JOHN CURTIN SCHOOL OF MEDICAL RESEARCH 630/1963

DEPARTMENT OF EXPERIMENTAL PATHOLOGY

ANNUAL REPORT 1962

Staff

Professor	F.C. Courtice, M.A., D.Phil., D.Sc., L.R.C.P., M.R.C.S., Hon. F.R.A.C.S., F.R.A.C.P., F.A.A.
Reader	G.B. Mackaness, M.B., B.S., M.A., D.Phil., D.C.P.
Senior Fellows	Bede Morris, B.V.Sc., D. Phil.;
	S.V. Boyden, B.Sc. (Vet.Sci.), Ph.D., M.R.C.V.S.
Senior Research Fellow	W.E. Stehbens, M.D., D.Phil., M.C.P.A. (from 6th June, 1962)
Research Fellows	A.K. Lascelles, M.V.Sc. (until 31st May, 1962)
	G. Woolley, M.Sc., Ph.D.
Visiting Fellow	M.A. Mishkel, B.Sc. (Med.), M.B., B.S., M.R.A.C.P. (supported by Life Insurance Medical Research Fund of Australia and New Zealand)
Research Assistants	A. Schmidt-Diedrichs, M.D. (until 16th November, 1962)
	R.J. North, B.Sc.
	M. Marcus (honorary; from 12th September, 1962)
Head Technician	J. Harding

Student and Teaching Activities

There are nine students proceeding to the Degree of Doctor of Philosophy: Mr. D.G. Garlick, B.Sc. (Med.), M.B., B.S. (commenced 2nd February, 1960); Mr. T.J. Heath, B.V.Sc. (commenced 27th June, 1960); Mr. R.B. Vaughan, M.B., B.S. (commenced 2nd January, 1961); Mr. D.S. Nelson, B.Sc. (Med.), M.B., B.S. (commenced 16th January, 1961); Mr. M.W. Simpson-Morgan, B.V.Sc. (commenced 16th January, 1961); Mr. V.P. Ackerman, B.A., M.B., B.S. (commenced 1st February, 1961); Mr. J.G. Hall, M.B., B.S., M.R.C.S., L.R.C.P. (commenced 14th August, 1961); Dr. C. Maureen Sass, M.D. (commenced 14th August, 1961) and Mr. E.P. Adams, B.Sc., M.Sc. (commenced 5th April, 1962).

Mr. A.K. Lascelles was awarded the degree of Doctor of Philosophy for a thesis entitled "Studies on Mammary Gland Lymph in relation to the secretion and resolution of milk."

Seminars given by members of the staff and also by visitors have been held within the Department.

Research Programme

The work in the Department during the year concerned several fields of experimental pathology, related especially to the pathogenesis of atherosclerosis, the functions of the lymphatic

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system in health and disease, disorders of lipid metabolism, cellular resistance to infection and various aspects of cell biology and immunology.

Long-term effects of injury of the arterial wall (Courtice, Schmidt-Diedrichs)

It was previously reported that lesions resembling those of experimental atherosclerosis develop in the wall of arteries that have been injured, provided the level of plasma cholesterol is appreciably increased. Further experiments were carried out to show the long-term changes, up to 6 or 9 months, that follow injury to the arterial wall. Two points have especially been studied: (i) the effect of restoring the cholesterol level to normal limits for several months in the lesions established in a hypercholesterolaemic animal and (ii) the effect of inducing hypercholesterolaemia one month after injuring the arterial wall of an animal on a normal diet, i.e. at a time when the intima of the injured areas had become considerably thickened, but did not contain lipid. The results showed that in the first group of experiments, the atherosclerotic lesions were still present nine months after injury and in the second group these lesions developed in the injured area but probably to a lesser extent than in the first group.

The removal of various lipoproteins from loops of doubly ligated arteries and veins (Schmidt-Diedrichs, Courtice)

Experiments have shown that the resolution of atherosclerotic lesions in the hypercholesterolaemic animal is very slow even when the lipoprotein levels in the plasma have been restored to normal. To study further the mechanisms concerned in lipid deposition and removal from the intima of arteries following injury, experiments have been designed to observe histologically the fate of different lipoproteins injected into loops of doubly-ligated portions of the carotid artery and facial vein of the rabbit. In loops of arteries, cholesterol-rich lipoproteins were mainly taken up by macrophages which were then gradually removed from the area, probably to a large extent by ingrowing vessels from the adventitia and media. In doubly-ligated veins the process of organization and removal of the lipid-laden macrophages was more rapid than in the case of arteries. This is probably due to the fact that vasa vasorum of the adventitia can more readily reach the intima. It seems that the rate of vascular-ization of the tissue containing the lipid-laden macrophages may play a significant role in their removal.

The pathogenesis of atherosclerosis and early intravascular thrombosis (Stehbens)

A study of the pathogenesis of atherosclerosis has been commenced. The emphasis is on the nature and causes of intimal proliferation which is an integral part of atherosclerosis, for it has a similar distribution and precedes lipid deposition in the arterial wall. The structure and the localisation of the intimal thickening in the cerebral and coronary arteries of infants is being examined. Comparative studies are also in progress.

An histological investigation of the intimal proliferation at the bifurcation of the renal artery in normal and hyper-cholesterolaemic rabbits is in progress preparatory to electron microscopy.

As intimal proliferation may result from injury, the reaction of endothelium to bacterial lipopolysaccharides and the conditions governing early thrombosis are being studied. Transparent ear chambers have been constructed for insertion in rabbits' ears for

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direct observation of intravascular thrombosis.

The size of the lipoproteins in plasma and lymph (Garlick and Courtice)

Chemical analysis has shown that the various components of the plasma lipoproteins are present in the lymph in experimental hypercholesterolaemia and hypertriglyceridaemia. The size of the lipoproteins in plasma and in lymph has been determined by electron microscopy after fixation in osmium tetroxide buffer. The lipoproteins of plasma and of lymph from the leg were first separated into their three main classes according to their density by means of the preparative ultracentrifuge. The results showed that the sizes of the three lipoprotein classes in the lymph were the same as in the plasma.

Exchange of lipoproteins across the capillary wall (Woolley, Garlick, Courtice and Marcus)

Previous investigations have shown that the gradient of permeability of the capillary wall to the different lipoprotein macromolecules runs roughly parallel to their size; chylomicrons which are the largest of the lipoproteins do not pass through the capillaries of the skin and muscle to any extent. When chyle or an artificial fat emulsion with complexes the same size as those of chyle are infused intravenously, the smaller lipoproteins are transferred across the capillary wall to a much less extent. The evidence obtained so far suggests that these smaller lipoproteins are to some extent adsorbed onto the larger complexes, thus reducing the transference. These experiments form part of investigations into the permeability of the vascular endothelium to macromolecules, especially lipoproteins, and the aetiology of the atherosclerotic lesions in arteries.

Haemodynamics and lymph production (Woolley and Courtice)

Studies on hepatic lymph production have been reported previously. These have led to the investigation of lymph formation in relation to organ haemodynamics. Preliminary work has been done on techniques for such study. This has centred around the use of an electromagnetic flowmeter by which means blood flows can be measured in non-cannulated blood vessels. Design and construction of miniaturized implantable flow-measuring probes has been undertaken with a view to their use in chronic experiments on unanaesthetized animals.

The nature of the sieve in the formation of lymph (Woolley)

Proteins and lipoproteins have been shown in a number of experimental preparations to enter the lymphatic system from the blood stream at rates proportional to their molecular size. Biophysical considerations seem to preclude the nature of the sieve being isoporous as previously believed. Electron microscopic studies elsewhere do not support the concept of the capillary wall as heteroporous with "pores" of sufficient size to permit sieving of macromolecules. Preliminary experiments in the rabbit hind leg preparation suggest that the ground-substance of the connective tissue plays an important role in macromolecular sieving. On the other hand, studies on lymph production from the rat thoracic duct and from the efferent popliteal lymphatic of the sheep hind leg have yielded equivocal results. The experiments so far have centred on the degradation *in vitro* of the ground-substance by the enzyme hyaluronidase.

Fat absorption in sheep (Adams, Heath and Morris)

The absorption of long chain fatty acids has been studied in lambs and in adult sheep with chronic intestinal lymphatic fistulae. Previous experiments had shown that there were

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significant differences between the patterns of fat absorption in young lambs and adult sheep. These differences were associated with the development of the rumen and the activities of the ruminal micro-organisms. Young lambs showed a pattern of fat absorption which was similar to monogastric animals whereas in adult sheep the absorption of fat introduced into the rumen extended over a period of several days.

The lipids of the intestinal lymph of adult sheep were examined by gas liquid chromatography. A characteristic feature of these lipids was the high concentration of C^{18} saturated fatty acids. These fatty acids are not characteristic of the lipids of pasture grasses. When maize oil, which contains a high content of C^{18} unsaturated fatty acids was given into the rumen, the lymph lipids recovered during the period of fat absorption showed a high proportion of C^{18} saturated and monounsaturated fatty acids and a greatly reduced content of C^{18} diene acids. When the rumen was bypassed and the maize oil given into the abomasum or small intestine, these changes did not occur, nor did they occur when maize oil was fed to young lambs. In view of these findings it was considered that the ruminal micro-organisms were responsible for the hydrogenation of a large part of the dietary unsaturated fatty acids.

The lipids of lymph collected from various regions of the body such as the liver, mammary gland, legs and uterus were also examined for their fatty acid content. A high content of C^{18} saturated fatty acids was characteristic of these lipids. The fatty acids of the plasma lipids of sheep were also found to contain a high proportion of C^{18} saturated acids. It appeared that the ruminal micro-organisms impressed their metabolic activities on the host animal in terms of the characteristically high content of C^{18} saturated fatty acids which were found in the lipids of sheep lymph and plasma.

Experiments were designed to establish the role of bile and pancreatic juice in the absorption of fat in sheep. Chronic fistulae of the bile and pancreatic ducts were established and one or both secretions diverted from the gut. It was found that in the absence of bile, essentially no absorption of fat occurred. When pancreatic juice was absent but bile was present a small amount of fat was absorbed from the gut. In view of the fact that extensive hydrolysis of triglycerides occurs in the rumen of sheep, it was concluded that the essential role of bile and pancreatic juice in fat absorption is not related to the action of pancreatic lipase in the small intestine and that bile acts in converting long chain fatty acids into a physical form suitable for absorption.

<u>The anatomy and function of the lymphatics of the reproductive tract of the ewe</u> (Sass and Morris)

The distribution of the lymphatics in the uterus and ovaries of the non-gravid and gravid uterus of the ewe has been studied by various histological techniques. The lymphatics of the uterus have been injected with various contrast media and their growth and enlargement followed during the course of pregnancy.

In order to obtain a measure of the function of the lymphatics of the reproductive tract in pregnancy, lymph has been collected from the main lymphatics draining the ovary, uterus and mammary gland. The flow of lymph from these tissues was followed over periods of weeks in conscious unrestrained pregnant and lactating ewes. The lymph flow from the non-gravid uterus was less than 10 ml/hr. During pregnancy there was a gradual increase in the rate of lymph flow and by the end of gestation period, the lymph flow from the uterus reached about 200 ml/hr. About

/24-48 hr. before

24-48 hr. before parturition, red cells appeared in the lymph. During the actual expulsion of the foetus, the lymph flow ceased as the uterus contracted, but following the birth of the lamb, the flow returned to pre-parturition levels. The lymph flow decreased over the 2 weeks following parturition as the uterus underwent involution. During the period of maximum lymph flow before parturition, the protein content of the uterine lymph was about 0.1-0.2 g%. It was thought likely that some absorption of fluid from the amnion and allantois may be occurring by way of the uterine lymphatics.

The initiation of milk secretion caused a sudden increase in the flow of lymph from the udder. In most sheep, the udder became oedematous in the 48 hr. after lambing. When this occurred the rate of lymph flow from the udder reached levels of the order of 100 ml/hr. With the onset of milk secretion, the oedema receded rapidly and the lymph flow decreased.

These experiments are being continued in an attempt to assess the role of the uterine and ovarian lymphatics in the exchange of materials between the blood and foetal fluids.

<u>The competitive oxidation of fat and carbohydrate in the intact animal</u> (Simpson-Morgan and Morris)

As a preliminary to studying the oxidation of fat and carbohydrate in the intact animal, a metabolism system was designed to measure continuously the excretion of $^{14}CO_2$ by unanaesthetized rats infused intravenously with various labelled substrates. An ionization chamber, vibrating reed and recorder combination were used for these studies. Initially, the kinetics of excretion of $^{14}CO_2$ were examined in rats infused intravenously with $NaH^{14}CO_3$. It was found that the excretion curve for $^{14}CO_2$ could be described by two exponential components with half-lives of 7 min. and 43 min. When a labelled material was infused, additional components appeared in the $^{14}CO_2$ excretion curve and these could be related to the oxidation of the particular labelled material.

When chylomicrons labelled with ^{14}C palmitic, oleic or linoleic acids were infused intravenously, the excretion of $^{14}CO_2$ reached a relative plateau after about 90 min. The oxidation of these three fatty acids when incorporated into chylomicron triglycerides followed a quantitatively and qualitatively similar course. Over rates of infusion of total fatty acids from 5-100 mg/hr. there was no change in the percentage of the infused fat oxidized "promptly" to CO_2. In rats starved for 24 hr. about 40% of the fat was oxidized to CO_2 as it was infused, and at the highest levels of infusion this amounted to 25-30% of the animals' total metabolism.

The oxidation patterns of the labelled chylomicrons were interpreted to mean that some 25% of the infused chylomicron fat entered a small pool of lipid which was metabolically very active; the half-life of the fatty acids in this pool was about 15 min. The remainder of the fat entered a much larger pool in which the half-life of the fatty acids was about 9 hr.

To test the effects of carbohydrate on fat metabolism, glucose was infused intravenously into rats at rates between 0 and 300 mg/hr. At the same time, labelled chylomicron fat was infused at rates between 50 and 100 mg/hr. The simultaneous infusion of glucose with the chylomicron fat caused the R.Q. of starved rats to rise from a mean of 0.72 to 0.76. This small increase in R.Q. meant that the proportion of the animals' total

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calories coming from carbohydrate increased from 4-18%. At the highest level of glucose infusion (300 mg/hr.) the oxidation of the labelled chylomicron fat was about 16% lower than when no glucose was infused. These results showed that although high rates of infusion of glucose decreased the oxidation of long chain fatty acids, the "sparing" effect was small and could be explained on a statistical basis. There was no evidence that carbohydrate was oxidized as a preferred substrate to fat when both these substrates were infused intravenously.

The lymph borne cells of the immune response (Hall and Morris)

The changes in the cell population in the efferent lymph from the popliteal node has been studied in unanaesthetized sheep given subcutaneous injections of various antigens into the lower part of the leg. The antibody content of the cells and of the lymph plasma was measured during the immune responses which followed the injections of the antigens.

Human serum globulin, chicken red cells and killed Salmonella typhi were the antigens used. The chicken red cells and Salmonella organisms were virtually all retained in the lymph node whereas human serum globulin for the most part passed through the node and was recovered in the efferent lymph. With all the antigens, a characteristic cellular response occurred in the efferent lymph. Initially there was an increase in the output of mature lymphocytes followed by the appearance of primitive stem cells and finally plasma cells. Antibody was detected in the cells and lymph plasma in experiments with particulate antigens and in the lymph in experiments with human serum globulin.

When a second dose of particulate antigen was injected, the response was always shorter and more vigorous than with the primary response. There was little difference between the primary and secondary response when human serum globulin was injected.

The cell population of afferent lymph was found to change in roughly the same way as that of efferent lymph when an antigen was injected into the leg. All the cell types which characterized the immune response in the efferent lymph were identified in the afferent lymph.

Studies on fat metabolism in choline deficient livers (Mishkel and Morris)

The metabolism of free fatty acids and chylomicron triglycerides has been investigated in the isolated perfused choline deficient liver of the rat. Previous studies on the gaseous metabolism of the perfused liver showed that the R.Q. of the perfused choline deficient liver was significantly lower than the normal liver. This suggested that endogenous fat was being oxidized extensively by the choline deficient liver.

When ^{14}C free palmitic acid or ^{14}C labelled chylomicron triglycerides were added to the perfusate, they were taken up by the choline deficient liver as rapidly as by the normal liver. About 50% of the free fatty acids and 6% of the chylomicron triglycerides were extracted in a single passage through the liver. The labelled fatty acids were also oxidized to $^{14}CO_2$ by the choline deficient livers as efficiently as by normal livers.

The labelled fatty acids taken up by the livers were rapidly esterified. In experiments with free fatty acids more of the label was recovered in the liver triglyceride fraction than in the

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liver phospholipids. In experiments with chylomicron triglycerides the reverse held. The perfused livers released considerable amounts of esterified fat into the perfusate. In normal livers at the end of 3 hr. about 80% of the residual activity in the perfusate was present as esterified fat. In experiments with choline deficient livers, significantly less of the labelled fatty acids was incorporated into the perfusate esterified fat. The retransport of esterified fatty acids was significantly higher in both normal and choline deficient fed livers than in normal and choline deficient starved livers.

Studies with ^{14}C acetate showed that there was no significant difference between the rate of synthesis of triglycerides from ^{14}C acetate in normal and choline deficient fed livers. The synthesis of choline containing phospholipids however was significantly reduced in the choline deficient livers. No evidence was obtained which suggested that impaired oxidation or increased lipid synthesis was responsible for the accumulation of fat in the liver in choline deficiency. There was evidence that retransport of esterified fatty acids was reduced by choline deficiency.

Cell biology and immunology (Boyden, Nelson and Vaughan)

Work is in progress on aspects of cellular interaction and on the mechanisms by which cells "recognise" and respond to cellular and particulate components of their environment. It has long been known, for example, that macrophages engulf and digest damaged and dead host cells including effete red cells. However, the mechanisms by which the macrophages distinguish between such cell debris and healthy cells of the tissues is unknown. A technique has been developed for studying this problem in vitro. It has been found that, under appropriate conditions, aged red cells adhere in large numbers to living macrophages, while fresh red cells do not. The mechanisms by which the macrophages "recognise" the effete red cells appears to be different from that by which they recognise foreign matter (such as red cells from other species, bacteria, etc.). In the latter case, serum "opsonins" are essential for adherence of the particle to the phagocyte to take place; in the case of effete red cells, however, the macrophage-red cell interaction takes place as effectively in the absence of serum factors as in their presence (Vaughan, Boyden).

Further studies have been carried out on the mechanisms of the chemotactic response of polymorphs. A method which was developed in this Department is being used to investigate the nature of the chemotactic principle for polymorphs which was found to be released following the interaction of serum with foreign matter. A recent finding is that damaged tissue is strongly chemotactic for rabbit polymorphs. Although this result is contrary to the reports of some other workers using less satisfactory techniques it agrees well with in vivo observations (Boyden).

The mechanism of allergy (hypersensitivity) of the "delayed" or "tuberculin" type remains a major problem in immunology. In a series of experiments aimed at the analysis of the allergic reaction on a cellular level, it has been found that the injection by any route of the specific antigen into an animal with delayed-type hypersensitivity causes the virtual disappearance within a few hours of macrophages normally present in the peritoneal cavity. This reaction is specific in the immunological sense and is apparently strictly related to the state of delayed-type hypersensitivity rather than to the presence of serum antibodies. It is hoped that it will lead to better understanding of this type of allergic reaction (Nelson and Boyden).

/Cellular

Cellular resistance to infection (Mackaness, Ackerman and North)

The mechanism of acquired immunity to Listeria monocytogenes infection in mice has been studied in greater detail. It was reported last year that the phagocytic cells of infected mice acquire antibacterial properties at the same time that the tissues become hypersensitive to Listeria antigens. During the year an attempt has been made to study the relationship between hypersensitivity and the acquisition of anti-Listeria activity by host phagocytes. At the same time a study has been made of the specificity of this cell-mediated form of acquired immunity.

It was found that the immune cells which arise during infections caused by three unrelated intracellular parasites all possess the ability to kill Listeria monocytogenes. It is clear therefore that the protection conferred upon an infected animal is not confined to the organism which promotes the change in the functional state of the host's phagocytes. However, the mechanism by which the cells acquire these non-specific antibacterial properties is itself a highly specific reaction. It bears the specificity of the hypersensitive state. This argues strongly in favour of the view that the cells become altered during the allergic (hypersensitivity) reaction.

The structure of Listeria-immune macrophages has been examined in the electron microscope. The immune cell possesses distinctive anatomical features. It is extremely rich in vesicular structures with the characteristic morphology of lysozomes. The latter are known to contain large quantities of degradative enzymes. These are discharged into the vacuoles which surround engulfed bacteria. It is possible, therefore, that the striking antibacterial activity of the cells from immunised animals is related to their enriched content of lysozomes.

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* = not a member of A.N.U.

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Other Activities

Professor Courtice spent six months study leave in the United States and England and also attended the Twelfth General Conference of Unesco in Paris as a member of the Australian delegation. Dr. G.B. Mackaness spent three months study leave visiting laboratories in England and the United States. Dr. S.V. Boyden attended a Conference at the W.H.O. headquarters in Geneva as a member of a group invited to advise on research in Immunopathology.

Professor Courtice, Dr. Morris, Dr. Boyden and Dr. Stehbens participated in a Symposium on vascular, lymphatic and cellular reactions to injury organized by Professor D.L. Wilhelm of the University of New South Wales and held in Sydney. Dr. Boyden read papers at a Symposium on Antibodies at the University of Adelaide and at the Walter and Eliza Hall Institute in Melbourne. Dr. Morris read a paper by invitation at the Annual Conference of the N.S.W. branch of the Australian Veterinary Association in Sydney and gave a lecture at the Royal Newcastle Hospital arranged by the University of Sydney.

Several members of the Department read papers at the meeting of the Australian Physiological Society in Melbourne and at A.N.Z.A.A.S. in Sydney. At the meeting of A.N.Z.A.A.S., Dr. Morris was chairman of a Symposium entitled "The function and formation of tissue fluids and lymph."

Mr. M.W. Simpson-Morgan's Research Fellowship with the Life Insurance Medical Research Fund of Australia and New Zealand was renewed for 1963.

Dr. W.E. Stehbens, Senior Lecturer in the Department of Pathology of the University of Sydney, was appointed to the position of Senior Research Fellow and took up his appointment in June.

Dr. G.B. Mackaness resigned his position as Reader in the Department as from 31st December to take a Chair within the Department of Microbiology of the University of Adelaide. Dr. Mackaness has been associated with the Department of Experimental Pathology since its inception. He was awarded an A.N.U. Scholarship to work with Sir Howard Florey in Oxford in 1948 and in 1951 he was appointed to a Research Fellowship. In 1953 he was appointed to a Senior Fellowship and he came to Canberra in 1954. He was made a Reader in 1958.

Dr. A.K. Lascelles resigned his position as Research Fellow in June to take up a position as Senior Research Fellow in the Faculty of Veterinary Science of the University of Sydney.

Dr. M.A. Mishkel was appointed as a Physician to the Prince Henry Hospital, Sydney within the Department of Medicine of the University of New South Wales. Dr. Mishkel has been working in the Department of Experimental Pathology for two years as a Visiting Fellow, supported by a grant from Merck, Sharp and Dohme for the first year and by the Life Insurance Medical Research Fund of Australia for the second year.