reagent (1.5 g. cupric sulphate, 6.0 g. sodium potassium tartrate, 300 ml. 10 per cent sodium hydroxide solution made up to 1 litre) and the colour allowed to develop for half an hour. For the determination of albumin, the precipitated globulins were removed from the remaining original mixture by filtration through Whatman No. 42 papers and 8 ml. of Biuret reagent was added to 2 ml. of the clear filtrate and the colour allowed to develop for half an hour. A calibration curve was obtained from solutions containing various concentrations of bovine serum albumin (see Fig. 6).

The optical density of the colour developed in this way was measured at 540 mu. Since the turbidity of plasma and lymph was minimal ether extraction was not necessary.

Correction of Biuret Optical Density for T-1824 Interference. Protein determinations were frequently carried out in plasma and lymph which contained appreciable

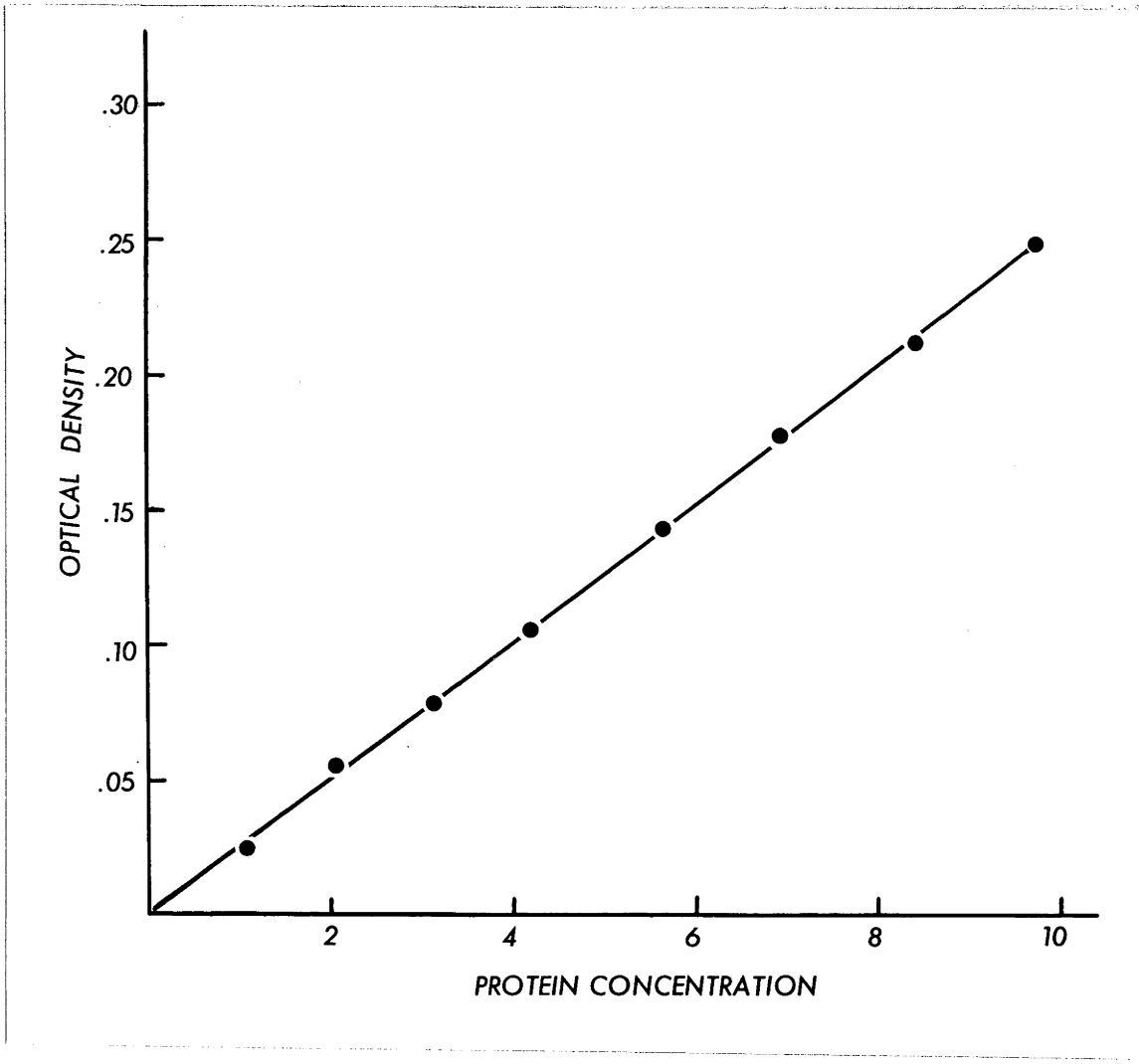

Figure 6.

The calibration line for the estimation of protein.

Wave length 540 mu.
Regression coefficient = 39.47
Calculation: Optical Density of unknown X 39.47 = concentration of protein in g. per cent.

concentrations of T-1824. It was found that the presence of T-1824 in the samples elevated the optical density attributable to protein alone. A correction factor was derived by measuring the O.D. at 540 mu. of a number of T-1824 standard solutions treated as in the Biuret method (see Fig. 7). Biuret protein estimations were carried out on solutions containing a standard concentration of protein to which standard amounts of T-1824 solution had been added. These experiments indicated that by application of the correction factor, protein concentrations of solutions containing protein and a wide range of T-1824 concentrations could be measured accurately.

Sodium and Potassium estimations were carried out using a Beckman Direct Reading Flame Photometer.

Glucose was measured by the colorimetric method of Nelson (1944).

Calcium was measured by a modification of the micro-titration method of Wilkinson (1957) using an EEL titration apparatus with a 707 filter and spot galvanometer. To a small EEL titration vessel was added 0.1 ml. of plasma or lymph, 2.5 ml. of freshly prepared murexide reagent (6 mg. ammonium purpurate to 100 ml. with ion free water) and 0.2 ml. 1 N. aqueous tetramethyl ammonium hydroxide.

Titration was carried out by means of an "Agla" micrometer syringe using E.D.T.A. solution (containing

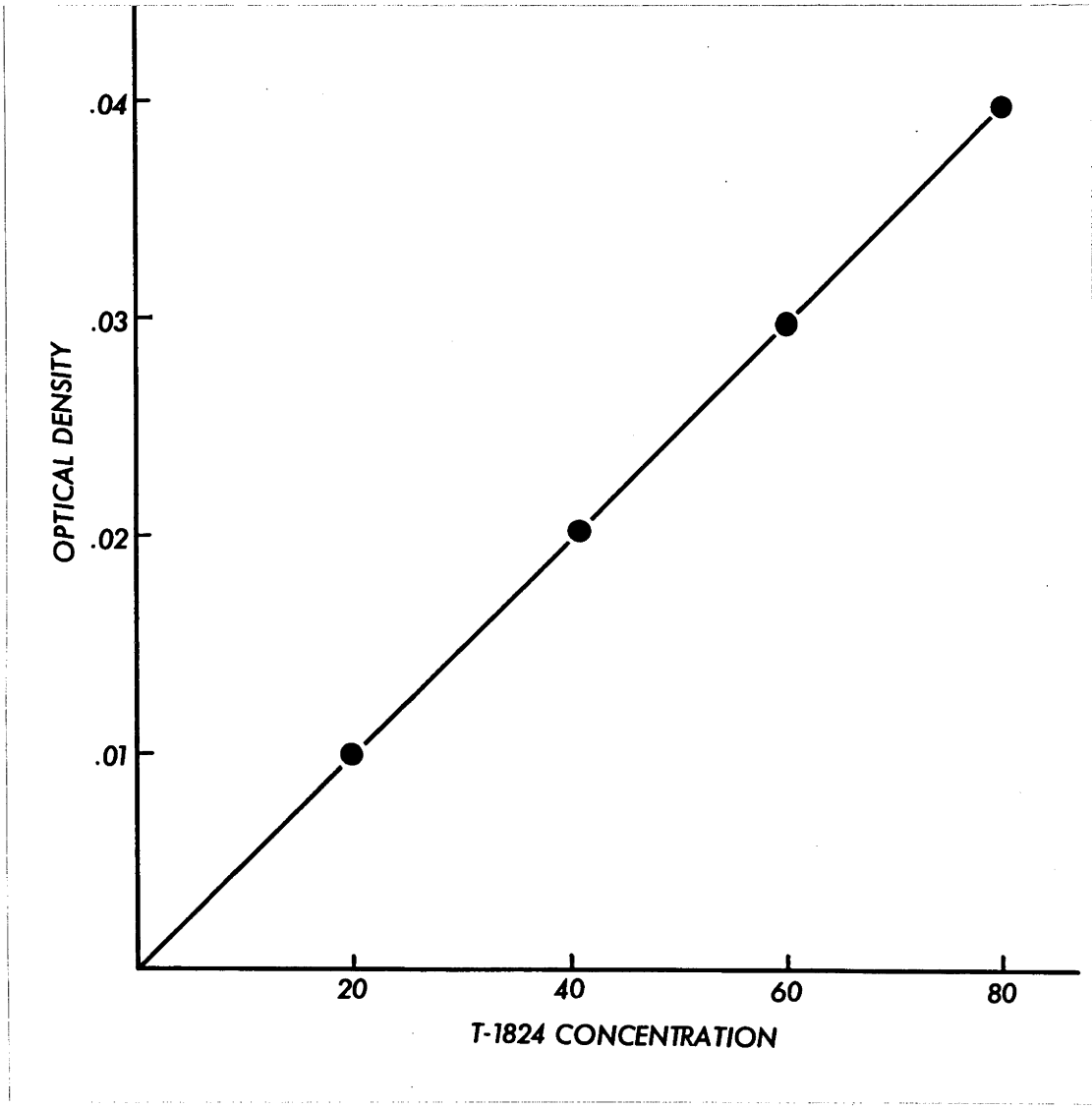

Figure 7.

Calibration line for correction of Biuret optical density in the presence of T-1824.

Wave length 540 mu.
Regression coefficient = 0.49.
Calculation: T-1824 concentration of sample (mg/litre) X 0.49 = Optical Density attributable to T-1824.

4.804 g. of the disodium salt of ethylene diamine tetra acetic acid per litre) which was introduced into the contents of the vessel through a fine polythene tube. The contents of the vessel were thoroughly mixed by the magnetic stirrer during the course of the titration. As each small increment of E.D.T.A. was added, the optical density as recorded on the spot galvanometer increased until a maximum reading was obtained and subsequent additions of E.D.T.A. actually resulted in a slight fall in optical density. The maximum optical density was taken as the end point. A standard solution of calcium (0.250 g. pure dry calcium carbonate in 6 ml. 1 N. hydrochloric acid, the excess acid driven off by boiling and the calcium chloride solution made up to 1 litre with distilled water) containing 10 mg. per 100 ml. was treated in a similar manner. This method gave very reproducible results and duplicate titrations agreed within ±1.5 per cent. Recovery of added calcium to serum samples varied between 98-100 per cent.

Magnesium. Magnesium in 0.1 ml. of plasma and lymph was estimated by the method of Orange and Rhein (1951). The proteins were precipitated by the addition of 1.1 ml. of 10 per cent trichloracetic acid solution. To 1 ml. of the supernatant was added 1 ml. of 0.1 per cent solution of polyvinyl acetate (Dupont's low viscosity type), 1 ml. titan yellow (7.5 mg. per 100 ml. water) and 2 ml.

7.5 per cent sodium hydroxide solution. The optical density was measured at 560 mu. A standard curve was derived from dilutions of a standard solution of magnesium (10.0935 g. magnesium ammonium phosphate in 1 litre of 0.1 N. hydrochloric acid solution) containing 1 mg. of magnesium per ml. (see Fig. 8).

Chloride Determinations were made by means of electrometric titration with silver ion (Cotlove, Trantham and Bowman, 1958).

T-1824 Estimations were carried out on 0.5 ml. samples of plasma and lymph using a modification of the method of Allen (1951). The dye was separated from the albumin by adding 1 ml. 25 per cent Teepol (Shell) to samples of plasma and lymph and after 10 minutes the mixture was poured onto a cellulose column (pulped Kleenex tissue) 1 cm. deep and the dye adsorbed onto the cellulose. The column was then washed successively with normal saline, ether and normal saline. The dye was eluted with 1:1 acetone water mixture and the volume of the eluate made up to 10 ml. The optical density of the T-1824 solution was read at 620 mu. A standard curve was obtained from a series of T-1824 solutions (see Fig. 9). Reproducible results were obtained with the method; duplicate determinations agreeing within $\pm$2 per cent and recovery of added dye varied between 97-100 per cent.

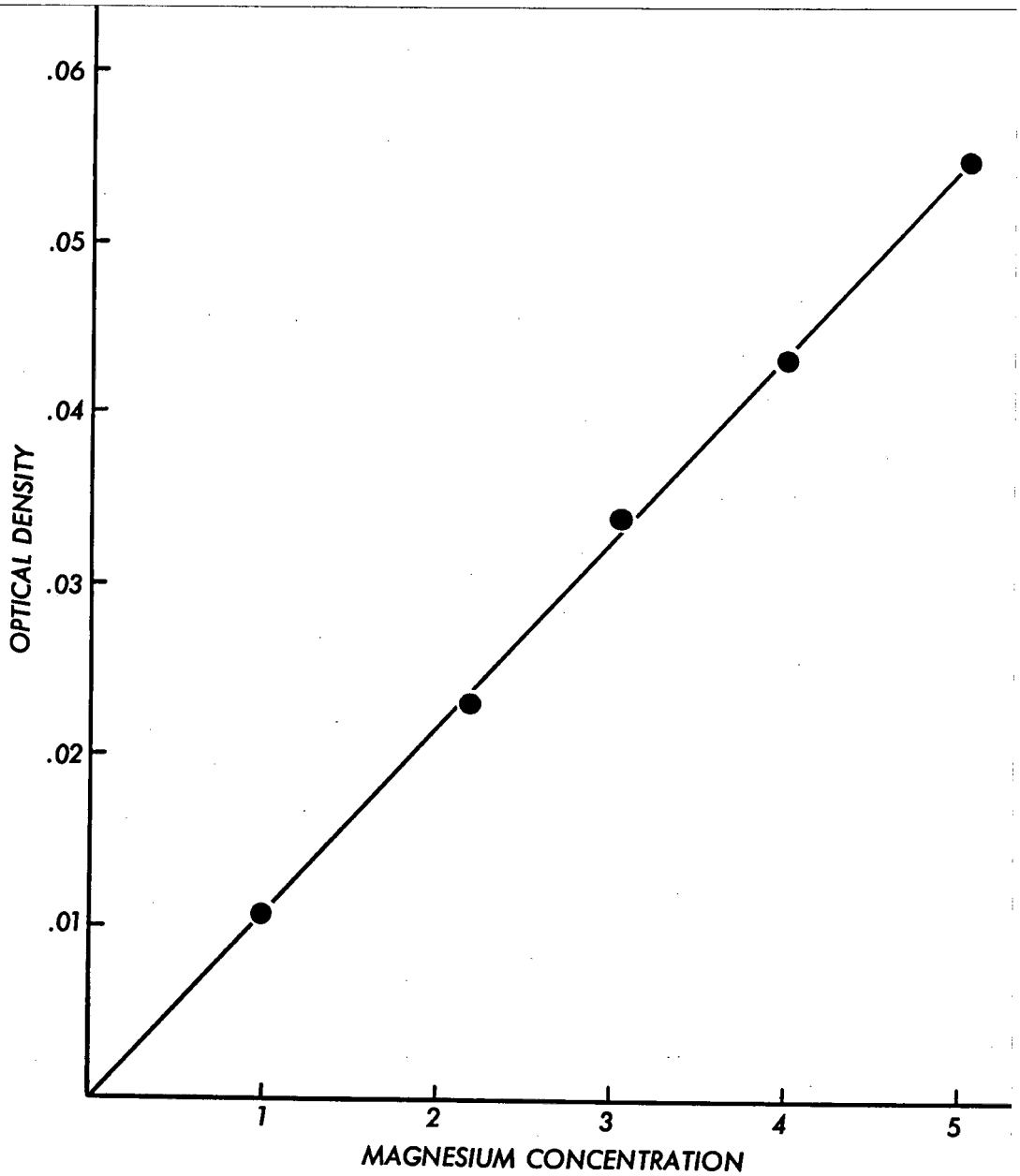

Figure 8.

The calibration line for the estimation of magnesium.

Wave length 560 mu.
Regression coefficient = 95
Calculation: Optical Density of unknown X 95 = magnesium concentration in mg. per cent.

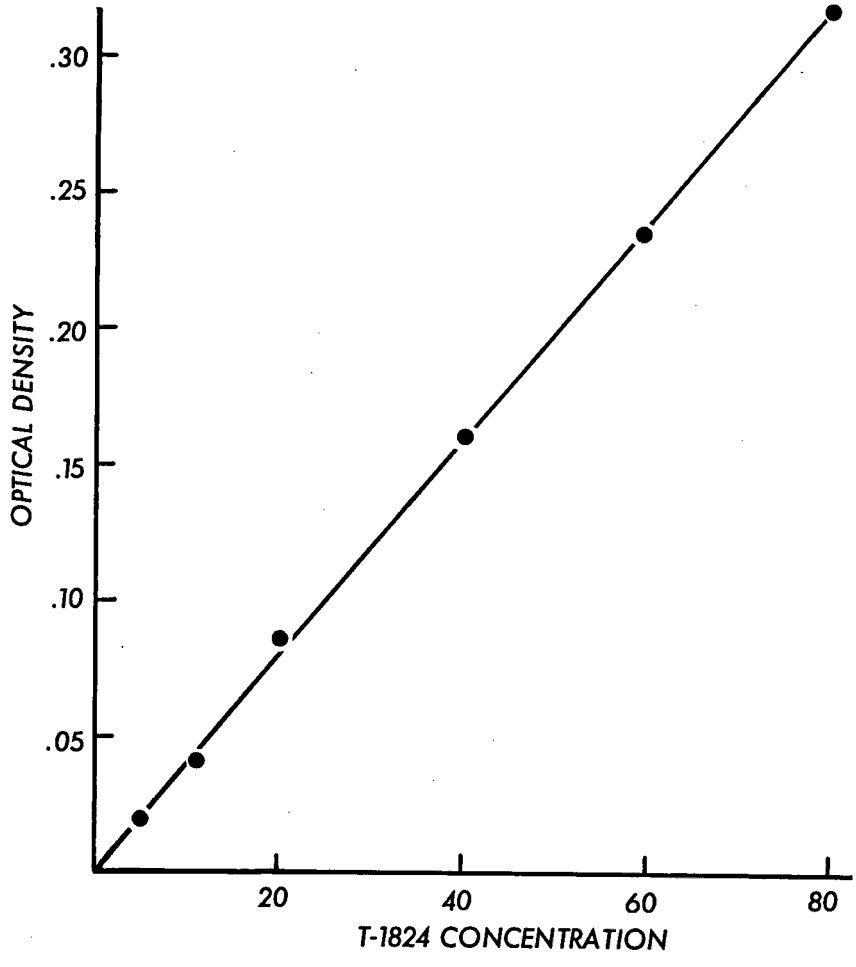

Figure 9.

The calibration line for the estimation of T-1824.

Wave length 620 mu.
Regression coefficient = 244
Calculation: Optical Density X 244 = T-1824 concentration in mg./litre.
of unknown

Fractionation of Casein for Specific Activity Measurements.

In one of the experiments in which ^{131}I casein was injected intravenously, casein was fractionated from the milk and its specific activity determined (cf. Rowland, 1938; Barry, 1952). To 10 ml. of the skim milk was added 80 ml. of water at 40°C and 1 ml. 10 per cent acetic acid. After standing 10 minutes, 1.0 ml. of 1 N. sodium acetate solution was added. The precipitated casein was recovered by centrifugation, washed in acetate buffer, pH 4.6 (50 ml. 0.2 N. acetic acid plus 5.0 ml. 0.2 N. sodium acetate) and again recovered by centrifugation. The precipitate was twice dissolved by the slow addition of 0.1 N. ammonium hydroxide and reprecipitated with 1 per cent acetic acid treated with hot ethanol 3 times to remove fat and then washed with ether and dried. The dried casein powder was then dissolved in borate buffer, pH 8.0 (3.0 ml. 0.05 M. sodium borate plus 7.0 ml. 0.2 M. boric acid). The solution contained approximately 1 g. casein in 100 ml. of borate buffer.

Production of Antisera against Skim Milk.

One ml. of ewe's skim milk was injected intravenously into each of 4 rabbits once a week for 5 weeks. 20 ml. of blood was taken from each rabbit 8 days after the last injection and the serum separated after the clot

had contracted. The antibody titre of the sera was determined by a tube precipitin test. 0.2 ml. of serum was added to precipitin tubes containing 0.2 ml. of a series of skim milk dilutions in normal saline and incubated in a water bath at 37°C for 20 minutes. Precipitation was present in all tubes at the lower dilutions, the heaviest precipitation being found in the tubes containing 1/10 to 1/20 dilutions of skim milk. This serum contained a sufficiently high titre of antibody for use in the gel diffusion precipitin test.

Specific Precipitation in Agar.

The modified Ouchterloney gel diffusion precipitin test described by Mansi (1957) was used to identify milk proteins in lymph. 25 ml. of a medium (15 g. Davis powdered agar, 16 g. sodium chloride, 50 ml. of 10 per cent phenol saline solution, 0.03 g. methyl orange made up to 1 litre with distilled water and dissolved by heating in an autoclave, then filtered through gauze) was poured into each Petri dish. Seven wells on each plate were cut out from the medium by means of an appliance which consisted of 7 stainless steel cutting tubes arranged so that 6 tubes formed a circle around and equidistant from a central 7th tube. The distance between the tubes was 0.75 cm. and each tube had a diameter of 0.5 cm. The tubes were fixed in perspex and rubber and were connected to a vacuum pump. After removal of the cylinders of

medium by means of this apparatus, the bottom of each well was sealed with a drop of melted medium.

The central well was filled with antiserum and the antigens (milk, 1/10 dilution, plasma and lymph) were placed in the peripheral wells. Plates were left at room temperature and read every 24 hours under strong reflected light.

Fractionation and Labelling of Casein.

Fat was removed from ewe's milk by centrifuging at 3,000 r.p.m. for 30 minutes at 0-5°C. Hydrochloric acid solution was added slowly to the skim milk which was stirred continuously. The pH was recorded using a Beckman direct reading pH meter and hydrochloric acid solution was added until a pH of 4.6 was obtained. Casein was thus precipitated and was separated from the whey by centrifugation. The whey was decanted and the precipitated casein was washed several times by resuspending in a large volume of water, the casein being recovered each time by centrifugation. The casein was then redissolved by resuspending the washed precipitate in a small volume of water and adding 0.5 N. sodium hydroxide until a pH of 7.3 was reached. The procedure of acid precipitation and washing was repeated and the casein again redissolved by the addition of alkali. Five ml. of casein solution (containing 0.1-0.15 g. casein) were made up to 10 ml. by the addition of 4 ml. calcium-magnesium saline and 1 ml.

freshly prepared 0.2 M. carbonate-bicarbonate buffer (pH 10). Approximately 0.25 mc. carrier free ^{131}I was added to a test-tube containing 2.0 ml. carbon tetrachloride, 2.5 ml. of 10^{-4} M. potassium iodide and 0.05 ml. 2 N. sulphuric acid. Hydrogen peroxide (0.2 ml.) was then added and the 2 phases were mixed by gentle shaking for half an hour. The water phase was then sucked off leaving the slightly pink carbon tetrachloride phase which was then washed twice by shaking with distilled water. Ten ml. of the previously prepared casein solution was added to the carbon tetrachloride containing the iodine and the 2 phases mixed very gently for 10 minutes. The casein solution was then transferred to another tube and was centrifuged to remove any insoluble matter. The centrifuged casein was dialyzed first against running water for 12 hours to remove free iodine and then against calcium-magnesium saline for 12-14 hours. ^{131}I casein of satisfactorily high specific activities (2-6 x 10^6 counts per mimute per ml.) was obtained with this method. Electrophoresis on cellulose acetate in Tris buffer (pH 8.9) showed 2 fractions, α - κ- and β-casein which were radioactive. The radioactive casein solution was sterilized by injecting through a cellulose filter of .45 u pore size (Millipore Filter Corp., Bedford, Mass., U.S.A.) into a sterile container, and kept in the refrigerator at $4^{o}C$. The radioactivity present in free form was less than 2 per cent

of the total activity.

This procedure did not produce uniform results as some preparations contained a varying percentage of apparently denatured protein. The suitability of each preparation was tested by injecting a tracer dose intravenously into a sheep to establish the behaviour of the labelled material in the circulation. Of a total of 12 preparations, 2 were found to contain a high proportion of denatured protein. Following the intravenous injection of these apparently denatured preparations it was found that most of the radioactivity was removed from the circulation in the first 2-3 minutes and after 10 minutes, the radioactivity remaining in the plasma was not bound to protein.

Radioactive Assay.

Radioactivity measurements were made at the completion of each experiment. These were carried out on 0.2 ml. samples using a thin mica end-window G.—M. tube. The samples were spread over circular plastic planchets 2 sq. cm. in area, together with 0.1 ml. of a plating fluid which contained 1 ml. of 1 in 1,000 Teepol, 5 ml. glycerine, 5 ml. 10 per cent trichloracetic acid, 0.5 g. tragacanth and water to 100 ml) and covered with a lens tissue the exact size of the planchet. The radioactivity bound to protein in the samples of plasma, lymph and milk was determined by measuring the radioactivity in

trichloracetic acid precipitates. To 0.2 ml. samples were added 2 ml. of water and 3 ml. of 12.5 per cent trichloracetic acid and the precipitated protein was collected on a circular disc of filter paper (Whatman No. 1) which fitted exactly into a perforated plastic planchet (2 sq. cm. in area). This planchet was housed in a specially constructed filtration funnel. Radioactivity in the free form was measured in the supernatant which was obtained after centrifugation of the protein precipitated from solution by trichloracetic acid. The supernatant was spread and dried in 0.2 ml. portions over a planchet containing a lens tissue of exact size. Protein containing and protein free fractions of urine samples were obtained in a similar way after the addition of carrier bovine albumin to the urine. A solution of purified casein (approximately 1 per cent) was prepared as described and the radioactivity in a 0.2 sample was measured. The radioactivity measurements were carried out after the contents of the plastic planchet had been evaporated to dryness. The activities of replicate samples agreed within 5 per cent.

Electrophoresis.

Zone electrophoresis was carried out on cellulose acetate (Oxoid electrophoresis strips) using a perspex casette described by Fazekas de St. Groth, Webster and Datyner (1962). Strips of cellulose acetate (2.5 cm. x 18cm.

were soaked first in buffer and then mounted horizontally within the apparatus on perspex carriers of appropriate size. A modified tris buffer (Aronsson and Grönwall, 1958) pH 8.9 was used. It had the following composition: tris-hydroxymethyl aminomethane ("Sigma 7-9") 78.65 g. E.D.T.A. (acid) 7.8 g. and boric acid 5.98 g. made up to 1 litre with water. Samples of plasma and lymph (approximately 1.5 u litres) were applied to the strips of cellulose acetate in a straight line by stroking with the micropipette. Separation of the protein fractions was carried out for 4 hours at 100 volts and approximately 0.5 milliamps per strip.

The protein fractions were fixed and stained by a modification of the method of Kohn (1958). The strips were fixed in 20 per cent sulphosalysilic acid and stained in 0.5 per cent amido black solution (0.5 g. amido black in 100 ml. of solution containing 45 per cent methanol, 45 per cent water and 10 per cent acetic acid). Excess dye was washed out by immersing in a solution containing 90 per cent methanol and 10 per cent acetic acid. The strips were then placed in water and later dried under an infra-red lamp. In order of their greatest mobility, the following fractions could be detected under these conditions: albumin, α_1, α_2, α_3, β_1, β_3, and γ globulins (nomenclature according to Perk and Lobl,

1960). In most separations the α_1 and α_2 globulins which comprise only a small proportion of the total protein were not clearly visible and clear separation of the β_1 and β_3 globulin was not achieved in many cases (see Fig. 10).

Bacteriology

Staphylococcus aureus. The strain of Staphylococcus aureus used was recently isolated from a field case of bovine gangrenous mastitis. It was received on a nutrient agar slope, and was subcultured in Brain-Heart Infusion broth (B.H.I.) Difco. It showed typical β-haemolysis and colonial morphology on horse blood agar.

Streptococcus agalactiae. This strain was also recently isolated from a field case. It was received in cooked meat medium and was subcultured in B.H.I. medium. It gave characteristic non-haemolytic colonies on blood agar.

Preparation of Inoculating Suspensions. Sub-cultures of the 2 strains of organism were made in B.H.I. medium and grown for 14 hours at 37°C. In the case of Streptococcus agalactiae the growth from 40 ml. of medium was centrifuged and resuspended in 10 ml. of B.H.I. in order to obtain a sufficient concentration of organisms for injection. The cultures were briefly dispersed in

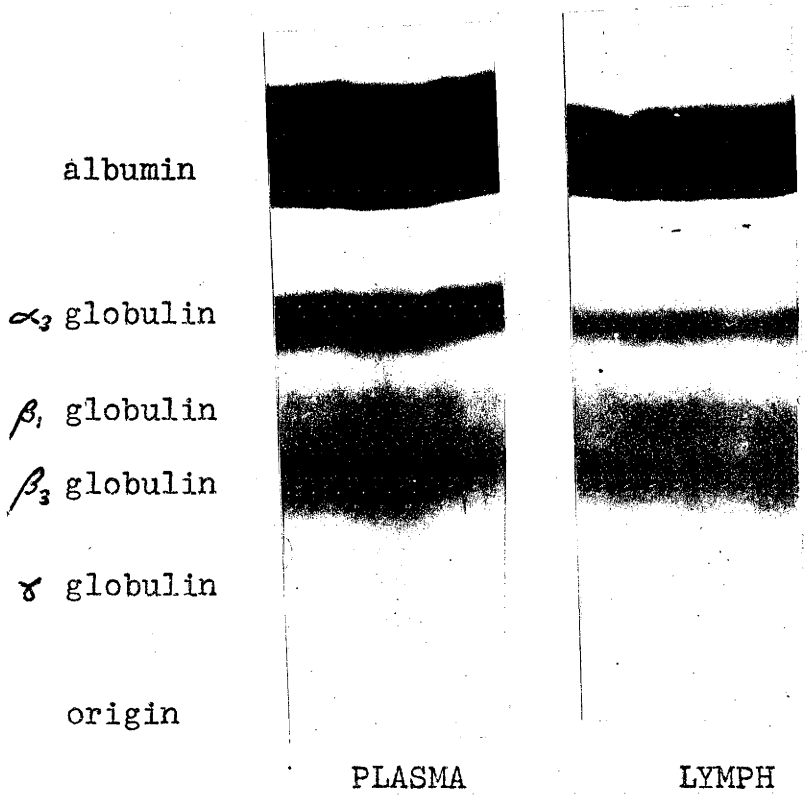

Figure 10.

Showing the electrophoretic patterns of plasma and mammary lymph. The lymph contains all the protein fractions present in plasma.

a Mullard (M.S.E.) ultrasonicator and counted in a Petroff-Hauser bacterial counting chamber. The right hand side of the udder was infected after the milk was stripped out. A sterile polythene cannula was introduced into the cavity of the gland and an inoculum containing approximately 5×10^8 bacteria was injected in 10 ml. of B.H.I. broth.

CHAPTER 3.

SURGICAL TECHNIQUES FOR THE COLLECTION OF LYMPH FROM THE MAMMARY GLAND OF UNANAESTHETIZED SHEEP

In order to study various aspects of lymphatic physiology, it is necessary to obtain animal preparations from which lymph can be collected over relatively long periods of time under physiological conditions. Bollman, Cain and Grindlay (1948) described a method of collecting thoracic duct, liver and intestinal lymph from unanaesthetized rats over a period of several days. The dog has also been used as an experimental subject for the collection of thoracic duct and liver lymph by several groups of workers (Cain, Grindlay, Bollman, Flock and Mann, 1947; Glenn, Cresson, Bauer, Goldstein, Hoffman and Healey, 1949; Grindlay, Cain, Bollman and Mann, 1950; Brown and Hardenbergh, 1951; Rampone, 1959). These techniques lead, in most cases, to a drastic loss of weight due to the continuous drainage of large amounts of proteins, fluid and electrolytes from the lymphatic fistulae.

In this chapter the surgical procedure which has been used to collect mammary lymph from sheep over periods

of weeks, under essentially physiological conditions, is described.

Anatomical Considerations.

The supramammary lymph nodes in the sheep are situated on each side of the udder above its posterior border usually as one or two large nodes. The efferent lymphatics ran laterally and anteriorly along the lateral surface of the udder together with the external pudendal vessels and nerves. In some animals a lymph node is located anteriorly along the course of the external pudendal vessels and when this is present the main mammary lymph duct arises as the efferent lymphatic from this node. The main lymph duct passes through the inguinal canal with the external pudendal artery and vein and joins the lumbar lymph trunk within the peritoneal cavity. The number and size of the main lymphatic ducts varies in different animals. In some animals only 1 large duct was found while as many as 7 ducts were found in other animals. In 3 animals of a total of 50 which were studied, most if not all the lymph from both glands was carried away in 1 or 2 large efferent trunks which were situated on the left hand side only.

Surgical Technique.

The skin was incised at the level of the inguinal canal and the loose areolar tissue between the medial surface of the thigh and the abdominal wall separated by blunt dissection. The external pudendal vessels were seen

ventral and anterior to the insertions of the adductor femoris and pectineus muscles and beneath the aponeurosis of the external and internal oblique abdominal muscles. This aponeurosis was cut through over the external pudendal vessels for about 2-4 cm. and the lymph trunks were dissected free from the accompanying vessels and adipose tissue and tied off close to the inguinal canal. The largest duct was cannulated with a transflex catheter (external diameter 1.3-1.75 mm.) which was tied securely in place. Fig. 11 shows the dissection of the mammary lymphatics. The operation was less extensive than this dissection as the skin incision was carried back to a point just behind the inguinal canal. The mammary lymph nodes were not exposed at the operation. It was important to cut through the aponeurosis of the external and internal oblique abdominal muscles parallel with the direction of the fibres otherwise the wound was difficult to suture. Unless the incision was closed correctly, the underlying peritoneum was left exposed and herniation of the peritoneal contents was likely to occur. The incision in the aponeurosis was carefully sutured and the catheter was led through a tunnel running dorsally and anteriorly under the skin to emerge through a stab wound in the skin, dorsal to and in front of the coxal tuber. The lymph was collected in a plastic bottle sutured to the skin of the flank. The day following the operation, the ewe was put back with

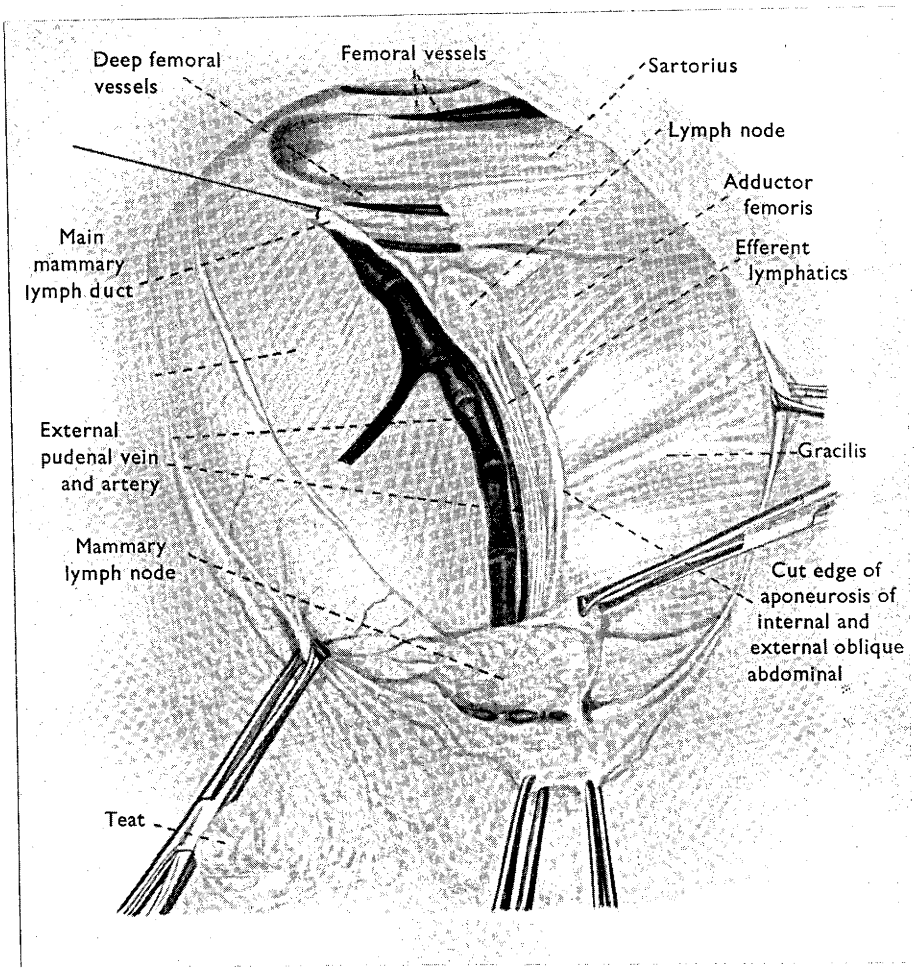

Figure 11.

The dissection of the mammary lymphatics. This dissection shows a single mammary lymph node situated in the substance of the udder with efferent lymphatics running to a second smaller node nearer the inguinal canal. The main mammary lymph duct arises as the efferent lymphatic from this node.

the lamb which was able to suckle without affecting the catheter. Fig. 12 shows a ewe in which the mammary lymph duct has been cannulated. In the experiments in which radioactive isotopes were used the ewes were kept in metabolism cages (see Fig. 13).

Area Drained by Mammary Lymph Duct.

In order to define the anatomical areas from which the lymph was derived, injections of T-1824 in saline were given subcutaneously in various regions around the udder. The lymph was observed subsequently for the appearance of dye. When a solution of T-1824 was injected into the skin in front of the udder, dye appeared in the mammary gland lymph 30-60 minutes later. Similarly, injections of T-1824 into the skin and subcutaneous tissues of the escutcheon and the vulva showed that lymph drained from these areas through the mammary lymph duct. When T-1824 was injected into the medial aspect of the thigh the dye appeared slowly in the lymph and only in very low concentration. When T-1824 was injected into one side of the udder or the surrounding tissues no dye appeared in the lymph draining the opposite half of the udder and it appeared that the lymph from each half of the udder drained, in most cases, separately through the mammary lymph ducts on the respective sides.

The surgical procedure described has been used to cannulate the mammary lymphatics in 50 ewes. In 10 of

Figure 12.

A ewe with a mammary lymphatic duct fistula. The photograph was taken 16 days after operation. The lamb is suckling from the half of the udder which is drained by the lymphatic fistula. Note the bottle almost filled with lymph.

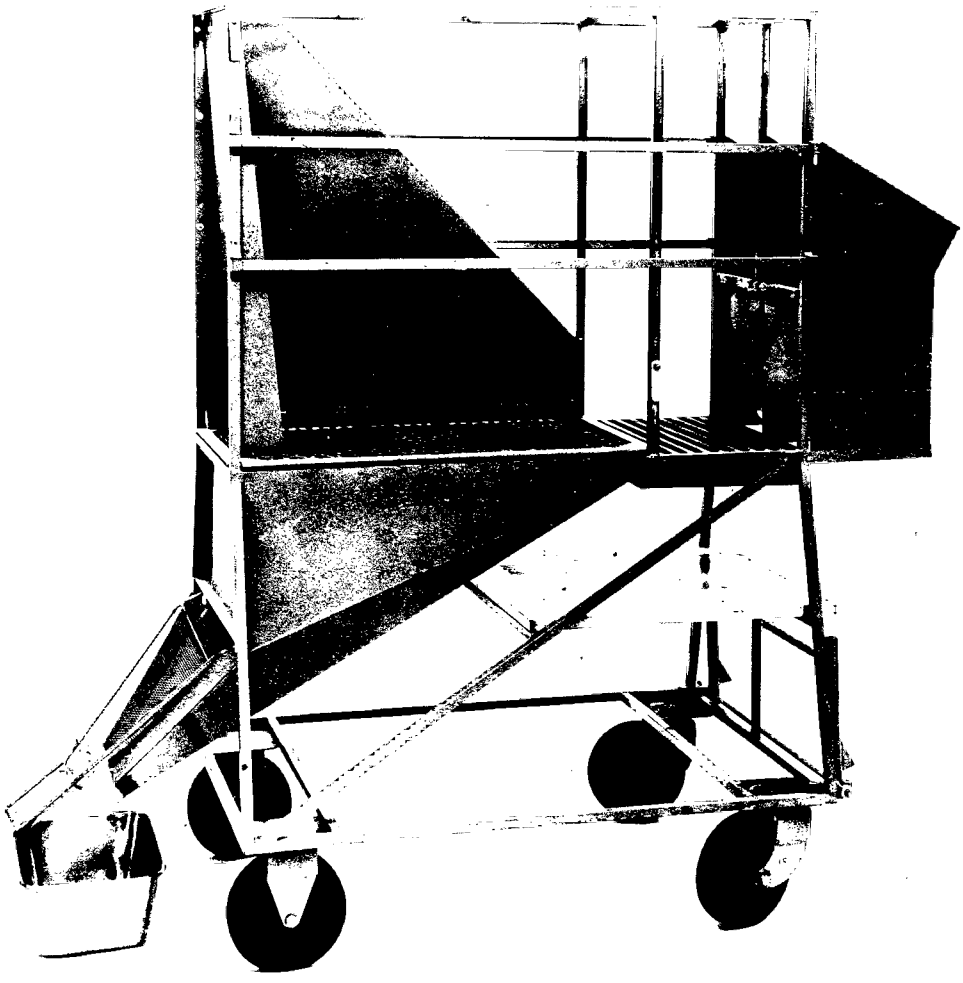

Figure 13.

The metabolism cage in which many of the experimental sheep remained during the course of experiments. The cage was constructed so that urine and faeces could be collected separately.

these the lymphatics on both sides of the udder were cannulated. Lymph flowed for periods up to 10 weeks at rates which varied from 0.7 to 50 ml. per hour. Clots formed occasionally in the catheters and they occurred more commonly immediately after the operation when the rate of flow was low. The clots could usually be removed by suction or by dislodging them with a fine brass wire passed up the catheter. At the end of the experiment the catheter could be pulled out of the duct and the ewe returned to the paddock. The lymph ducts closed spontaneously.

CHAPTER 4.

THE FLOW AND COMPOSITION OF LYMPH FROM THE MAMMARY GLAND IN MERINO SHEEP

It was considered that comparisons between the composition of the plasma and lymph in lactating and non-lactating animals might provide an indication of any specific changes which occur in the lymph in response to lactation. Since it has been shown that part of the lymph which is collected from the mammary lymph duct comes from tissues other than the udder it was necessary also to obtain some measure of the contribution of lymph from these areas. An attempt was made to estimate the size of the tissue fluid pools drained by the mammary lymph duct in both lactating and dry ewes and thus to obtain a measure of the extent to which lactation increases the volume of interstitial fluid in the mammary gland through which metabolic exchanges between blood and milk must occur. Four of the ewes used for these experiments were in early lactation, one was in mid-lactation, two were at the end of lactation and three were completely dry. The ewes were kept in the same pen as their lambs and the lambs could suckle at will. The lactating ewes were dried off after

the initial data had been collected and further observations were made on the rate of lymph flow and on the transfer of protein between the plasma and mammary lymph in these animals when dry.

Lymph Flow and Composition.

The rate of lymph flow was low immediately after the operation but increased when the animals recovered from the anaesthetic and stood up and walked around. Table I shows the average hourly rate of flow of lymph from each ewe. The rate of lymph flow was correlated with the stage of lactation and with the amount of milk produced. Ewe 5 was a very heavy milker and produced almost a litre of lymph in 24 hours. This ewe lost considerable weight during her lactation and reared a large lamb. The lymph flow from this ewe when she was dried off 2 months after the operation was only about 10 per cent of the flow during lactation.

The flow rate varied considerably throughout the day and was related to the ewe's activities. When the ewes were lying quietly, the rates of flow were lower than when they were moving about and feeding. There was a dramatic increase in lymph flow when the lambs suckled the ewes or when their udders were massaged. The increase in flow occurred promptly and was maintained over a period of 2-5 minutes.

The concentrations of the various constituents

TABLE I.

Mean and standard errors of rates of flow of lymph from the mammary lymph duct measured over a 24-hr. period. Maximum rates of flow recorded in the lactating ewes during suckling are also given.

Sheep No.	1		2	3		4	5		6	7	8
Stage of Lactation	Middle	Dry	Dry	Early	Dry	Early	Early	Dry	Dry	Dry	Dry
Mean rate of lymph flow during 24-hr. period.	8.4	1.9	5.1	19.9	2.6	21.4	40.9	5.5	4.2	5.0	2.3
ml. / hr.	±0.80	±0.16	±0.43	±0.96	±0.27	±1.03	±3.6	±0.55	±0.26	±0.35	±0.18
Maximum rate of lymph flow recorded during suckling	46.0	-	-	95.0	-	80.0	180.0	-	-	-	-
ml. / hr.	-	-	-	-	-	-	-	-	-	-	-

in the plasma and lymph are given in Table II. The concentrations of esterified fatty acids, phospholipid and cholesterol were all higher in plasma than in lymph. The concentrations of esterified and free fatty acids were highest in the plasma of those ewes in early lactation. As there was little change in the plasma phospholipid or cholesterol levels in these ewes, the increase in esterified fatty acids was probably due to an increase in triglycerides. Although the concentration of free fatty acids was highest in the lymph of those ewes in early lactation, the difference between the concentration of free fatty acids in the plasma and lymph in these ewes was much larger than in dry ewes.

The total protein concentration of the lymph was lower than that of the plasma and the ratio of albumin to globulin was higher in lymph than in plasma. The concentration of protein in the lymph of lactating ewes was lower than in the lymph of the dry ewes and in general, protein concentration was negatively correlated with the rate of lymph flow. In the dry ewes, the plasma protein concentration was higher and the ratio of albumin to globulin lower than in ewes in early lactation. The output of protein in the lymph from both mammary glands amounted to as much as 1.2 g. per hour in the lactating ewes.

TABLE II.

The concentrations of various constituents in the plasma and the mammary gland lymph of ewes. Results are given for 8 individual sheep. "P" indicates plasma values and "L" lymph values.

Sheep No. / Stage of Lactation		1 Middle	2 Dry	3 Early	4 Early	5 Early	6 Dry	7 Dry	8 Dry
Free fatty acids mg. per cent	P	6.5	10.1	32.2	24.3	33.0	10.5	8.4	7.7
	L	4.6	8.7	14.5	14.1	17.9	8.8	8.3	6.1
Volatile fatty acids mg. acetate per cent	P	-	8.9	5.6	5.8	7.6	9.3	9.2	6.6
	L	-	8.5	5.7	5.3	6.3	7.1	7.8	5.5
Total esterified fatty acids mg. per cent	P	99.4	108.9	156.2	151.7	283.1	75.9	118.2	84.8
	L	28.5	59.0	37.8	53.2	75.6	21.3	49.0	28.2
Phospholipids mg. per cent	P	108.5	99.2	144.1	103.3	121.2	95.4	130.5	101.2
	L	54.1	51.5	42.3	42.4	39.5	34.4	37.2	51.2
Cholesterol mg. per cent	P	55.6	106.5	106.6	81.3	112.1	105.6	121.1	109.4
	L	17.3	37.1	32.0	25.4	26.6	44.4	49.6	41.0
Protein g. per cent	P	7.81	9.15	7.87	7.71	6.90	8.46	8.45	8.09
	L	3.24	5.51	2.96	2.39	1.46	4.78	4.34	5.09
A/G Ratio	P	0.9	0.7	1.2	0.7	0.9	0.7	0.8	1.0
	L	1.5	0.9	1.5	1.1	1.3	0.8	1.4	1.1

TABLE II (cont.)

Sheep No. / Stage of Lactation		1 Middle	2 Dry	3 Early	4 Early	5 Early	6 Dry	7 Dry	8 Dry
Glucose mg. per cent	P	80.1	78.6	-	-	-	-	-	-
	L	80.7	75.0	-	-	-	-	-	-
Sodium mEq/l.	P	148.0	144.0	144.0	148.0	142.0	145.0	144.0	142.0
	L	145.0	145.0	143.0	146.0	140.0	143.0	140.0	140.0
Potassium mEq/l.	P	4.2	4.4	4.6	4.6	4.3	4.4	4.6	4.9
	L	4.0	4.4	4.4	4.7	4.0	4.2	4.5	4.6
Calcium mEq/l.	P	4.7	5.6	4.5	4.3	4.1	4.6	5.0	4.8
	L	4.1	4.5	3.6	3.5	3.1	4.0	4.5	4.4
Magnesium mEq/l.	P	1.4	1.4	1.9	1.9	2.0	2.5	2.3	2.4
	L	1.3	1.1	1.6	1.8	1.6	1.6	1.7	2.1
Chloride mEq/l.	P	102.3	109.0	112.2	114.4	113.2	108.2	111.6	112.0
	L	111.1	114.2	116.2	115.6	112.0	113.4	115.9	116.6
Inorganic Phosphate mEq/l.	P	3.1	2.9	3.6	3.0	3.2	3.3	2.7	2.5
	L	4.4	2.4	2.6	3.0	3.7	2.7	2.6	3.0

The concentrations of calcium and magnesium were significantly lower in the lymph than in the plasma. This held for both lactating and dry ewes. There was no significant difference between the concentrations of glucose, sodium or potassium in the plasma and lymph. In all ewes, the chloride concentration was higher in lymph than in plasma. The concentration of inorganic phosphate in lymph showed no consistent trend, being higher in the lymph of some ewes and higher in the plasma of others.

The Exchange of Albumin between the Plasma and Mammary Gland Lymph.

To obtain an estimate of the volume of the interstitial fluid pool in the udder, labelled proteins were injected into the blood stream of ewes with lymphatic duct fistulae and the rate of replacement of the protein in the interstitial pool by the labelled protein was followed. Experiments were carried out on both lactating and dry ewes. Albumin was labelled with either T-1824 or ^{131}I. In order to label the animal's own plasma albumin 150 mg. of T-1824 in 10 ml. of saline were injected intravenously. Twenty microcuries of ^{131}I H.S.A. in 10 ml. of saline were injected intravenously in other ewes. In some ewes however, both T-1824 and ^{131}I H.S.A. were injected together. The concentration of T-1824 and albumin was determined in each plasma and lymph sample which were collected at various intervals after the injection of dye and specific

activities were expressed as mg. T-1824 per g. of albumin. The radioactivity in the plasma and lymph samples was also measured and specific activities were expressed as counts per minute per g. of albumin.

The rate of disappearance of T-1824 and ^{131}I from the plasma of a lactating ewe is shown in Fig. 14. T-1824 had a significantly shorter circulating half-life than the ^{131}I, the calculated values being about 5 and 15 hours respectively. With both labels, there was a rapid initial loss from the plasma during the first 3-4 hours followed by a slower, almost linear disappearance during the next 12 hours.

The label appeared rapidly in the lymph and could be detected within 30 minutes of the injection. The rate of transfer was very much higher in lactating ewes than in dry ewes. In the lactating ewes, the maximum concentration of label (T-1824 or ^{131}I) and the maximum specific activity of the lymph albumin occurred about 6-9 hours after injection. In dry ewes these maxima were reached several hours later.

It was considered that when the specific activity of the albumin in the lymph equalled or exceeded that of the plasma, the albumin present initially in the interstitial fluid pool drained by the mammary lymph duct had been replaced completely by newly filtered albumin. In the lactating ewes, equilibration occurred 8-12 hours

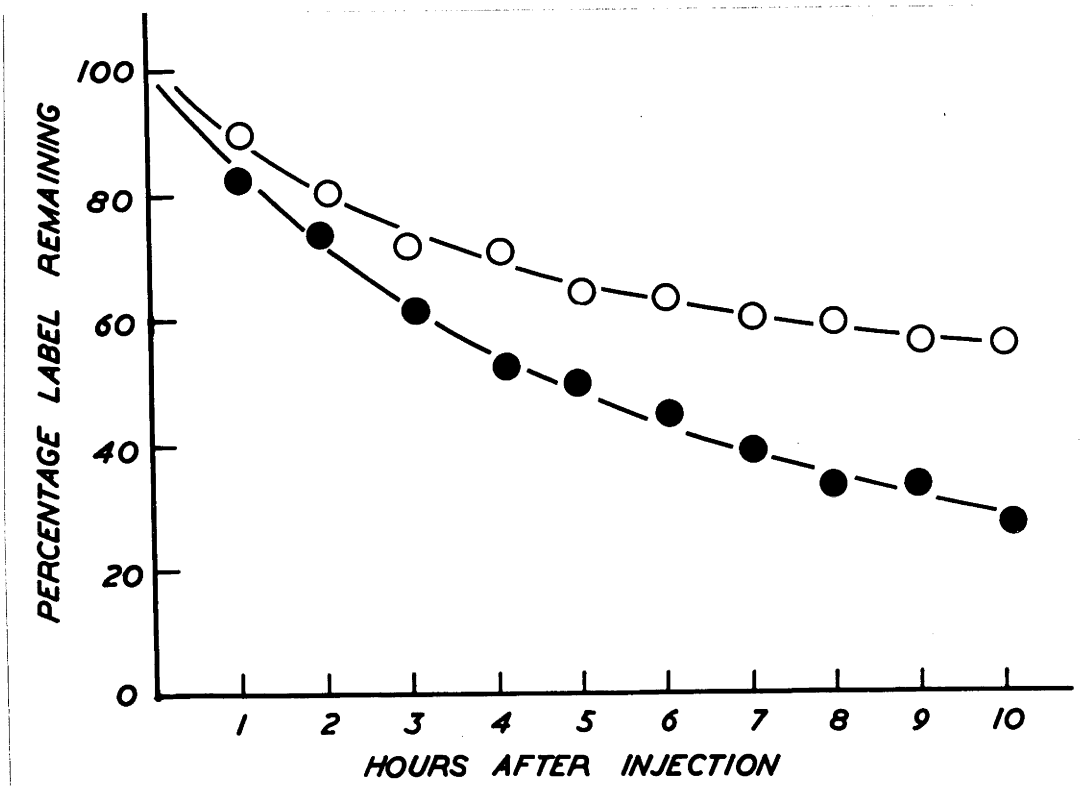

Figure 14.

The disappearance of T-1824 and ^{131}I H.S.A. from the plasma following their injection intravenously into a lactating ewe.

O-O ^{131}I H.S.A.

●-● T-1824

after injection, whereas in dry sheep it took 24-30 hours. In some cases the specific activity curves for plasma and lymph did not meet and equilibration was taken to have occurred when the two curves had approached one another closely and were decreasing in parallel. It was sometimes difficult to determine the point of equilibration in dry sheep and this was estimated in some cases by extrapolation.

Estimates of the size of the interstitial pool of the udder were made in some sheep during lactation and subsequently when they were dried off. Figs. 15 and 16 show the specific activity curves for plasma and mammary lymph from a ewe determined at the height of her lactation and again 4 weeks later after she was dried off. Although the T-1824 and the ^{131}I disappeared from the circulation at different rates, the time taken for the specific activities of the albumin in the plasma and lymph to equilibrate was essentially the same for both labels (see Figs. 17 and 18).

The Estimation of the Size of the Interstitial Pool drained by the Mammary Lymph Duct.

The amount of T-1824 or ^{131}I which was collected each hour in the lymph could be determined by measuring the volume of lymph collected during the hour and its concentration of T-1824 or ^{131}I. The amount of newly filtered albumin in the lymph collected each hour was

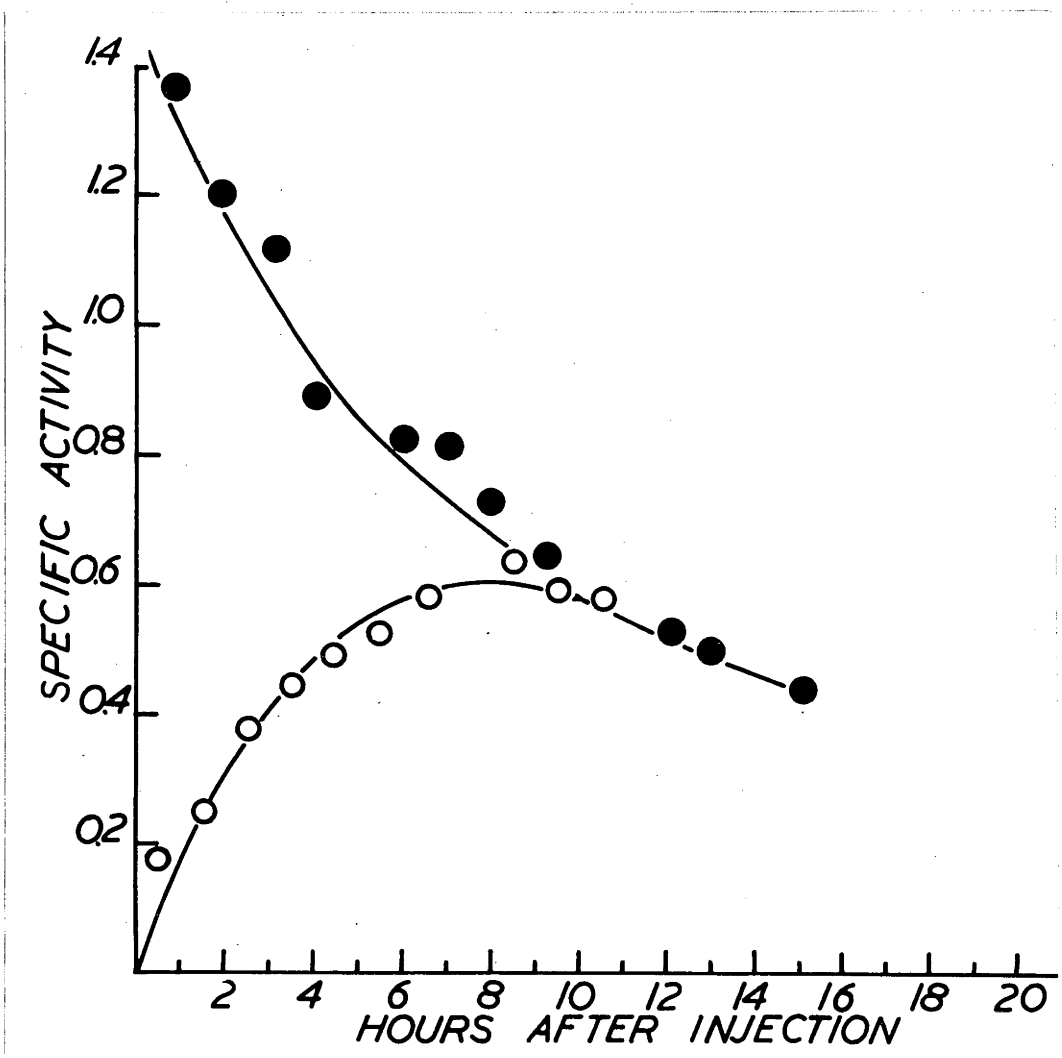

Figure 15.

The specific activities of the plasma and mammary lymph following the intravenous injection of T-1824 into a ewe in early lactation.

●-● Plasma

0-0 Lymph

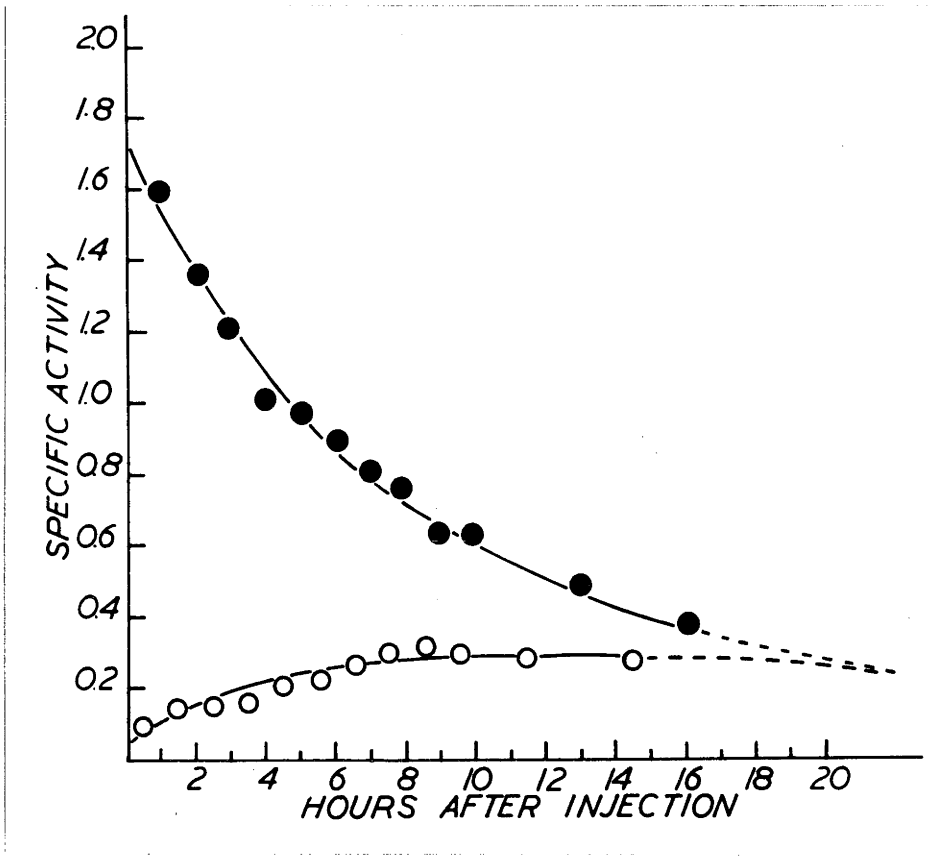

Figure 16.

The specific activities of the plasma and mammary lymph following the intravenous injection of T-1824 into a dry ewe.

●-● Plasma

0-0 Lymph

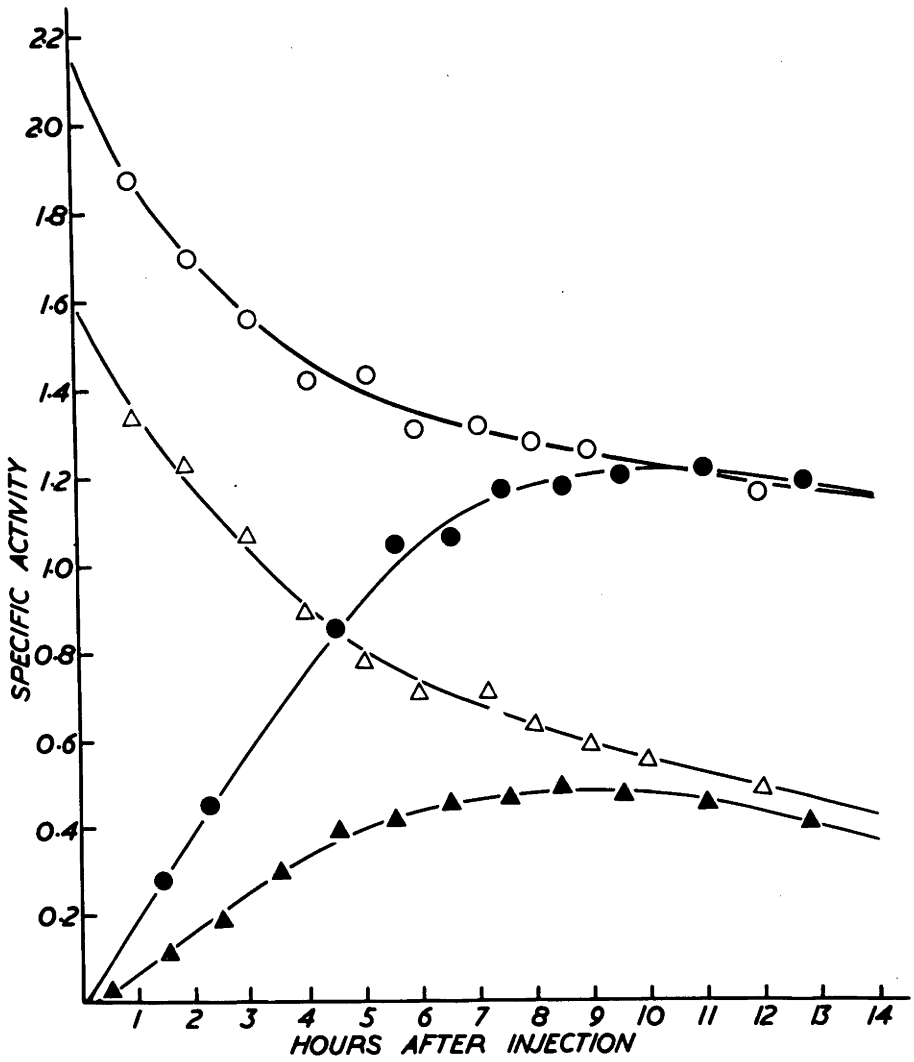

Figure 17.

The specific activities of plasma and mammary lymph following the intravenous injection of T-1824 and ^{131}I H.S.A. into a ewe in early lactation.

O-O	S.A. ^{131}I H.S.A.	Plasma
●-●	S.A. ^{131}I H.S.A.	Lymph
△ - △	S.A. T-1824	Plasma
▲ - ▲	S.A. T-1824	Lymph

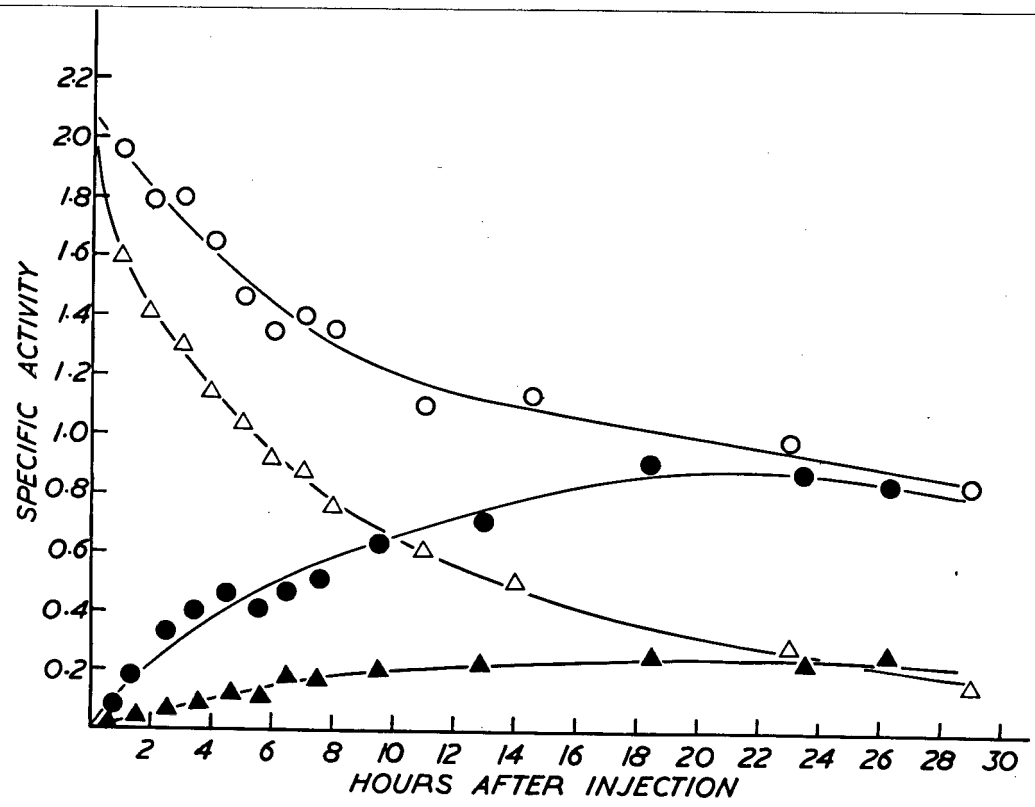

Figure 18.

The specific activities of plasma and mammary lymph following the intravenous injection of T-1824 and ^{131}I H.S.A. into a dry ewe.

O-O	S.A. ^{131}I H.S.A.	Plasma
●-●	S.A. ^{131}I H.S.A.	Lymph
△ - △	S.A. T-1824	Plasma
▲ - ▲	S.A. T-1824	Lymph

calculated by relating the amount of label collected in the lymph to the mean specific activity of the plasma during the same period of time as follows:

$$\text{Amount of newly filtered albumin (g.)} = \frac{\text{amount of label collected in lymph}}{\text{mean plasma specific activity}}$$

The sum of the hourly estimates of newly filtered albumin collected in the lymph between the time the label was injected and the time equilibration occurred between the specific activities of the plasma and lymph was thought to give a reasonable estimate of the amount of albumin required to replace completely the albumin originally present in the interstitial pool.

Table III sets out estimates of the amount of albumin present in the interstitial pool drained by the mammary gland lymphatics together with the volume of the interstitial fluid calculated from the concentration of albumin in the lymph. Essentially similar results have been obtained for both T-1824 and ^{131}I.

DISCUSSION

The volume of lymph collected from mammary lymph ducts of ewes at the height of their lactation varied between 400-950 ml. per day. In anaesthetized cats and dogs, it has been shown that essentially all of the thoracic duct lymph comes from the intestines and the liver

TABLE III.

The estimates of the amount of filtrate albumin required to replace the albumin in the interstitial pool drained by the mammary lymphatic duct of one side. Calculated volumes of the interstitial pool are also given together with the mean albumin concentration of the lymph during the period of equilibration.

Sheep No.	1		2	3		4	5		6
Stage of Lactation	Middle	Dry	Dry	Early	Dry	Early	Early	Dry	Dry
Filtrate albumin required to replace that in interstitial pool	0.62	0.29	1.02	1.72	0.44	2.05	1.88	1.05	1.24
Mean albumin concentration of lymph g. per cent	1.40	1.49	2.68	1.68	2.35	1.26	0.83	1.68	2.18
Size of interstitial pool (ml.)	44.0	19.0	38.0	102.00	19.0	163.0	227.0	63.0	57.0

(Morris, 1956a). It is evident however that in lactating animals with well-developed mammary glands, the volume of lymph formed in this tissue represents an appreciable part of the total thoracic duct lymph flow. The sheep produces between 2,000 and 4,500 ml. of thoracic duct lymph per day and between 200 and 500 ml. of liver lymph per day (Lascelles and Morris, 1961). If it is assumed that each half of the udder contributes an equal volume of lymph, it can be calculated that something of the order of 20-30 per cent of the total thoracic duct lymph flow can come from the mammary glands. The amount of protein leaving the circulation and returning to the plasma via the mammary gland lymph in the lactating ewe is of the same order as that transported in the liver lymph.

The free and esterified fatty acid content of the plasma of ewes in early lactation was raised significantly. Further observations have been made on the concentration of lipids in the plasma and mammary lymph of ewes and these have shown that the highest concentration of lipids occur in those ewes which are high milk producers. It may be that depot fats are being mobilized in these animals to contribute fatty acids to the milk. The large plasma-lymph gradient for free fatty acids in lactating ewes when compared with dry ewes suggests that the mammary gland is extracting free fatty acids from the capillary filtrate. Esterified fatty acids, cholesterol

and phospholipids occurred in mammary gland lymph in lower concentrations than in the plasma and the differences in the protein concentration and A/G ratio are similar to those observed between plasma and lymph draining other areas of the body (cf. Yoffey and Courtice, 1956).

Heyndrickx and Peeters (1958a, 1958b; 1960) and Heyndrickx (1959) have studied the composition of udder lymph and plasma which they obtained from freshly slaughtered cows. They concluded that there were significant differences in the concentration of substances such as calcium, potassium and lactate when compared with published data for lymph collected from other regions of the body. However, the composition of mammary gland lymph obtained from conscious lactating goats (cf. Linzell, 1960) did not support these findings. The composition of mammary gland lymph of sheep is almost identical to that of goats. Linzell suggested that the composition of the lymph obtained by Heyndrickx and Peeters may have been influenced by the period of anoxia which is unavoidable when lymph samples are collected from post-mortem material and this suggestion is supported by the results of the experiments reported here.

The Interstitial Fluid Pool in the Mammary Gland.

A significant increase occurred in the size of the interstitial pool and the volume of lymph draining from the udder during lactation. The maximum specific activity

reached in the lymph of the lactating ewes was significantly higher than that in dry ewes, and this must be due to an increased rate of protein leakage per unit volume of interstitial fluid. The fractional rate of replacement of the interstitial pool was consequently much greater in the lactating than in the dry gland. Protein leakage from the capillaries is a function of capillary permeability, the area available for filtration and capillary pressure. It seems likely that in the lactating mammary gland, the increased rate of leakage of protein per unit volume of interstitial pool would be associated with an increased filtration pressure and filtration area. It is known that an increase in venous pressure occurs in the mammary veins during lactation (cf. Linzell, 1959) and the great increase in the size of the mammary gland and in its blood supply during lactation would be consistent with an increase in the size of the filtration area. The question of whether any changes occurred in capillary permeability cannot be answered.

It was thought that the transient changes in the rate of flow which occurred when the ewes were suckled or their udders massaged was due to an increased propulsion of lymph through the lymphatics rather than to any change in the rate of lymph production. However, Linzell (1960) claimed that this response did not occur in lactating goats.

These different findings may well be related to the differences in the techniques used for collecting lymph. Linzell cannulated the mammary lymph duct close to the superficial supramammary node. In the experiments described in this thesis the lymph duct was cannulated just before it entered the inguinal canal so that a considerable length of the duct passed beneath the aponeurosis of the external and internal oblique abdominal muscles before it reached the point of cannulation. This aponeurosis is often under considerable tension particularly when the animal is standing and this may lead to partial obstruction of the duct under normal conditions and to the accumulation of lymph in the peripheral lymphatics. It was noted that in many ewes, the flow of lymph was spasmodic and this was related to the animal's movements. The duct in its course beneath the muscular aponeurosis is massaged by the movement of the udder. The flow rate response to any factor which increased the propulsion of already formed lymph would therefore be expected to be greater in cases where the lymph flow had been partially obstructed.

The method used for estimating the size of the interstitial fluid and protein pools drained by the mammary lymph duct is valid only if certain conditions are fulfilled (Morris, 1956b). The difference in the rate of disappearance of T-1824 and ^{131}I albumin from the circulation suggests that the T-1824 albumin complex may

not be stable in the circulation of sheep and that some differential breakdown of the complex occurs with loss of dye to the tissue proteins. This would lead to an over-estimate in the size of the interstitial pool and the error involved would be greatest in dry ewes where equilibration occurred slowly.

The estimates of the size of the interstitial pool in dry ewes were larger than would be expected on the basis of the mass of mammary tissue present. This was due, at least in part, to the fact that lymph was coming from areas other than the udder. The difference in the size of the pools in lactating and dry ewes would be due largely to the increased mass of mammary tissue which develops during lactation. Since there was a five- to ten-fold increase in the rate of lymph flow during lactation it follows that the relative contribution of lymph from tissues other than the udder would be decreased by approximately the same amount.

CHAPTER 5.

THE REMOVAL OF MILK CONSTITUENTS FROM THE MAMMARY GLAND

The mammary gland of a well fed merino ewe is capable of producing 1.5 litres of milk per day (cf. Davies, 1958). If suckling is stopped suddenly, large amounts of casein, whey proteins, milk fat and lactose, all of which are not normally found elsewhere in the body, will accumulate in the gland. During the next 5-7 days most of the accumulated milk has been removed. In this chapter an attempt has been made to determine the role the lymphatics may play in the removal of the constituents of milk from the mammary gland. Initial investigations were concerned with the changes in the lymph flow and in the composition of plasma and lymph in ewes after suckling was stopped early in lactation. In addition, attempts were made to determine whether or not milk proteins were present in the lymph coming from the udder in these conditions.

The first series of experiments was carried out on 3 ewes in early lactation. The mammary lymph duct was cannulated in these ewes and after they had recovered from

the operation their lambs were taken from them. The udders of these ewes did not become very distended at any stage. Five to six days after suckling was stopped, each udder had become markedly atrophied and only a small volume of milk remained in the gland. Tables IV, V and VI give details of the lymph flow and composition of plasma and lymph in these 3 ewes after suckling was stopped. The lymph from the ewes remained clear throughout the 6-8 days of the experiment. There was a pronounced fall in the rate of flow of lymph and in the concentration of free fatty acids in plasma and lymph. There was no suggestion however, that the absorption of the milk from the udder affected either the flow or the composition of the lymph collected.

In another group of 3 ewes, suckling was stopped very early in lactation and the lymph which is normally clear became distinctly milky in appearance 30-48 hours after suckling was stopped. By this time, the udder had become very distended with milk. The milkiness appeared suddenly and persisted for as long as 2 days in one sheep and for only about 4 hours in the other 2. Microscopic examination of the milky lymph under dark-ground illumination revealed the presence of particulate fat. Samples of this lymph were tested by the specific precipitation technique in agar and protein peculiar to

TABLE IV

Some measurements of the flow and composition of mammary lymph and the composition of the plasma of a lactating ewe at various times after suckling was stopped. The ewe had lambed 3-4 weeks previously. The udder was moderately distended with milk 30-48 hours after suckling was stopped. The lymphatic cannula became blocked shortly after the collection of the last lymph sample. "P" indicates plasma values and "L" indicates lymph values.

Hours after suckling stopped		0	12	24	48	96	168
Lymph flow rate ml. per hour		27	24	24	20	12	5
Protein g. per cent	P	7.8	7.7	7.7	7.8	7.9	7.8
	L	2.2	2.0	2.1	2.4	2.5	2.9
Total esterified fatty acids mg. per cent	P	172.6	175.5	170.2	168.4	160.9	164.2
	L	47.3	48.1	42.3	44.6	40.9	37.1
Phospholipids mg. per cent	P	120.0	122.5	118.4	121.9	116.4	113.2
	L	38.2	36.7	38.8	39.2	40.5	44.9
Cholesterol mg. per cent	P	104.4	104.0	105.4	96.3	108.9	106.7
	L	35.9	33.4	36.8	35.0	37.5	38.4
Free fatty acid mg. per cent	P	25.7	24.3	22.8	22.9	16.2	7.1
	L	11.9	11.5	12.2	11.0	8.0	5.7
Potassium mEq/l.	P	4.6	4.5	4.5	4.4	4.6	4.5
	L	4.4	4.5	4.6	4.3	4.5	4.6
Calcium mEq/l.	P	4.3	4.4	4.3	4.5	4.6	4.5
	L	3.5	3.5	3.6	3.5	3.7	3.6
Inorganic phosphate mEq/l.	P	3.6	3.5	3.5	3.6	3.4	3.5
	L	2.8	2.9	3.0	2.9	3.0	2.9

TABLE V

Some measurements of the flow and composition of mammary lymph and the composition of the plasma of a lactating ewe at various times after suckling was stopped. The ewe had lambed 4 weeks previously. The udder was distended with milk 24-48 hours after suckling was stopped. "P" indicates plasma values and "L" indicates lymph values.

Hours after suckling stopped		0	12	24	48	96	192
Lymph flow rate ml. per hour		23	20	17	11	12	6
Protein g. per cent	P	8.1	8.0	7.9	8.1	8.0	7.9
	L	2.4	2.3	2.5	2.8	2.8	3.0
Total esterified fatty acids mg. per cent	P	157.3	158.6	156.3	146.0	141.8	138.9
	L	45.6	46.2	44.7	46.4	42.7	43.7
Phospholipids mg. per cent	P	108.2	114.8	109.7	107.5	112.4	109.5
	L	56.0	49.3	57.7	56.1	49.5	59.6
Cholesterol mg. per cent	P	95.2	101.7	92.3	89.5	91.6	98.9
	L	27.1	29.8	31.9	37.0	35.4	34.1
Free fatty acids mg. per cent	P	20.2	22.3	18.4	16.3	9.2	5.9
	L	8.6	8.4	6.8	7.2	5.8	4.1
Potassium mEq/l.	P	4.2	4.5	4.3	4.4	4.6	4.5
	L	4.0	4.5	4.4	4.2	4.5	4.3
Calcium mEq/l.	P	4.8	4.6	5.1	4.9	5.3	4.9
	L	3.9	4.0	4.2	4.1	4.4	4.3
Inorganic phosphate mEq/l.	P	3.3	3.1	3.2	3.0	3.1	3.3
	L	3.4	2.9	2.8	3.3	3.5	3.4

TABLE VI

Some measurements of the flow and composition of mammary lymph and the composition of the plasma of a lactating ewe at various times after suckling was stopped. The ewe had lambed 8 weeks previously. The udder was only slightly distended with milk 30-40 hours after suckling was stopped. The lymphatic cannula became blocked shortly after the collection of the last sample. "P" indicates plasma values and "L" indicates lymph values.

Hours after suckling stopped		0	12	24	48	96	144
Lymph flow rate ml. per hour		16	15	15	13	8	4
Protein g. per cent	P	8.4	8.3	8.2	8.3	8.4	8.4
	L	2.8	2.7	3.0	3.3	3.2	3.5
Total esterified fatty acids mg. per cent	P	151.3	148.9	151.5	145.4	142.8	139.3
	L	51.3	47.7	50.4	48.5	50.1	45.9
Phospholipids mg. per cent	P	101.7	107.6	108.3	105.4	107.1	103.2
	L	61.0	58.7	59.6	59.2	61.4	54.1
Cholesterol mg. per cent	P	96.4	99.8	102.7	97.3	94.3	99.4
	L	33.2	35.4	34.8	31.0	32.5	29.7
Free fatty acids mg. per cent	P	17.8	18.8	16.4	14.3	11.2	7.1
	L	9.0	9.4	8.6	7.9	7.1	5.6
Potassium mEq/l.	P	4.4	4.5	4.4	4.5	4.3	4.5
	L	4.5	4.4	4.6	4.5	4.3	4.2
Calcium mEq/l.	P	4.5	4.4	4.6	4.5	4.6	4.7
	L	3.7	3.6	3.9	3.6	3.6	3.8
Inorganic phosphate mEq/l.	P	3.1	3.1	3.2	3.1	3.0	3.2
	L	3.2	3.3	3.2	3.2	3.0	3.1

milk was identified (see Fig. 19). Milk protein was identified only in samples of lymph which showed visible opalescence. When the lymph cleared precipitation tests in agar were negative. The concentrations of total esterified fatty acids, calcium and inorganic phosphate were approximately 10 per cent higher in the milky lymph than in the clear lymph collected at other times during the course of the experiment.

The results indicated that under certain circumstances, some of the fat and proteins of milk may appear in the lymph coming from the mammary gland. The fact that milk constituents appeared in mammary lymph when the udder was grossly distended suggested that this phenomenon may be associated with actual rupture of some acini and milk ducts. The rupture of these elements has been recorded in distended mammary glands of rats (Selye, 1934, 1954; Benson and Folley, 1957). It appears then that the macromolecules of milk, such as casein micelles and particles of milk fat, are carried away by the lymphatics once they have entered the interstitial fluid. The higher levels of calcium and phosphate which were found in the milky lymph may have been due to the presence of casein micelles with which calcium and phosphate are associated in milk. Only a small proportion of the accumulated milk appears in the mammary lymph in obvious form. The failure at most times to observe any

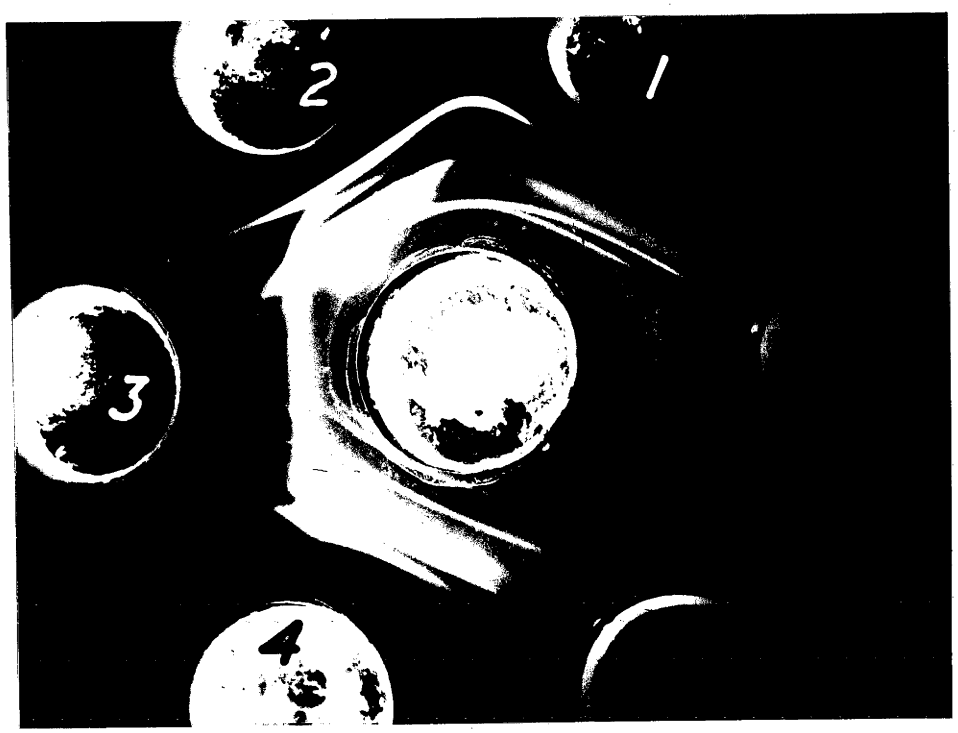

Figure 19.

Lines of precipitation between the central well containing ewe skim milk antiserum and the surrounding wells containing (1) non-milky lymph, (2) milky lymph, (3) diluted milk, and (4) wether's serum. A broad line of precipitation can be seen opposite the well containing milk. This line crosses all the precipitation lines opposite the well containing wether's serum and represents the precipitation between specific antibodies and protein in milk not found in plasma. This broad milk protein line is continuous with a line (often seen divided) opposite the well containing the milky lymph. No such line was found opposite the well containing the non-milky lymph.

alteration in the flow and composition of lymph which could be attributed to the absorption of milk suggested that the bulk of the milk is removed from the gland by processes which do not involve the regional lymphatics.

The Absorption of Labelled Proteins from the Mammary Gland.

The following section describes some experiments which were carried out to determine the way in which proteins are removed from the mammary gland. As a sensitive method for the detection of very small amounts of protein was required, radioactive proteins were used in these experiments. The radioactive protein (either ^{131}I H.S.A. or ^{131}I casein) was injected into the mammary gland cistern of lactating ewes in separate experiments. Each gland was milked out immediately prior to the injection of the labelled protein. Suckling was stopped and plasma, lymph and urine samples were collected during the next 1-2 weeks and the radioactivity in these samples was measured. The doses of ^{131}I H.S.A. and ^{131}I casein were adjusted so that approximately 6×10^6 counts per minute were introduced into the mammary gland. In all, 8 experiments were carried out in this way, 5 with casein and 3 with serum albumin. Similar experiments were carried out in which ^{131}I H.S.A. was introduced into the gland cistern of 3 ewes 5-7 days after suckling was stopped when involution of the gland had occurred.

The behaviour of casein in the body fluids was also studied. A dose of radioactive casein containing

approximately 2 x 10^6 counts per minute was injected intravenously into each of 3 lactating ewes with mammary lymph duct fistulae. The radioactivity in plasma, lymph, milk and urine was measured during the next 24 hours.

The Removal of Albumin from the Secreting Mammary Gland. After the introduction of ^{131}I H.S.A. into the mammary gland cistern, radioactivity appeared in the lymph; the level of radioactivity increased to a maximum and then fell exponentially. The time course of the appearance of the radioactivity varied considerably and seemed to be related to the degree of distention which occurred in the mammary gland. Figs. 20 and 21 show the rate of removal of the radioactivity from the mammary gland by mammary lymphatics in a ewe in early lactation and in a ewe in mid-lactation. It can be seen that the mammary lymph of the ewe in early lactation had high levels of radioactivity 24-36 hours after the introduction of the radioactive albumin into the mammary gland. Since all the radioactivity was bound to protein it appeared that the ^{131}I H.S.A. was removed from the mammary gland without being broken down. The percentage of the original activity present in the extracellular fluid of the body at the end of the experiment together with the percentage of the original activity collected in the lymph and urine are shown in Table VII. The estimation of the amount of radioactivity remaining in the extracellular fluid at the end of the experiment was

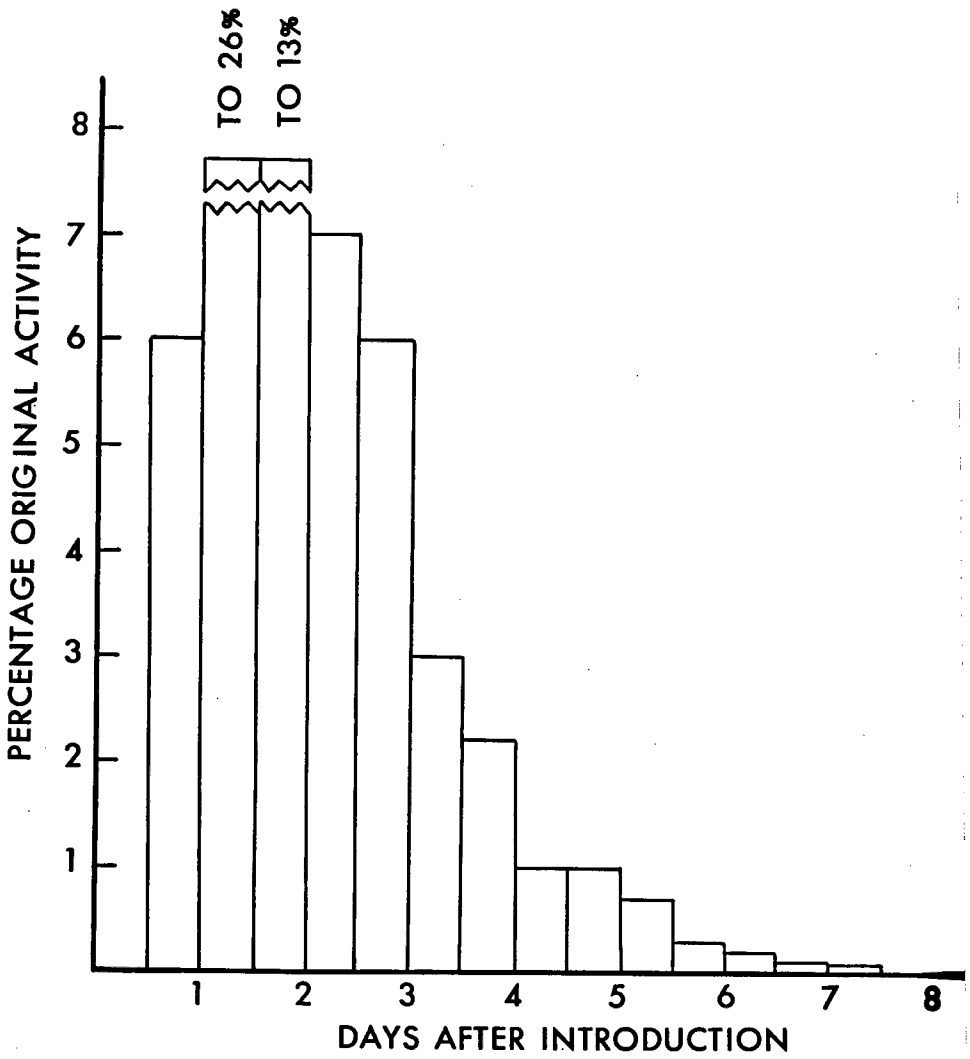

Figure 20.

The percentage of the original radioactivity collected in the lymph following the introduction of ^{131}I H.S.A. into the mammary gland cistern of a ewe in early lactation.

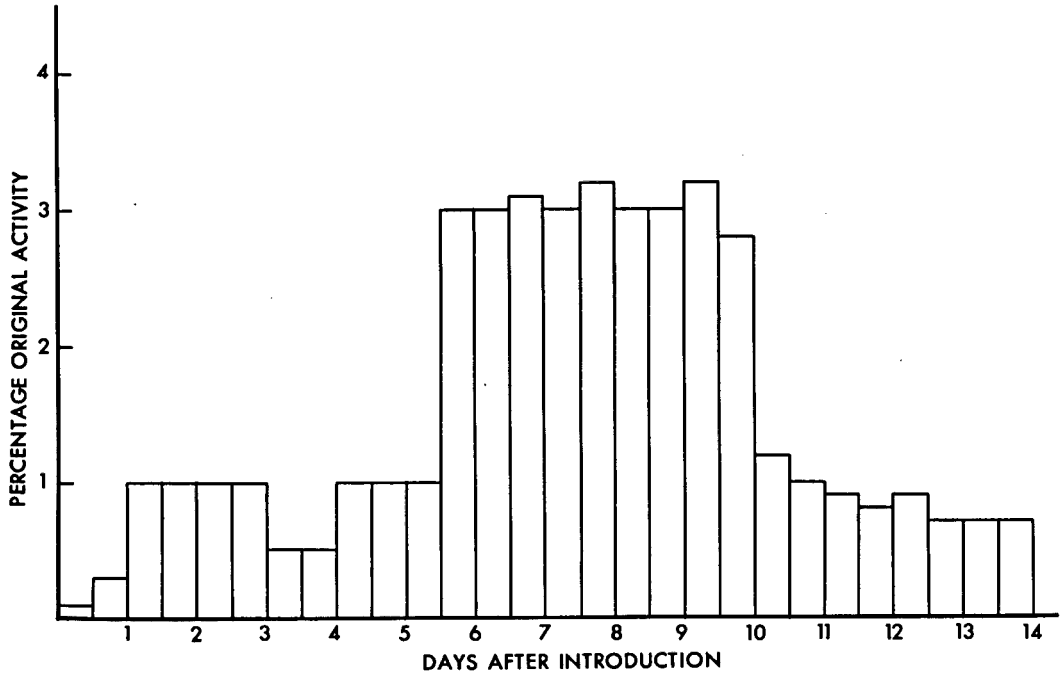

Figure 21.

The percentage of the original radioactivity collected in the lymph following the introduction of ^{131}I H.S.A. into the mammary gland cistern of a ewe in mid-lactation.

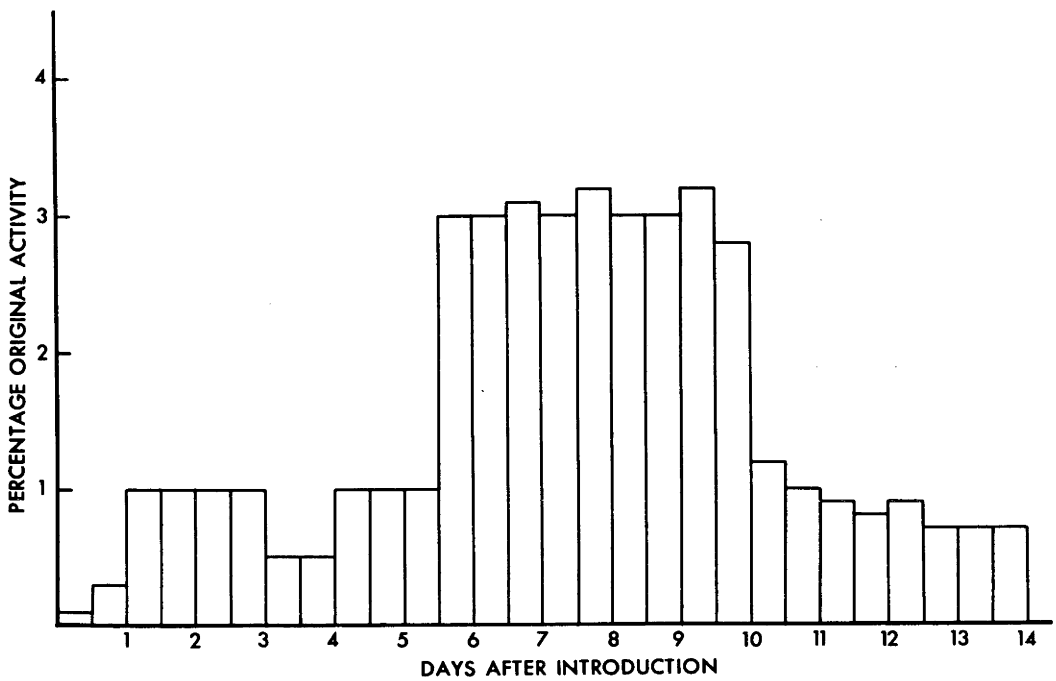

Figure 21.

The percentage of the original radioactivity collected in the lymph following the introduction of ^{131}I H.S.A. into the mammary gland cistern of a ewe in mid-lactation.

TABLE VII

The percentage of the original radioactivity collected in the lymph and urine and the estimated percentage remaining in the extracellular fluid of the body following the introduction of ^{131}I H.S.A. into the mammary gland cistern of lactating ewes.

Experiment	Duration of Experiment (days)	Percentage Original Activity			
		Collected in Lymph	Collected in Urine	In Extracellular Fluid at end of Experiment	Total Recovered
1	7	68	0	1	69
2	14	38	15	14	67
3	9	43	9	10	62

made on the assumption that the plasma volume was 5 per cent and the interstitial fluid volume was 15 per cent of the body weight (cf. Gamble, 1954). It was also assumed that the level of radioactivity in the interstitial fluid was half that in the plasma. The radioactivity in the plasma was bound to protein but was always very low relative to that in the lymph. The radioactivity which appeared in the urine however, was not precipitated with trichloracetic acid and was presumably in the form of iodide or iodotyrosine. In these experiments the amount of radioactivity recovered varied between about 60-70 per cent.

The Removal of Albumin from the Involuting Mammary Gland. When ^{131}I H.S.A. was injected into glands which were undergoing involution it was removed from the gland largely by way of the lymphatics (see Fig. 22). The rate of removal of the albumin was much more rapid than from undistended glands (cf. Fig. 21). The percentage of the original activity collected in the lymph and urine and the estimated percentage of the original activity present in the extracellular fluid of the body at the end of the experiment is shown in Table VIII.

The Removal of Casein from the Mammary Gland. After ^{131}I casein was introduced into the gland, a low level of radioactivity often appeared in the lymph after 12-24 hours. The level of radioactivity in the plasma slowly increased

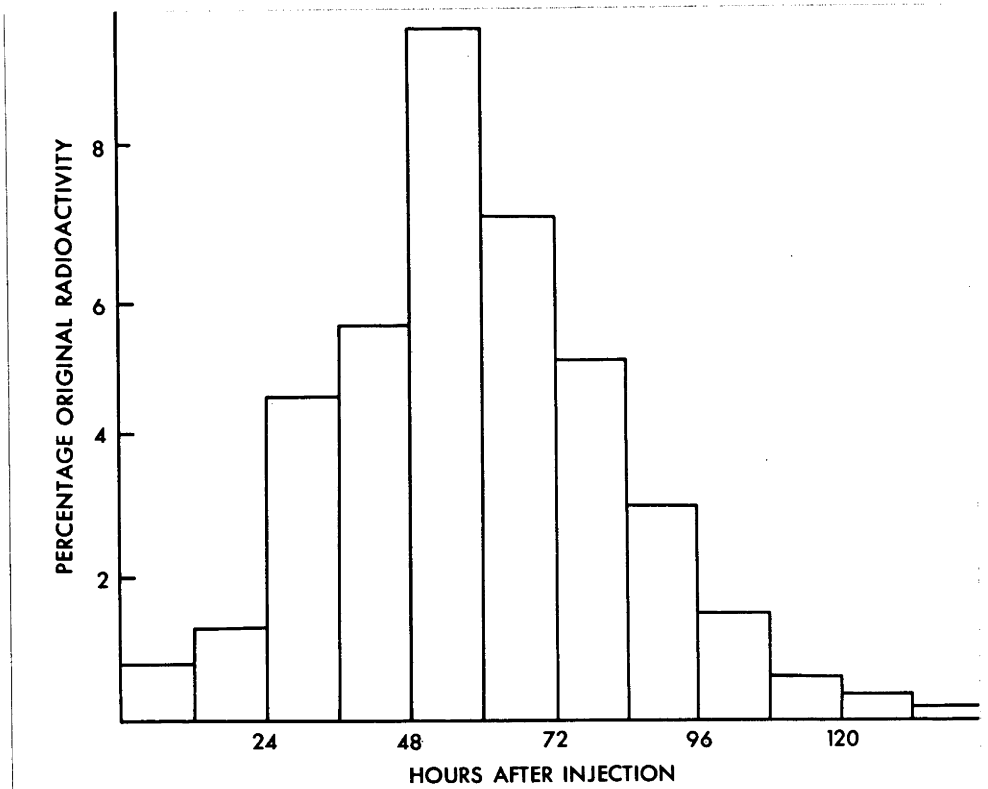

Figure 22.

The percentage of the original radioactivity collected in the lymph following the introduction of ^{131}I H.S.A. into the mammary gland cistern of a lactating ewe. The lamb was removed from the ewe 6 days before the radioactive protein was injected.

TABLE VIII

The percentage of the original radioactivity collected in the lymph and urine and the estimated percentage remaining in the extracellular fluid of the body following the introduction of ^{131}I H.S.A. into the mammary gland cistern about 6 days after suckling stopped.

Experiment	Duration of Experiment (days)	Percentage Original Activity			
		Collected in Lymph	Collected in Urine	In Extracellular Fluid at end of Experiment	Total Recovered
1	7	24.6	18.0	19.6	62.2
2	8	52.3	14.3	15.2	81.8
3	6	46.2	8.3	10.6	65.1

and after 48-96 hours, was equal to or in some cases exceeded the level in the lymph. Table IX shows the results of 1 of 5 experiments of which the results were essentially similar. The radioactivity in both the plasma and the lymph was almost entirely bound to protein. However, in one experiment which was conducted over a period of 14 days, radioactivity which was not bound to protein appeared in the plasma and in the lymph on the 13th and 14th days. In one experiment in which the udder became grossly distended with milk, the lymph suddenly became milky in appearance 32 hours after suckling was stopped. The radioactivity in the milky lymph was 10-15 times that of the lymph in the 31st hour and during the next few hours as the lymph cleared, the radioactivity decreased. The percentage of the original radioactivity introduced into the mammary gland cistern which was collected in the lymph and urine and which was present in the extracellular fluid of the body at the end of the experiment, is given in Table X. The results show that the lymphatics played a minor role in the removal of the radioactive casein from the mammary gland. Approximately half the radioactivity in the urine was bound to protein throughout the first 7 days of the experiment. The proportion of radioactivity bound to protein decreased during the next few days.

TABLE IX

The radioactivity in plasma and lymph following the introduction of ^{131}I casein into the mammary gland cistern of a ewe in mid-lactation.

Hours after Injection	Radioactivity (counts/min.) in 0.2 ml. sample		Hours after Injection	Radioactivity (counts/min.) in 0.2 ml. sample	
	Lymph	Plasma		Lymph	Plasma
3	1	0	96	22	22
6	20	0	120	22	22
12	20	0	144	19	24
24	4	4	168	18	21
48	4	5	192	20	23
72	11	8	240	14	17
			288	8	7

TABLE X

The percentage of the original radioactivity collected in the lymph and urine and the estimated percentage remaining in the extracellular fluid of the body following the introduction of ^{131}I casein into the mammary gland cistern of ewes in various stages of lactation.

Experiment	Duration of Experiment (days)	Percentage Original Activity			
		Collected in Lymph	Collected in Urine	In Extracellular Fluid at end of Experiment	Total Recovered
1	12	4	27	36	67
2	10	6	28	29	63
3	7	4	30	18	52
4	9	6	21	24	51
5	14	5	33	27	65

A similar dose of radioactivity of an apparently denatured preparation of ^{131}I casein was injected into the mammary gland cistern of a ewe; almost all the radioactivity found in the plasma, lymph and urine during the next 9 days was not bound to protein. The rate and time of appearance of the label in the plasma, lymph and urine was similar to that in the experiments described above.

The Behaviour of ^{131}I Casein in the Circulating Blood.

As radioactive casein appeared to enter the blood stream following its absorption from the udder, it was considered necessary to investigate the behaviour of radioactive casein in the circulation after its intravenous injection. The level of radioactivity in the plasma and in the lymph of a lactating ewe following the intravenous injection of ^{131}I casein is shown in Fig. 23. The radioactivity disappeared from the plasma rapidly, the circulating half-life being a little less than 30 minutes. The radioactivity appeared rapidly in the lymph and after approximately 1 hour, the level in the plasma and the lymph was equal. Between 70-90 per cent of the radioactivity in the plasma and lymph was bound to protein. Very high levels of radioactivity were found in the milk collected after the ^{131}I casein was injected intravenously and it was estimated that 15-20 per cent of the injected radioactivity was removed by the mammary gland

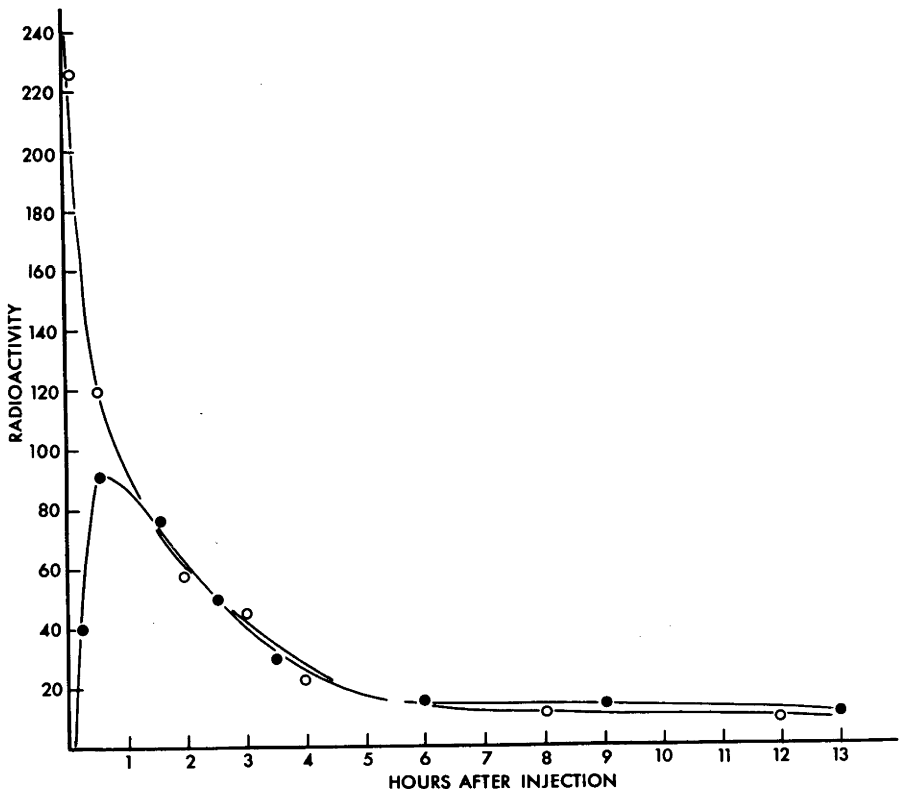

Figure 23.

The radioactivity in the plasma and lymph following the intravenous injection of ^{131}I casein into a ewe in mid-lactation.

and secreted into the milk. Significant levels of radioactivity in the milk occurred after 2-3 hours and these were 3-5 times greater than the highest level in the plasma. All the radioactivity in the milk during the first 2-3 hours was bound to protein. A sample of milk was collected from a ewe 2 hours after the intravenous injection of ^{131}I casein and the milk proteins were fractionated. All of the radioactivity in the milk sample was found in the casein fraction. A decreasing proportion of radioactivity bound to protein was found in the milk collected between 3 and 24 hours after the injection.

DISCUSSION

The results indicate that the epithelium within the mammary gland is permeable to human serum albumin. Once the albumin found its way into the interstitial fluid of the udder, it was taken up by the lymphatics. It has already been pointed out that one of the functions of the lymphatic system is to return protein which collects in the interstitial fluid to the general circulation. Serum albumin occurs normally in milk in low concentration (Peskett, 1932) and it appears that the mammary lymphatics would play an important role in returning proteins of this type to the general circulation following their entry into the interstitial fluid from the milk. The same conditions

may apply to serum globulins which are also present in milk.

In the experiments with serum albumin, a low level of radioactivity which was bound to protein was found in the plasma of 2 ewes towards the end of the experimental period. The rate of flow of lymph diminished rather rapidly in these ewes and this was thought to be due to clots forming within the catheters. This in turn may have caused a back pressure of lymph which would lead ultimately, to the development of collateral lymphatics (cf. Carlsten and Olin, 1952). These collaterals may have carried some protein into the blood stream in these experiments.

The difference in the rate of removal of albumin by the mammary lymphatics in the two experiments shown in Figs. 20 and 21 was thought to be due, at least in part, to the difference in the degree of distention of the mammary glands in the 2 ewes. In the first example (see Fig. 20), in which a large proportion of the albumin was removed in the first 72 hours, the udder became very distended with milk and the lymph became milky 24-30 hours after the experiment began. The contractile elements associated with the acini and ducts of the gland would exert pressure on the fluid within the gland and as the acini and ducts became distended with milk the pressure would tend to increase. An increase in pressure within

the cavity of the mammary gland may have caused an increased rate of transfer of albumin from the milk into the interstitial fluid. It was thought that the milkiness of the lymph was probably associated with actual rupture of the acini. Although this phenomenon may have contributed to an early appearance of radioactivity in the tissue fluid or lymph, it was responsible for the appearance in the lymph of only a small proportion of the total radioactive protein injected. The experimental results also suggested that changes which occur during involution greatly influenced the rate at which the ^{131}I H.S.A. was removed from the mammary gland. Although the concentration of serum albumin is low in normal milk, it appears to increase greatly in concentration in the milk as the gland involutes (McMeekin, 1952). Thus concurrent with the changes which occur during involution there appears to be a much more ready transfer of serum albumin across the mammary epithelium in both directions.

It was not possible to account for more than 70 per cent of the radioactivity introduced into the udder. This was probably due to the fact that a significant amount of radioactivity still remained in the gland at the end of the experiment. Some of the ^{131}I H.S.A. which entered the blood would be metabolised and a proportion of the free iodide released would be taken up by the thyroid gland and would not be measured.

After the introduction of ^{131}I casein into the mammary gland, radioactivity appeared in the lymph and at 48-96 hours, the level of radioactivity in the plasma and lymph was equal. The radioactivity in the plasma and lymph was bound to protein and it seems that the ^{131}I labelled protein was casein. When a denatured radioactive casein preparation was introduced into the mammary gland cistern most of the radioactivity which subsequently appeared in the plasma, lymph and urine was not bound to protein. The site of breakdown of the denatured casein cannot be decided. This may have occurred within the mammary gland or after its absorption into the general circulation. In view of these results the possibility that the normal radioactive casein preparations were first broken down and the liberated iodide or iodotyrosine then attached to or incorporated with another protein (within the thyroid gland for example) seems very remote.

The results show that the ^{131}I labelled casein was not absorbed to any extent by way of the lymphatic system and this suggests that it was removed from the cavity of the mammary gland in a form in which it was able to enter the blood stream directly. The casein in milk has been shown to occur in various physico-chemical forms. Most of the casein is present in the form of micelles which vary in size between 30 mu. and 300 mu. (Nitschmann, 1949; Hostettler and Imhoff, 1951a, 1951b). A small proportion

of the casein is also thought to exist in soluble form as small aggregates and monomers. The 3 physico-chemical forms are presumed to be in equilibrium. The importance of calcium in the formation of the micelles is well recognized and the alteration of the distribution of casein between the soluble and micellar forms by the removal and addition of calcium has been described by von Hippel and Waugh (1955).

The resolution of casein precipitated from acid solution, by moving boundary electrophoresis into α, β and γ peaks was observed by Mellander (1939). More recently a new component called κ-casein has been identified (von Hippel and Waugh, 1955; Waugh and von Hippel, 1956). These workers described α-casein as occurring in skim milk in the α- κ-complex. It is generally agreed that at room temperature, at neutral pH and in the presence of sodium chloride, α and β-casein are aggregated. As the pH is raised, the casein dis-aggregates and at pH 11 to 12 the casein is found in the soluble monomeric form (McMeekin and Peterson, 1955; von Hippel and Waugh, 1955). Likewise, κ-casein is highly aggregated at neutral pH and is disaggregated into monomers at pH 12 (Wake, 1959). Molecular weights of and β-casein are approximately 25,000 and 17,000 respectively (MacKenzie and Wake, 1959). The molecular weight of the κ-casein monomer was found to be

approximately 26,000 (Wake, 1959).

It would appear that the ^{131}I casein preparations used in the experiments described in this chapter were in the physico-chemical forms of monomers and aggregates. Their physico-chemical forms would change following introduction into the mammary gland. At the lower pH of milk and in its characteristic electrolyte environment, the casein would be expected at least to aggregate more heavily and it seems probable that much of it would form micelles.

Thus the ^{131}I casein which was probably in the form of large aggregates and micelles after introduction into the mammary gland, appeared to be absorbed as small protein molecules. These may have been casein monomers. It is significant in this regard that after ^{131}I casein was injected intravenously, it left the plasma rapidly and the radioactivity in plasma and lymph was equal one hour after the injection. These results demonstrated the rapid transfer of the ^{131}I casein preparation between blood and lymph and suggest that the casein in the circulation was present in the monomeric form. The fact that high levels of ^{131}I casein occurred in the milk following its intravenous injection also showed that the casein in the circulation readily entered the mammary gland. It also indicates that after the casein molecule

had diffused into the milk, which has a low pH and characteristic electrolyte environment, it was sequestered there as aggregates or micelles. It may be that as the calcium concentration of the milk decreases and as the pH increases during the period in which milk is being absorbed from the gland (Petersen and Rigor, 1932b) there is a gradual alteration in the physico-chemical forms of casein in the milk. The equilibrium reaction, casein micelles $\rightleftharpoons$ casein aggregates $\rightleftharpoons$ casein monomers, would tend to favour the formation of casein monomers. The casein monomer diffuses into the interstitial fluid of the mammary gland and from there into the general circulation.

It is clear that some of the constituents of milk are removed preferentially by the lymphatics. Serum albumin is removed largely by way of the lymphatics while casein molecules appear to be absorbed directly into the blood stream. The rate of absorption of both albumin and casein from the mammary gland is slow and appears to be related to the bulk movement of fluid.

CHAPTER 6.

THE FUNCTION OF THE MAMMARY LYMPHATICS IN STAPHYLOCOCCAL AND STREPTOCOCCAL MASTITIS IN THE SHEEP

The mammary gland is constantly exposed to the risks of infection. The interior of the gland is in direct communication with the exterior by way of the streak canal. It is through this streak canal that bacteria may gain entry into the interior of the gland. It was considered that the inflammatory reaction resulting from the growth of bacteria within the gland would lead to changes in the character of the tissue fluid.

The functioning of regional lymphatics during inflammatory reactions has been studied by several workers (cf. Menkin, 1950; Yoffey and Courtice, 1956). Since the lymph from any tissue is thought to be of approximately the same composition as the tissue fluid from which it is derived, the effects of various degrees of injury on the composition of the tissue fluid can be measured by collecting lymph from the affected area. In general it has been found that in mild inflammation a marked exudation occurs initially, and this is accompanied by

a parallel increase in lymph flow and pressure. Menkin has suggested that if the local inflammation or necrosis is of sufficient intensity the lymphatics become blocked. As a result, when foreign proteins, bacteria or particulate substances are injected into the inflamed site they tend to be confined to the area but when such substances are injected intravenously they tend to accumulate in the inflamed area more readily than elsewhere. The extent to which these changes occur in the inflamed mammary gland has been investigated in the sheep by a method which permits continuous collection of lymph from both glands for long periods of time. Experimental infections were induced in one gland, leaving the other gland to serve as a control. The bacteria used were Staphylococcus aureus and Streptococcus agalactiae. These organisms give rise to 2 clinically recognized forms of mastitis which differ distinctly in their intensity. Thus Derbyshire (1958a) has described the intense oedema, necrosis and accompanying gangrene which occurs in the mammary gland of the goat infected with large numbers of virulent staphylococci. On the other hand, the injection of streptococci into the cavity of the mammary gland produces a less marked systemic reaction and only moderate local changes in the gland. These rapidly subside, leaving the gland fibrosed and atrophied (Pattison and Holman, 1951).

Four ewes were infected with Staphylococcus aureus and 3 with Streptococcus agalactiae in 7 separate experiments. Observations were made on the flow and composition of the lymph, on the transfer of T-1824 labelled albumin from plasma to lymph and on the absorption of ^{131}I H.S.A. from the infected mammary gland. The general clinical condition, temperature and pulse rate of the ewes were recorded twice daily during the course of the experiments. Particular attention was paid to the clinical condition of the udder. Milk samples were collected from both glands immediately before the start and at the completion of the experiments. No samples of milk were taken during the course of the experiment. The physical appearance of the milk was noted, its pH measured and its leucocyte content determined by the modified Breed smear technique (Howell, Pattison, Holman and Smith, 1954) or directly in a haemocytometer after dilution with white cell diluting fluid.

The experimental mastitis caused by the introduction of staphylococci into the cistern of the mammary gland of the ewe closely resembled the disease described in the goat by Derbyshire (1958a). In 1 animal, an acute non-gangrenous mastitis occurred; in the other 3, a typical gangrenous mastitis developed and sloughing of the skin occurred 36-72 hours after the start of the infection.

The reaction to the introduction of Streptococcus agalactiae into the milk cistern of the mammary gland of the ewe was somewhat different from that described for goats by Pattison and Holman (1951). Moderately acute signs of inflammation of the gland were observed within 4-6 hours after the injection and these persisted for 12-36 hours. The acute inflammation was followed by signs of a continuing sub-acute inflammatory change which was characterized by induration and atrophy of the glandular substance. The highest temperatures were recorded during the first 12 hours and in 1 ewe only was there evidence of a subsequent crisis. Marked systemic symptoms did not occur in any of the ewes.

The milk which was drawn from both sides of the udder before the injection of the bacteria and from the normal control side at the end of the experiment was of normal physical appearance. Leucocyte counts were within normal limits (less than 1×10^6 cells per ml.) and the pH of the milk samples varied between 6.5 and 6.8. The milk taken from the infected gland was obviously abnormal in appearance. This was particularly so in staphylococcal mastitis and the leucocyte content of the infected milk was as high as 500×10^6 cells per ml. The pH varied between 7.0 and 7.4. There was no indication in any of the experiments that the control gland became infected at any stage.

Lymph Flow and Composition.

Staphylococcal Mastitis. The changes in the flow rate and protein concentration of lymph in 2 of the ewes with staphylococcal mastitis are shown in Fig. 24. In all cases the lymph flow from the infected side began to increase within 4 hours after the injection of the bacteria. As the gland became more inflamed and oedematous, the flow increased and reached a maximum at 6-12 hours after the injection. The flow rate at this time was 3-5 times greater than the initial rate. From this point onwards, the flow rate decreased, despite an increase in the degree of oedema in the udder and surrounding tissues. The rate decreased very rapidly in those ewes in which the mastitis progressed to necrosis and gangrene (see Fig. 24). The flow rate of lymph from the control side decreased during the first few hours, but rose subsequently to rates which in 2 cases exceeded those from the infected side.

The protein concentration of the lymph from the infected side increased during the first 12 hours after the injection of the bacteria. The maximum concentration of protein in the lymph occurred about 12 hours after the injection and was 36-104 per cent higher than the original level. The protein concentration then decreased; the rate of decrease was more rapid in the ewes with non-

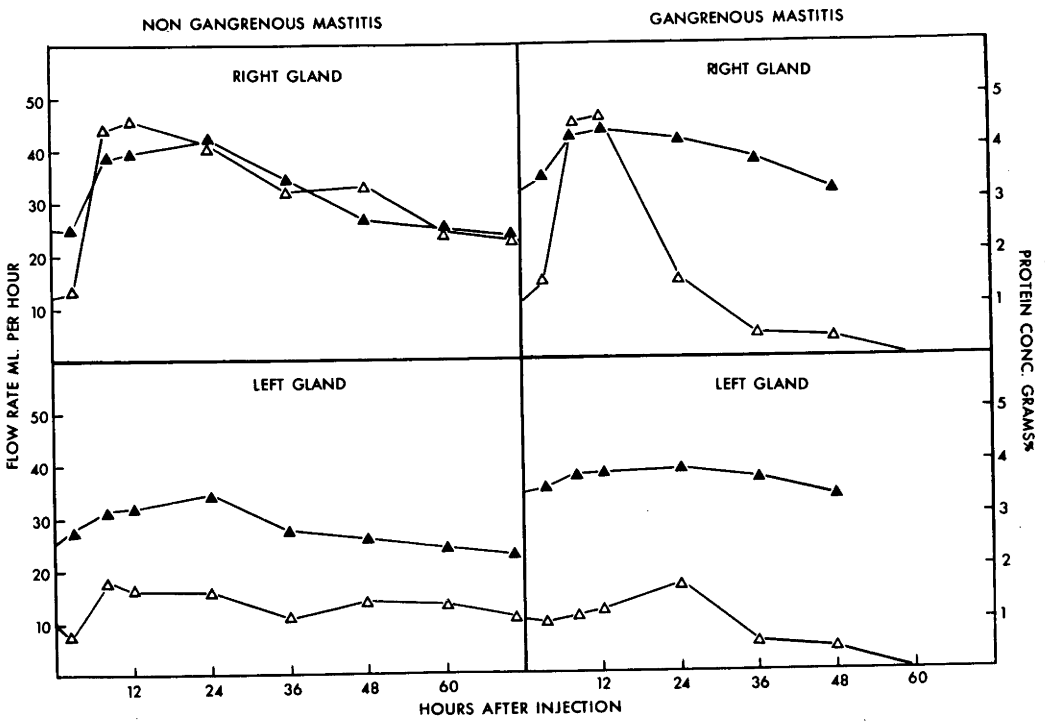

Figure 24.

The changes in the flow rate and protein concentration of lymph from the udder of two ewes with mastitis. Lymph was collected from both the right and left gland after a culture of Staphylococcus aureus was injected into the cavity of the right mammary gland.

△ - △ Flow rate

▲ - ▲ Protein concentration

gangrenous mastitis (see Fig. 24). The changes in the protein concentration of the lymph from the control gland were less marked than those observed in the lymph from the inflamed gland (see Fig. 24).

During the course of the infection there was a decrease of 15-25 per cent in the plasma protein concentration and a 15-20 per cent decrease in the A/G ratio. The A/G ratio of the lymph fell by 25-40 per cent on the inflamed side and to a lesser extent on the control side. The A/G ratio of the lymph however, was at all times higher than the plasma value.

The changes in the concentration of various constituents in the plasma and lymph from 3 ewes with experimental staphylococcal mastitis are given in Tables XI, XII and XIII. The phospholipid, esterified fatty acid and cholesterol concentrations in plasma and lymph were somewhat variable but in general were related to the protein concentration. The concentration of total esterified fatty acids decreased more than the other lipids and this suggested that the decrease was related to the triglyceride content. There was a sharp decrease in the concentration of free fatty acids in both plasma and lymph and the plasma-lymph gradient of free fatty acids was somewhat lower towards the end of the experiment. The plasma calcium concentration was slightly reduced during

TABLE XI

The concentration of various constituents in the plasma and lymph from a ewe with staphylococcal mastitis. The lymph was collected independently from both mammary glands. A culture of virulent staphylococci was introduced into the cistern of the right gland and an acute non-gangrenous mastitis developed subsequently. "P" indicates plasma values, "L_1" values for lymph from the control gland and "L_2" values for lymph from the infected gland.

Hours after injection of bacteria		0	12	24	48	72	120
Protein g. per cent	P	8.72	8.14	7.62	7.28	6.91	7.34
	L_1	2.61	3.18	3.36	2.66	2.44	2.38
	L_2	2.52	3.86	4.10	2.64	2.50	2.44
A/G ratio	P	.72	.68	.64	.68	.62	.68
	L_1	.84	.80	.78	.82	.79	.82
	L_2	.90	.78	.69	.76	.72	.82
Total esterified fatty acids. mg. per cent	P	152.1	146.2	138.6	142.0	128.1	127.9
	L_1	37.6	42.6	43.4	34.7	30.1	29.7
	L_2	38.2	46.6	37.3	30.5	32.7	26.1
Phospholipids mg. per cent	P	98.7	96.7	88.2	96.8	86.1	72.9
	L_1	33.8	36.2	41.6	32.2	29.3	26.0
	L_2	35.7	40.6	49.0	29.1	26.4	27.3
Cholesterol mg. per cent	P	120.4	112.0	114.7	107.2	106.3	115.2
	L_1	25.0	25.9	27.5	24.2	18.6	19.1
	L_2	25.6	27.8	29.2	18.7	19.8	20.4
Free fatty acids mg. per cent	P	18.6	16.8	15.1	10.9	10.4	7.0
	L_1	9.1	8.5	8.0	6.6	6.1	5.1
	L_2	8.9	8.6	7.9	6.8	5.6	4.8
Calcium mEq/l.	P	4.4	4.6	4.4	4.6	4.5	4.7
	L_1	3.6	3.5	3.8	3.7	3.6	3.7
	L_2	3.7	3.8	3.7	3.8	3.8	3.6
Inorganic phosphate mEq/l.	P	3.2	3.9	3.8	5.4	4.8	3.6
	L_1	3.3	3.6	3.8	4.8	3.4	3.7
	L_2	3.2	3.8	3.6	4.6	3.5	3.6

TABLE XII

The concentration of various constituents in the plasma and lymph from a ewe with staphylococcal mastitis. The lymph was collected independently from both mammary glands. A culture of virulent staphylococci was introduced into the cistern of the right gland and a gangrenous mastitis developed subsequently. "P" indicates plasma values, "L_1" values for lymph from the control gland and "L_2" values for lymph from the infected gland.

Hours after injection of bacteria		0	12	24	48	72
Protein g. per cent	P	7.62	7.55	6.80	6.11	6.26
	L_1	1.81	2.60	2.91	2.74	1.94
	L_2	1.78	2.94	3.62	3.12	2.12
A/G ratio	P	.99	.92	.83	.91	.84
	L_1	1.42	1.26	1.21	1.14	1.08
	L_2	1.51	1.21	1.10	1.06	1.14
Total esterified fatty acids. mg. per cent	P	171.0	164.1	156.2	136.2	118.1
	L_1	55.2	61.4	64.6	43.5	54.9
	L_2	53.4	68.2	75.1	56.0	45.2
Phospholipids mg. per cent	P	98.1	95.2	99.1	71.2	75.9
	L_1	39.2	43.5	48.2	41.9	30.0
	L_2	40.3	47.9	59.7	50.4	33.1
Cholesterol mg. per cent	P	97.2	88.1	84.3	91.5	83.4
	L_1	21.4	23.2	28.6	26.4	19.7
	L_2	20.6	28.6	36.3	29.7	23.6
Free fatty acids. mg. per cent	P	26.8	21.1	16.1	14.2	12.8
	L_1	12.8	11.4	8.8	7.5	7.4
	L_2	12.3	10.1	7.9	7.5	6.9
Calcium mEq/l.	P	5.6	5.5	5.2	5.5	5.4
	L_1	4.4	4.5	4.6	4.6	4.4
	L_2	4.3	4.3	4.6	4.6	4.5
Inorganic phosphate mEq/l.	P	3.5	3.6	3.4	3.6	3.5
	L_1	3.3	3.4	3.5	3.5	3.4
	L_2	3.2	3.5	3.4	3.5	3.4

TABLE XIII

The concentration of various constituents in the plasma and lymph from a ewe with staphylococcal mastitis. The lymph was collected independently from both mammary glands. A culture of virulent staphylococci was introduced into the cistern of the right gland and a gangrenous mastitis developed subsequently. "P" indicates plasma values, "L_1" values for lymph from the control gland and "L_2" values for lymph from the infected gland.

Hours after injection of bacteria		0	12	24	48
Protein g. per cent	P	8.30	8.0	7.60	6.88
	L_1	3.31	3.75	3.70	3.29
	L_2	3.20	4.36	4.06	3.24
A/G ratio	P	.91	.84	.72	.75
	L_1	1.26	1.00	.98	1.06
	L_2	1.30	.80	.84	.78
Total esterified fatty acids. mg. per cent	P	138.9	126.6	128.4	77.2
	L_1	44.2	50.2	46.2	32.4
	L_2	41.6	65.1	55.4	36.2
Phospholipids mg. per cent	P	104.2	98.2	95.4	92.1
	L_1	44.2	55.3	48.0	34.2
	L_2	43.6	59.7	51.3	36.8
Cholesterol mg. per cent	P	101.2	94.4	96.6	87.6
	L_1	31.6	36.1	37.7	27.3
	L_2	30.4	39.6	35.1	28.9
Free fatty acids. mg. per cent	P	22.1	13.4	13.8	8.4
	L_1	9.8	6.7	7.1	6.4
	L_2	10.1	6.8	6.5	5.6
Calcium mEq/l.	P	5.1	5.2	5.3	5.4
	L_1	4.6	4.6	4.7	4.6
	L_2	4.4	4.3	4.5	4.5
Inorganic phosphate. mEq/l.	P	3.7	3.8	3.6	3.5
	L_1	3.4	3.0	3.7	3.4
	L_2	3.5	3.7	3.6	3.8

the course of the infection and the plasma-lymph gradient was also slightly reduced. The inorganic phosphate concentrations were very variable and no consistent trend was evident (see Tables XI, XII and XIII).

Streptococcal Mastitis. The flow rate and protein concentration of lymph in a ewe with streptococcal mastitis is shown in Fig. 25. There was a sharp increase in the flow rate and in the protein concentration of lymph from the infected gland within 4 hours of the injection of the bacteria into the milk cistern. By about 12 hours after the injection the flow rate had increased to a level of 2-3 times higher than the initial rate. The flow then decreased and 6-24 hours later it was less than the pre-injection level (see Fig. 25). At this time atrophy and induration of the gland were evident. The protein concentration increased to a maximum level 12-24 hours after the injection of the bacteria and thereafter it decreased slowly. The maximum protein concentration was 35-70 per cent higher than the initial concentration. The flow rate of lymph from the control gland decreased steadily while the protein concentration increased; these changes were presumably attributable to the gradual atrophy of the gland. The plasma protein concentration and A/G ratio did not change significantly during the course of the experiments. Apart from this,

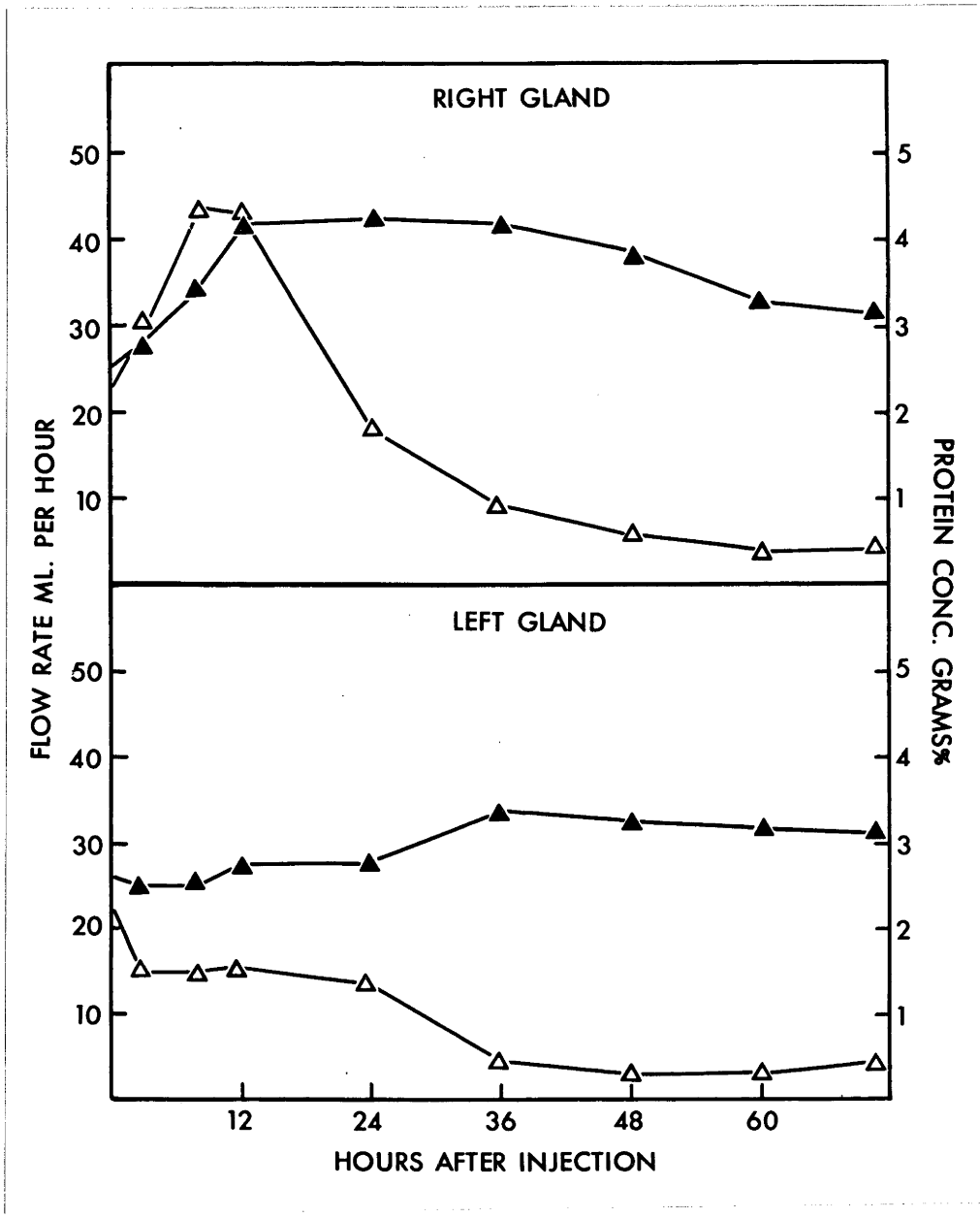

Figure 25.

The changes in the flow rate and protein concentration of lymph from the right and left mammary glands of a ewe. A culture of Streptococcus agalactiae was injected into the cavity of the right hand side mammary gland.

△ - △ Flow rate

▲ - ▲ Protein concentration

the changes in the composition of the plasma and in the lymph collected from the infected gland showed trends similar to, but less marked than those found in the ewes with staphylococcal mastitis. The changes in the concentration of the various constituents of plasma and lymph in 3 ewes with experimental streptococcal mastitis are given in Tables XIV, XV and XVI.

The Transfer of Albumin from Plasma to Lymph in Ewes with Mastitis.

T-1824 was injected intravenously into ewes with mastitis and the rate of transfer of T-1824 labelled albumin between the plasma and mammary lymph was determined.

Staphylococcal Mastitis. The specific activity of the albumin in the plasma and the lymph draining the control and inflamed glands of an udder infected with Staphylococcus aureus are depicted in Fig. 26. Initially the rate of transfer of labelled albumin was more rapid on the inflamed side. In the 3 animals in which a gangrenous mastitis developed the specific activity of the lymph of the control side increased sharply in 6-9 hours after the onset of the infection and by the 12th hour the specific activities of the lymph on each side were roughly equal. The half-life of the T-1824 in the plasma of the ewes with staphylococcal mastitis was variable but in general was less than that in the ewes with streptococcal mastitis. Twenty-four hours after the injection of the

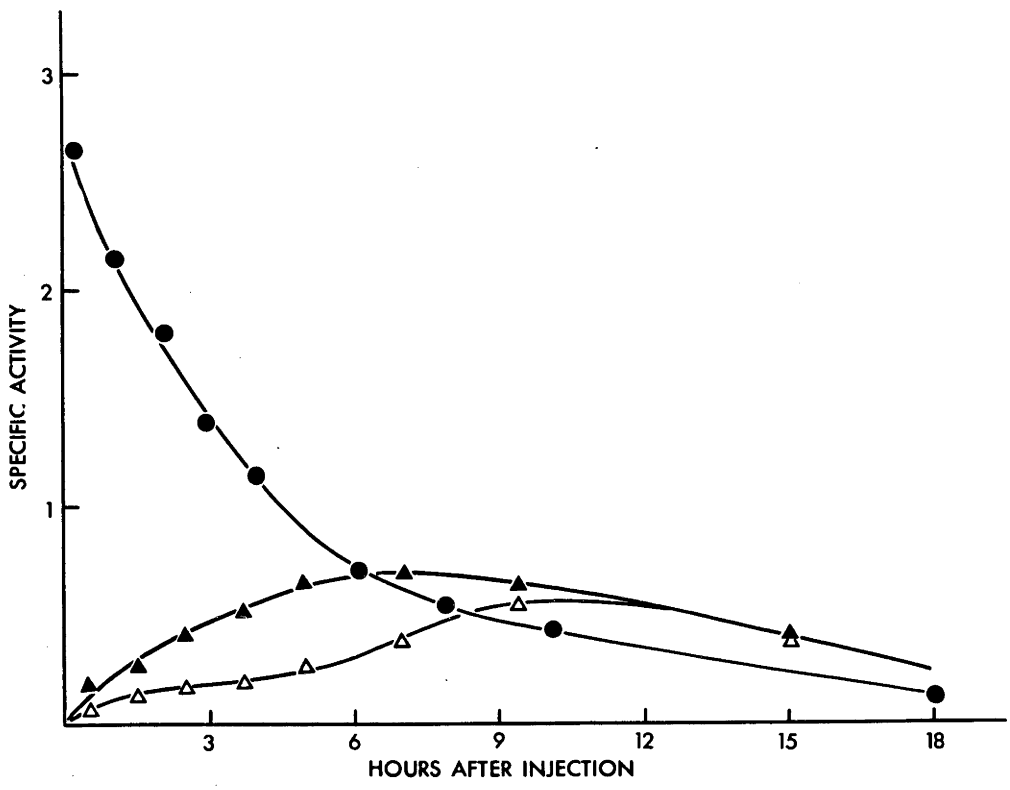

Figure 26.

The specific activity of the plasma and the lymph from each side of the udder following the intravenous injection of T-1824 into a ewe with mastitis. A culture of Staphylococcus aureus was injected into the right hand side mammary gland.

●-● Plasma

▲-▲ Lymph from right (inflamed) gland

△-△ Lymph from left (normal) gland

TABLE XIV

The concentration of various constituents in the plasma and lymph from a ewe with streptococcal mastitis. The lymph was collected independently from both mammary glands. A culture of virulent streptococci was introduced into the cistern of the right gland and a sub-acute mastitis developed subsequently. "P" indicates plasma values, "L_1" values for lymph from the control gland and "L_2" values for lymph from the infected gland.

Hours after injection of bacteria		0	12	24	48	96	144
Protein g. per cent	P	7.82	7.91	7.86	7.72	7.82	8.02
	L_1	2.60	2.68	2.75	2.75	3.14	3.10
	L_2	2.55	4.18	4.22	3.84	3.64	3.14
A/G ratio	P	1.22	1.10	1.20	1.26	1.21	1.22
	L_1	1.41	1.46	1.48	1.42	1.31	1.51
	L_2	1.52	1.31	1.40	1.36	1.41	1.44
Total esterified fatty acids. mg. per cent	P	176.2	171.4	164.8	172.2	164.4	167.2
	L_1	62.4	61.9	63.7	59.4	58.4	57.1
	L_2	65.1	69.4	75.6	75.6	70.2	61.2
Phospholipids mg. per cent	P	121.2	123.4	120.8	119.5	120.6	110.6
	L_1	36.3	37.1	40.5	41.0	39.8	41.2
	L_2	39.1	43.2	41.4	44.2	39.6	39.6
Cholesterol mg. per cent	P	113.7	116.2	112.2	113.9	114.3	110.0
	L_1	35.5	37.7	36.4	39.6	36.1	35.8
	L_2	34.9	38.2	37.6	38.9	31.0	38.6
Free fatty acids. mg. per cent	P	25.6	20.1	18.0	15.8	13.4	14.4
	L_1	11.3	10.1	8.8	8.1	7.2	7.9
	L_2	12.4	10.3	9.2	8.1	7.6	7.9
Calcium mEq/l.	P	5.2	5.0	5.1	4.9	5.2	5.1
	L_1	4.4	4.3	4.3	4.4	4.4	4.4
	L_2	4.5	4.5	4.6	4.4	4.6	4.1
Inorganic phosphate mEq/l.	P	3.0	2.9	3.2	3.0	3.3	3.1
	L_1	2.9	2.9	3.1	3.1	3.0	2.9
	L_2	2.8	2.8	3.2	3.0	3.2	2.8

TABLE XV

The concentration of various constituents in the plasma and lymph from a ewe with streptococcal mastitis. The lymph was collected independently from both mammary glands. A culture of virulent streptococci was introduced into the cistern of the right gland and a sub-acute mastitis developed subsequently. "P" indicates plasma values, "L_1" values for lymph from the control gland and "L_2" values for lymph from the infected gland.

Hours after injection of bacteria		0	12	24	48	72	120
Protein g. per cent	P	7.51	7.39	7.28	7.40	--	7.44
	L_1	1.60	1.55	1.72	1.84	1.78	1.81
	L_2	1.62	1.98	2.10	2.04	1.87	1.81
A/G ratio	P	.74	.77	.73	.84	--	.73
	L_1	1.10	1.01	.98	1.07	1.04	1.09
	L_2	1.04	.92	.88	.96	.93	.99
Total esterified fatty acids. mg. per cent	P	192.1	181.4	178.3	160.6	--	121.2
	L_1	61.3	62.4	58.9	54.3	57.1	42.1
	L_2	59.7	65.3	63.8	56.2	48.4	54.6
Phospholipids mg. per cent	P	127.1	124.3	120.9	122.5	--	126.1
	L_1	35.9	33.7	36.5	38.6	34.2	33.1
	L_2	37.2	41.3	42.4	38.1	33.2	35.1
Cholesterol mg. per cent	P	108.6	108.0	110.3	106.4	--	105.9
	L_1	24.6	22.9	23.2	21.1	18.5	18.0
	L_2	21.3	26.1	25.6	20.8	19.5	17.2
Free fatty acids. mg. per cent	P	30.8	27.7	23.1	24.2	--	10.6
	L_1	13.2	11.6	11.2	10.4	7.6	5.9
	L_2	12.1	11.6	10.8	10.6	8.4	6.1
Calcium mEq/l.	P	4.5	4.5	4.3	4.4	--	4.4
	L_1	3.7	3.7	3.7	3.6	3.5	3.4
	L_2	3.6	3.5	3.8	3.7	3.5	3.6
Inorganic phosphate mEq/l.	P	2.8	2.9	3.1	2.9	--	3.0
	L_1	2.7	2.8	3.1	3.0	3.0	3.1
	L_2	2.8	2.7	3.2	3.1	3.0	3.2

TABLE XVI

The concentration of various constituents in the plasma and lymph from a ewe with streptococcal mastitis. The lymph was collected independently from both mammary glands. A culture of virulent streptococci was introduced into the cistern of the right gland and a sub-acute mastitis developed subsequently. "P" indicates plasma values, "L_1" values for lymph from the control gland and "L_2" values for lymph from the infected gland.

Hours after injection of bacteria		0	12	24	48	72	120
Protein g. per cent	P	7.71	7.80	7.67	7.54	7.68	7.80
	L_1	3.12	3.08	3.24	3.46	3.52	3.68
	L_2	3.04	--	4.10	3.81	3.86	3.76
A/G ratio	P	.86	.94	.82	.85	.78	.82
	L_1	.98	1.06	.96	1.00	.94	1.05
	L_2	1.06	--	.98	.95	1.04	1.02
Total esterified fatty acids. mg. per cent	P	152.1	156.6	111.4	142.2	138.9	131.3
	L_1	48.7	51.3	29.1	32.3	21.4	33.2
	L_2	49.9	--	51.7	26.2	29.1	35.7
Phospholipids mg. per cent	P	104.6	106.2	101.9	99.6	98.2	70.2
	L_1	38.3	37.9	28.6	35.4	36.9	35.3
	L_2	41.0	--	43.2	29.7	28.3	31.2
Cholesterol mg. per cent	P	65.4	66.7	61.3	60.4	58.3	62.9
	L_1	20.2	21.6	18.7	16.9	18.3	19.2
	L_2	19.6	--	22.7	18.3	17.2	18.1
Free fatty acids. mg. per cent	P	22.2	20.3	16.5	14.9	14.0	8.1
	L_1	12.1	10.2	9.8	8.3	8.5	5.9
	L_2	12.2	--	8.8	8.5	7.9	6.5
Calcium mEq/l.	P	4.7	4.4	4.6	4.6	4.5	4.7
	L_1	4.1	4.2	4.5	4.3	4.1	4.2
	L_2	4.0	--	4.9	4.2	4.0	4.1
Inorganic phosphate mEq/l.	P	3.4	2.8	3.9	3.2	2.7	2.9
	L_1	3.3	2.7	4.0	3.4	2.9	3.0
	L_2	3.0	--	3.8	3.3	2.7	2.9

T-1824, the proportion which still remained in the plasma was significantly less in the ewes with staphylococcal mastitis than in ewes with streptococcal mastitis. Within 7-14 hours after infection, the specific activity of the lymph from both glands reached levels higher than those in the plasma; the levels remained elevated above plasma levels throughout the remaining period of observation.

Streptococcal Mastitis. The pattern of albumin transfer between plasma and lymph in ewes with streptococcal mastitis differed somewhat from that seen in the ewes with staphylococcal mastitis. As with staphylococcal mastitis, the labelled albumin appeared more rapidly in the lymph from the inflamed gland (see Fig. 27). The specific activity of the lymph became equal to that of the plasma 2½-10 hours after the injection of the T-1824 but thereafter the specific activity of the lymph from the infected gland and the plasma remained approximately equal. The specific activity of the lymph from the control gland reached that of the plasma 17-24 hours after the injection of the T-1824.

The Absorption of ^{131}I H.S.A. from the Inflamed Mammary Gland.

To follow the absorption of protein from the mammary gland during mastitis, ^{131}I H.S.A. was introduced into the cistern of the mammary gland 4-24 hours after the introduction of the bacteria. The radioactivity in

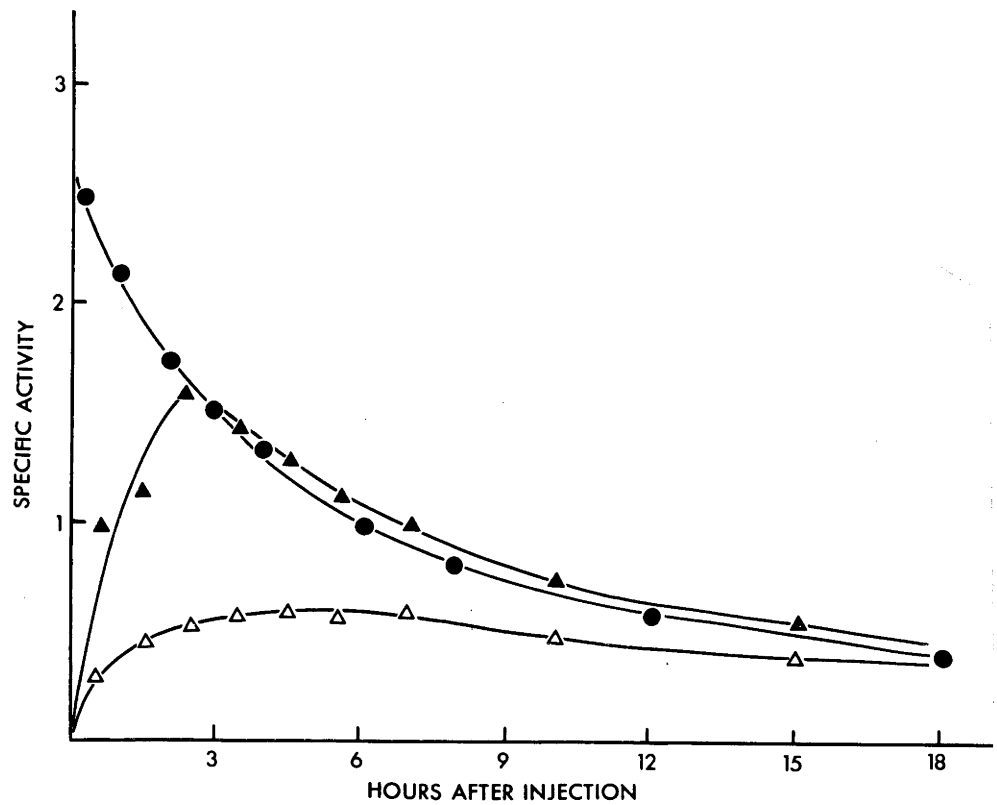

Figure 27.

The specific activity of the plasma and lymph from each side of the udder following the intravenous injection of T-1824 into a ewe with mastitis. A culture of Streptococcus agalactiae was injected into the right hand side mammary gland.

●-● Plasma

▲-▲ Lymph from right (inflamed) gland

△-△ Lymph from left (normal) gland

the plasma, lymph and urine was measured at intervals during the next few days.

Staphylococcal Mastitis. The rate of absorption of ^{131}I H.S.A. which had been introduced into the cistern of the mammary gland was very variable. In one case, the labelled protein was introduced 24 hours after the introduction of the bacterial culture, when obvious symptoms of necrosis of the skin of the udder were evident. During the following 16 hours of the experiment practically no labelled protein was recovered in the lymph, blood or urine. The animal was then slaughtered since extensive sloughing of the skin of the udder had occurred and the flow of lymph from both glands had almost stopped. In the remaining 3 ewes with staphylococcal mastitis the ^{131}I H.S.A. was introduced into the mammary gland cistern 4-6 hours after the bacteria. Radioactivity appeared in the lymph during the first hour after its injection into the gland and from this time onwards the level of radioactivity in the lymph increased rapidly. Initially, very low levels of radioactivity were found in the lymph from the control side. However, in 2 of the ewes which subsequently developed gangrenous mastitis, after 6-24 hours the radioactivity in the lymph from the control gland was higher than that from the inflamed gland. The details of the absorption of the labelled protein in 1 of the ewes with staphylococcal mastitis are shown in Fig. 28. The percentage of the

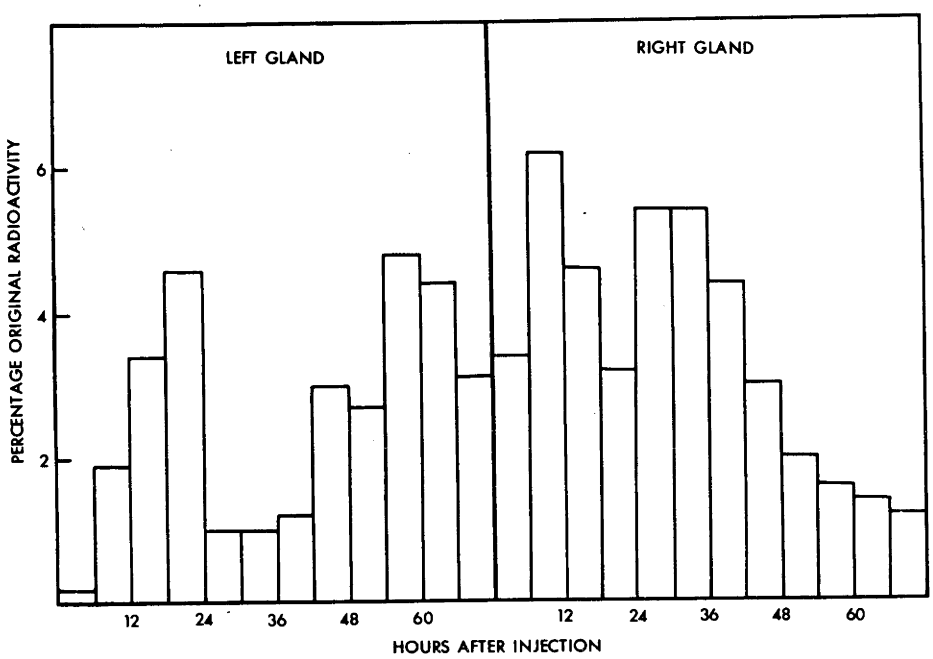

Figure 28.

The percentage of the original radioactivity collected in the lymph from both sides of the udder following the injection of ^{131}I H.S.A. into the cavity of the right hand side mammary gland of a ewe. A culture of Staphylococcus aureus had been injected into the same gland 4 hr. before.

original activity collected in the lymph and urine and the estimated percentage of the original activity present in the extracellular fluid of the body at the end of the experiment is given in Table XVII. In the ewe which developed acute non-gangrenous mastitis the proportion of the original radioactivity collected in the lymph of the normal side was only a small fraction of that which was collected from the inflamed side (see Table XVII).

Streptococcal Mastitis. ^{131}I H.S.A. was introduced into the mammary gland cistern 4-6 hours after the introduction of the bacteria. Fig. 29 gives details of the absorption of labelled protein in a ewe with streptococcal mastitis. The percentage of the original activity collected in the lymph and urine and the estimated percentage of the original activity present in the extracellular fluid of the body at the end of the experiment is given in Table XVIII. Radioactivity appeared in the lymph in high concentrations during the first hour after its injection and the maximum rate of absorption of the labelled protein by the lymphatics occurred 6-24 hours later. Only a small proportion of the radioactivity which was injected into the infected gland was absorbed by the lymphatics of the control gland.

Variable amounts of radioactivity were found in the plasma of all the ewes during the course of the experiments. The level of radioactivity in the plasma

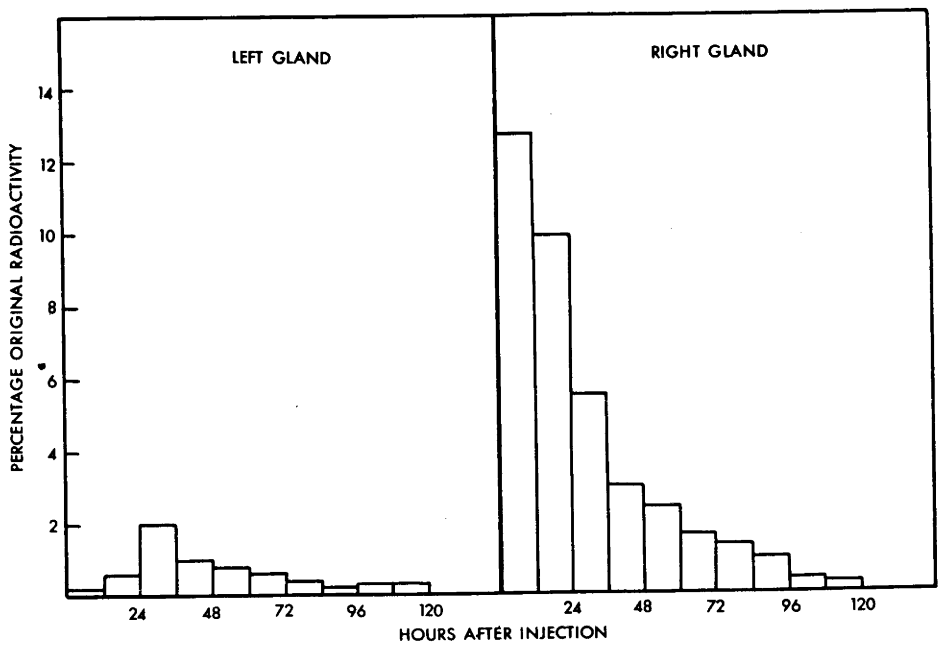

Figure 29.

The percentage of the original radioactivity collected in the lymph from both sides of the udder following the injection of ^{131}I H.S.A. into the cavity of the right hand side mammary gland of a ewe. A culture of Streptococcus agalactiae had been injected into the same gland 4 hr. before.

TABLE XVII

The percentage of the original radioactivity collected in the lymph and urine and the percentage remaining in the extracellular fluid of the body following the introduction of ^{131}I H.S.A. into the mammary gland cistern of lactating ewes with staphylococcal mastitis. The results of 4 separate experiments are given.

Type of Mastitis	Duration of Experiment (hours)	Percentage of Original Activity				
		Collected in Lymph (control gland)	Collected in Lymph (infected gland)	Collected in Urine	In Extracellular fluid at end of Experiment	Total Recovered
Gangrenous mastitis	72	15.6	21.0	5.0	14.6	56.3
" "	48	0.001	0.002	0	0.001	0.004
" "	96	26.8	18.5	4.6	25.2	75.11
Non-gangrenous mastitis	120	3.2	40.6	9.6	8.0	61.4

TABLE XVIII

The percentage of the original radioactivity collected in the lymph and urine and the percentage remaining in the extracellular fluid of the body following the introduction of ^{131}I H.S.A. into the mammary gland cistern of lactating ewes with streptococcal mastitis. The results of 3 separate experiments are given.

Type of Mastitis	Duration of Experiment (hours)	Percentage of Original Activity: Collected in Lymph (control gland)	Collected in Lymph (infected gland)	Collected in Urine	In Extracellular fluid at end of Experiment	Total Recovered
Streptococcal mastitis	144	1.7	40.7	1.9	20.2	63.3
" "	120	6.4	38.0	8.1	18.3	70.8
" "	120	0.7	17.2	2.2	18.1	38.2

however was always only a small fraction of that in the lymph from the inflamed gland. The radioactivity in the plasma and lymph samples was bound to protein while that in the urine was not protein bound.

DISCUSSION

The Rate of Flow and Composition of Lymph in Mastitis.

The decrease in the flow rate of lymph which occurred about 12 hours after the injection of staphylococci may, as was suggested by Menkin (1950), be due to lymphatic blockage. This decrease, which was very striking in one case, occurred in the presence of a large amount of local oedema. At the same time the flow rate and protein concentration of lymph on the control side increased. This suggested that as the lymphatic drainage of the inflamed gland became obstructed, collateral lymphatics opened up. The anatomical studies by El Hagri (1945b) on the udders of a large number of cows have shown that the lymphatic drainage of the two halves of the udder occurs independently in 77 per cent of cases. If, as appears to be the case, the anatomical arrangement of the lymphatics of the udder of cows is similar to that of ewes, it seems that the ramifications of the lymphatic ducts in the udder can be realized only when obstructions occur in part of the system. A large reserve of lymphatics may not normally carry any lymph and this may not be appreciated in

anatomical preparations using conventional techniques of dye injection.

When a tissue undergoes extensive damage, the protein content of the lymph draining from the area increases. Thus lymph draining from burned areas has a protein concentration close to that of plasma (cf. Yoffey and Courtice, 1956). In the case of the lymph draining from the udders of ewes with severe mastitis the protein concentration in the lymph did not increase to the extent that it approximated plasma protein concentration. This difference was probably due to the fact that a significant proportion of the mammary lymph collected was formed in tissues not directly involved in the inflammatory process. Thus the lymph collected was probably a mixture of lymph of normal composition derived from areas such as the perineum and skin around the gland, with lymph of changed composition derived from areas of acute inflammation. For similar reasons, the A/G ratio in the lymph never reached that of plasma as it was found to do in lymph draining areas of thermal burn (Perlman, Glenn and Kaufmann, 1943).

There was no evidence that blockage of lymphatics was a feature of streptococcal mastitis. The decrease in flow which occurred in the absence of oedema was probably associated with the prompt resolution of the inflammatory process and atrophy of the residual mammary

tissue which was not destroyed in the inflammatory process. The decrease in flow rate was probably attributable to a decrease in filtration area which might be expected to accompany the atrophy of the gland.

The Transfer of Albumin from Plasma to Mammary Lymph in Ewes with Mastitis.

The results indicate that when labelled albumin was injected intravenously into ewes with staphylococcal mastitis, it left the circulation more rapidly than it did in normal ewes or in ewes with streptococcal mastitis. This was probably due to the rapid transfer of labelled albumin into a greatly expanded interstitial pool in the inflamed region of the udder in this type of mastitis. The significant decrease in the plasma protein concentration during the course of the experiment is consistent with this finding.

The fact that the albumin specific activity curves of the lymph of the 2 glands remained well above that of the plasma after the curves crossed suggests that there was a considerable delay between the time the labelled protein molecules filtered from the blood stream to the time they appeared in the lymph. This may have been due to some mechanism which hindered the transfer of protein from the interstitial fluid to the lymph as well as to an increase in volume of the interstitial fluid pool. The tendency for the specific activity of lymph from the normal side

to increase after the first few hours is further evidence in support of the view that in this type of mastitis the lymphatic drainage of the 2 glands is not independent.

The results of the experiments in the ewes with streptococcal mastitis indicate that less labelled albumin was transferred from the plasma to the interstitial pool of the mammary gland, but that the filtered labelled albumin was transferred more readily from the interstitial fluid to the lymph. The relatively small volume of the interstitial fluid pool and the absence of obstruction of the lymphatics were probably responsible for the ready transfer of the labelled albumin from the interstitial fluid to the lymph.

The Absorption of ^{131}I H.S.A. from the Inflamed Mammary Gland.

Again, the results of the experiments suggested that blockage of the lymphatics occurred in staphylococcal mastitis. In one case the blockage was almost complete. However, since in this ewe it was shown that dye-labelled albumin continued to be transferred from plasma to lymph, it follows that the blocked lymphatics appeared to drain chiefly the acini and ducts of the gland. The lymphatics draining adjacent tissues, where the inflammatory reaction was probably less severe, must have remained patent. The fact that a considerable proportion of the injected ^{131}I H.S.A. was removed by the lymphatics of the normal side

in 2 of the ewes again points to the participation of collateral lymphatics in the removal of lymph from the damaged area.

In general, the absorption of ^{131}I H.S.A. from the mammary glands of ewes with mastitis occurred much more rapidly than in ewes with normal undistended secreting glands (see Chapter 5). It was shown also that when ^{131}I H.S.A. was injected intravenously into a ewe with mastitis, the radioactive protein appeared in the milk in much higher concentrations on the infected side than on the control side. The rate of transfer of ^{131}I H.S.A. across the mammary epithelial barrier would thus appear to be increased in both directions under conditions of inflammation.

In experiments in which radioactive protein was injected into the mammary gland, the proportion of the injected radioactivity which was collected in the urine plus that calculated to be in the extracellular fluid of the body at the end of the experiment, was very variable. A similar variability was reported in the experiments describing ^{131}I H.S.A. absorption from the normal mammary gland (see Chapter 5). It was suggested then that this radioactivity, which was at all times bound to protein, was probably returned to the general circulation by way of other lymphatics rather than directly into the blood stream.

Because of this variability it was impossible to decide whether a significant direct transfer of ^{131}I H.S.A. occurs between the mammary interstitial fluid and the blood under conditions of inflammation.

It has been suggested that the oedema of the ventral abdominal wall and udder which is a characteristic feature of staphylococcal mastitis is due to the occlusion of the mammary veins by thrombi (Derbyshire, 1958b). The results of the present experiments indicate that blockage of lymphatics is a characteristic feature and this would undoubtedly contribute to oedema formation.

CHAPTER 7.

GENERAL DISCUSSION

Lymph Composition in relation to Milk Secretion.

The mammary gland is a specialized secretory tissue which is capable of secreting characteristic substances, casein, whey proteins, milk fat and lactose. These substances are synthesized principally by the epithelial cells of the acini and possibly the intercalary ducts. This region is richly supplied with blood capillaries (Turner, 1952). The synthesis of these constituents of milk depends in part on the availability of precursor substrates such as glucose, acetate, amino acids etc. which circulate in the blood and interstitial fluids of the body.

The milk proteins, casein β-lactoglobulin and α-lactalbumin are known to be synthesized almost completely from the free amino acids found in the blood (Barry, 1958) while the milk fat fatty acids are synthesized largely from acetate, β-hydroxybutyrate, free fatty acids and triglycerides (Glascock, 1958; Garton, 1960). Glucose appears to be the main precursor for lactose and milk fat glycerol (Folley, 1956). In addition to de novo synthesis

the mammary gland is also capable of extracting and concentrating various constituents of the plasma such as calcium, potassium, inorganic phosphate, iodine and several of the vitamins. These substances are consequently found in milk in greater concentrations than in plasma.

The continuous extraction of these precursors from the interstitial fluid surrounding the alveoli and intercalary ducts would tend to lower their concentration in the tissue fluid. It is known however, that molecules of low molecular weight exchange very rapidly in both directions across the capillary endothelium (cf. Pappenheimer, 1953) and thus the concentration of most of the milk precursors of small molecular weight, in the tissue fluid or lymph would not be expected to differ significantly from that of venous blood. The experimental results have indicated that this is the case.

It has been shown that acetate and β-hydroxybutyrate are almost the sole precursors of all the shorter chain fatty acids of milk fat (Popjak, French and Folley, 1951; Popjak, French, Hunter and Martin, 1951; Rogers and Kleiber, 1956). There is however an appreciable proportion of long chain fatty acids in milk and it is not decided what proportion of these fatty acids are synthesized from acetate and β-hydroxybutyrate. That dietary fat may contribute to milk fat synthesis was

first demonstrated by Aylward, Blackwood and Smith (1937). More recently this was confirmed by Glascock, Duncombe and Reinius (1956) in experiments in which labelled free fatty acids and triglycerides were fed by mouth to cows. These workers concluded that the contribution of dietary fat to milk fat fatty acid synthesis was not more than 25 per cent. There is also indirect evidence that depot fats may contribute to milk fat synthesis under certain conditions. In this regard it is well known that a high producing dairy cow loses a considerable amount of body fat during her period of lactation. Furthermore, the increase in the degree of unsaturation of milk fats in dairy cows in inanition has been shown to be due to an increase in the concentration of oleic acid which is probably derived from the fat depots (Smith and Dastur, 1938).

The results presented in Chapter 4 of this thesis clearly show that the level of free fatty acids and probably triglycerides in plasma and lymph are much higher in ewes in early lactation than at other times during the lactation period. These high levels suggest that there is a mobilization of depot fat in response to lactation. It is interesting in this regard that under conditions where milk secretion stops, such as in mastitis or when the lamb is removed from a milking ewe, the concentration of free fatty acids in the plasma and lymph falls.

The exact mechanism for the mobilization of

depot fat under these conditions is not known. It is possible that the production of volatile fatty acids such as acetate and butyrate in the rumen, cannot meet the demands of the actively metabolizing mammary gland in early lactation and alternative substrate is mobilized from the body's energy stores. The mechanism responsible for the mobilization of depot fat is not known, but it is possible that some hormone mechanism is involved. Chalmers, Pawan and Kekwich (1960) have recently isolated a hormone, "starvation hormone", from the urine of humans and sheep. This hormone, which causes transient hypoglycemia, ketonemia and increased mobilization and catabolism of fat with depletion of body fat when injected into mice, might be involved in the mobilization of depot fat in ewes in early lactation. The mechanism of fat mobilization in ewes in early lactation may be more direct than this and may be related to the high blood levels of one or more of the hormones which are involved in lactogenesis or galactopoiesis such as prolactin or growth hormone. Reiss (1947) has claimed that injections of prolactin were capable of mobilizing body fat in rats, but as it is doubtful that the hormone preparation used for these experiments was pure, these results must be treated with caution.

The gradient of the concentration of free fatty acids between plasma and lymph has been found to be much higher in ewes in early lactation than at other stages in

the lactation period. This suggests that free fatty acids are readily taken up from the interstitial fluid of the mammary gland by the secretory epithelium thus lowering their concentration in the lymph.

Free fatty acids are known to be transported in the blood for the most part bound to albumin thus forming a water soluble complex (cf. Fredrickson and Gordon, 1958). In view of the very short circulating half-life of free fatty acids in the blood (Havel and Fredrickson, 1956; Laurell, 1957) relative to that of albumin it is thought that the fatty acid-albumin complex dissociates and the fatty acid is transferred across the endothelial cells independently, probably recombining with albumin as it enters the tissue fluid. Free fatty acids are probably transferred across the capillary membrane of the mammary gland of the ewe independently and the rate of transfer is probably rapid as in other species. In view of the large gradient which exists between plasma and lymph in ewes in early lactation it is thought that the rate of exchange of free fatty acids between plasma and lymph may be significantly slower than that of molecules such as acetate. The concentration of acetate in plasma and lymph was found not to alter significantly even in ewes in early lactation when the extraction of acetate by mammary tissue was high.

The concentration gradient of free fatty acids between plasma and lymph is considerably lower in ewes in

in the dry state than in ewes in early lactation. No satisfactory explanation can be advanced however to explain why this gradient in the dry ewe, even though it is small, should exist at all.

Lymph Flow and Protein Concentration.

The rapid flow rate of lymph from the lactating gland is thought to be due to an increase in filtration area and filtration pressure. The lymph protein output is increased under these conditions but the protein concentration is decreased. The evidence thus does not suggest alteration in the capillary permeability. On the other hand the increased flow of lymph from the inflamed mammary glands is accompanied by a rise in protein concentration and reduction in the A/G ratio. An increase in capillary permeability is undoubtedly associated with this response.

When lambs were removed from ewes in early lactation, the flow of lymph from the gland decreased. This decrease in lymph flow appeared to be associated with engorgement of the udder with milk. There did not appear to be any reabsorption of fluid from the milk cisterns ducts or acini by the mammary lymphatics under these conditions. It is possible that in the turgid udder the tension within the substance of the glands may cause the collapse of both blood and lymphatic capillaries with a consequent reduction in filtration area and in lymph flow. This phenomenon was observed by Jeffers (1935) and Silver

(1956) in histological sections of the mammary glands of recently weaned rats. As involution progresses and the glandular elements atrophy and degenerate there is a concomitant reduction in a number of capillary beds and the blood flow through the udder decreases. At the same time there is probably a fall in capillary hydrostatic pressure (Linzell, 1959). These factors undoubtedly would be responsible for the reduction in lymph flow and protein output observed during this period. At the same time there is an increase in protein concentration which is probably caused by a relatively greater concentration of the capillary filtrate.

The Resolution of Milk Constituents.

Petersen and Rigor (1932a) studied the pressure changes which occurred in the cavity of the mammary gland when milk was allowed to accumulate. The pressure in the mammary gland reached a maximum 24-36 hours after milking was stopped. It was concluded that milk secretion ceased and absorption began when the intra-mammary pressure reached 25 mm. of mercury. The results of the experiments presented in Chapter 5 of this thesis have shown when labelled serum albumin is introduced into the gland cistern of udders which become distended with milk, most of it is removed rapidly by way of the lymphatics. The absorption of the albumin appears to accompany the absorption of fluid from the gland; the highest rates of absorption under these conditions occur

between 24 and 48 hours after suckling is stopped when the udder is maximally distended. It appears that acini are likely to rupture in these circumstances and thus provide direct communications between the gland cavity and the interstitial fluid. This must occur infrequently and to only a limited extent as it seemed that not more than a few per cent of the material in the udder is absorbed by this means.

The rate of absorption of serum albumin was much slower from undistended glands. Such was the case when suckling was stopped in those ewes in a later stage of lactation. The bulk of the serum albumin was removed between the 5th and 10th days after suckling was stopped when the mammary gland was undergoing involution. Histological studies on the process of involution in rats have been carried out by a number of workers (Emmel, Weatherford and Streicher, 1926; Jeffers, 1935; Silver, 1956). The first definite signs of involution occur between the 3rd and 5th days when degenerative changes in the epithelial cells of the acini are evident and leucocytes infiltrate the spaces between the alveoli and between the alveolar epithelial cells. The alveoli soon lose their identity as the epithelial cells degenerate and disappear. As the alveolar tissue regresses, there is an expansion of connective tissue elements which fill in the spaces left

by the acini and smaller ducts. Thus when intra-mammary pressure is low, the bulk of the absorption of serum albumin and also of the fluid occurs when these degenerative processes are taking place. Similarly, injury to the secretory epithelium as in mastitis leads to more rapid absorption of albumin.

The results have suggested that when rupture of the secretory epithelial membrane occurs, casein micelles, along with the other constituents of the milk are transferred to the interstitial fluid. In view of their large size it is probable that any casein micelles which enter the interstitial fluid can only be removed by way of the lymphatic system. However, most of the casein appears to be removed after disaggregation of the micelles. The size of the disaggregated casein micelle (probably monomeric casein) is sufficiently small to allow their direct transfer to the blood stream and the lymphatics thus play a minor role in the absorption of casein.

Milk fat particles have been found in the milky lymph when the udder is grossly distended with milk. Again it would be expected that all the fat particles which entered the interstitial fluid would be absorbed by the regional lymphatics. However most of the accumulated milk fat is removed either very slowly or in a form which does not involve the lymphatics. It may be that milk fat is broken down by the involuting mammary epithelial cells or

that it is taken up and metabolized or carried away in wandering cells which are seen in large numbers in involuting mammary tissue (Okada, 1957). The glycerol and fatty acids produced if intracellular degradation of milk fat occurs, would equilibrate rapidly with the blood passing through the gland. There is thus no change in the composition of lymph which indicates a preferential removal of milk constituents by the lymphatics except when the udder becomes grossly distended with milk. The removal of serum albumin and possibly milk globulin involves the lymphatic system. However these constituents are present in milk in low concentration and are removed too slowly to cause significant alteration in the protein content of lymph.

SUMMARY OF EXPERIMENTAL RESULTS

Chapter 3.

The surgical technique for collecting lymph from the mammary lymph duct of ewes has been described. The procedure described has been used on 50 ewes and in 10 of these the lymphatics of both sides were cannulated. In most of the ewes the lymph from the 2 halves of the udder appeared to drain independently. However in 3 ewes the great bulk of the lymph formed in the udder was drained by 1 or 2 large lymphatic trunks situated on the left hand side. Lymph from the surrounding skin and escutcheon were shown to be at least partially drained by the mammary lymphatics.

Chapter 4.

An investigation has been made on the flow and composition of lymph collected from the mammary lymph duct of lactating and dry merino ewes. Lymph flowed at the rate of 20-40 ml. per hour in ewes in early lactation and 2-6 ml. per hour in dry ewes. The rate of lymph flow was highest in those ewes producing the most milk. Maximum rates of flow of 50-180 ml. per hour were recorded for short periods of time in lactating ewes when they were suckled or when they were milked. In the lactating ewes, the mammary lymph ducts contributed up to 20-30 per cent of the total thoracic duct lymph flow.

In general, the composition of udder lymph was similar to the data reported for the composition of lymph collected from areas such as the head, neck and limbs in other animals. In lactating ewes there was a large plasma/lymph concentration gradient for both free fatty acids and triglycerides and this suggested that these substances were being utilized by the mammary gland.

Estimates were made on the size of the interstitial fluid pool drained by the mammary lymph duct in lactating and dry ewes. Values of 100-220 ml. for ewes in early lactation and 20-60 ml. in dry ewes were found. These findings have been discussed in relation to the exchange of substances between the plasma and the mammary gland during the synthesis and secretion of milk.

Chapter 5.

A number of experiments were carried out to determine the role played by the lymphatic system in the lactating mammary gland of the ewe in the absorption of milk constituents. Initial investigations, which were concerned with changes in composition of the lymph when the lamb was removed from its mother early in lactation indicated that no significant change occurred unless the gland became grossly distended with milk. In 3 ewes, the normally clear lymph became distinctly milky in appearance when the udder became very distended with milk. This was thought to be caused by rupture of acini so

that milk enters the interstitial fluid. Protein peculiar to milk (probably casein) and fat particles have been identified in the milky lymph.

Experiments were carried out subsequently to determine the way in which labelled serum albumin and casein were removed from the mammary gland after suckling was stopped. ^{131}I H.S.A. and ^{131}I casein were introduced into the mammary gland cistern through a polythene cannula and lymph, plasma and urine samples were collected during the next 7-14 days and the radioactivity measured.

^{131}I H.S.A. was absorbed from the mammary gland without being broken down and was removed largely by way of the lymphatics. The rate at which absorption of albumin occurred appeared to be related to the condition of the glandular epithelium and the hydrostatic pressure which developed in the mammary gland. It also appeared that ^{131}I casein was absorbed from the mammary gland and entered the interstitial pool without being broken down. The experimental evidence suggested that the absorption of casein was associated with disaggregation of the casein micelle into its constituent casein molecules and these were readily transferred from the interstitial fluid directly into the blood stream, probably as a casein monomer. The lymphatics thus play an insignificant role in the absorption of the bulk of the casein.

The behaviour of ^{131}I casein when introduced intravenously was studied in lactating ewes with mammary lymph duct fistulae. The rapid rate at which casein left the blood and entered the lymph, milk and urine suggested that the casein in the blood and extravascular fluid existed as a small molecule. The results of the experiments are discussed in relation to the physico-chemical forms in which casein exists in the body fluids.

Chapter 6.

Experiments were carried out to examine some aspects of the function of mammary lymphatics in experimental staphylococcal and streptococcal mastitis in ewes. Lymph was collected continuously from both glands of the udder. Mastitis was induced in one gland and the other gland served as a control. Observations were made on the flow rate and composition of lymph, the rate of transfer of albumin labelled with T-1824 from plasma to lymph and the rate of absorption of ^{131}I H.S.A. from the cavity of the inflamed gland.

There was a rapid increase in the flow rate and protein concentration of lymph of the inflamed side within a few hours after the injection of the bacteria. A rapid decrease in flow occurred 12-24 hours after the injection of the bacteria, and this was seen particularly in those ewes which developed gangrenous mastitis. This decrease

in flow occurred during the accumulation of local oedema fluid. The rate of absorption of ^{131}I H.S.A. from less severely inflamed glands was much more rapid than observed previously in the normal undistended gland. The experimental results suggested that blockage of the lymphatics was a characteristic occurrence in ewes with gangrenous mastitis. Under these conditions collateral lymphatics appeared to function and the lymphatic drainage of the 2 halves of the udder was no longer independent.

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