THE EFFECT OF ANTILYMPHOCYTIC ANTISERUM
ON THE IMMUNE RESPONSE TO KIDNEY HOMOGRAFTS

by

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"...to reckon him who taught me this art equally dear to me as my parents, to share my substance with him..."

The Hippocratic Oath

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"... whoever carefully views the lymphatic system must be convinced that, as it explains and points out the cure of many diseases, it deserves the attention of the practitioners of the healing art. And as it is so generally diffused through the animal kingdom, it strongly claims the regard of those who wish to inquire philosophically into the animal economy."

William Hewson
1774
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PART I

INTRODUCTION
"What is now proved was once only imagined."

William Blake.

The role of the lymphocytes in the homograft rejection has been established. It has shown that thoracic duct drainage and consequently small lymphocytes depletion suppresses, to a marked degree, the primary immune response (but not the secondary) to sheep erythrocytes and tetanus toxoid in rats. The degree of such suppression was dependent on the degree of depletion (Gowans, McGregor, Cowen and Ford 1962). It has also been shown that depletion of 1-2 billion lymphocytes by thoracic duct drainage in rats prolonged skin homograft survival across a strong antigenic barrier by only 3-7 days (Woodruff and Anderson 1963; 1964). Although lymphocytes depletion by thoracic duct drainage effectively prolonged skin homograft survival to 74-344 days in non-inbred albino rats (small antigenic disparity) similar results to those of Woodruff and Anderson were obtained when homografts were performed across a stronger antigenic barrier (McGregor and Gowans 1964). By implanting skin homografts (4-6 mm. diameter) in rats under the renal capsule together with thoracic duct drainage, appreciable prolongation to 3½ months (controls 13 days), without cellular infiltration, was obtained (Mayer and Dumont 1963).
Thoracic duct drainage was similarly attempted in dogs. Applying this procedure together with skin homografts, slight prolongation of the homograft survival was attained (mean 14 days; control 7 days) (Samuelson, Fisher and Fisher 1963). The effect of thoracic duct drainage on renal homografts was consequently studied (Singh, Vega, Makin and Howard 1965). Six of the 18 dogs survived more than 20 days; the longest for 35 days (mean 13-14, for 2 groups). Similarly the effect of lymphocytes depletion by such a procedure was studied in man (Tunner, Carbone, Blaylock and Irvin 1965). This procedure prolonged skin homograft survival to fivefold the control value (mean 45 days; control 9 days). It also depressed some immediate and delayed skin hypersensitivity reactions in some cases.

Small lymphocytes depletion by thoracic duct drainage was one mode of approach to abrogate the immunological responsiveness of the recipient towards a homograft.

Another approach to overcome this problem was adopted by Woodruff, Woodruff and Forman (see Woodruff 1960). They used for the first time an antilymphocytic serum (rabbit-anti-rat lymph node cells). The antiserum was effective in lowering the blood lymphocyte count to a marked degree within two hours. Despite its daily administration, the antiserum was without effect in prolonging the survival of skin and endocrine tissue (ovary and suprarenal) homografts. They also noted that the blood lymphocyte count after a temporary period of suppression rose to the pretreatment level by the tenth day. The effect of the antiserum on the
blood lymphocytes was similar to that obtained in guinea pigs injected with antiserum prepared in rabbits with guinea pig lymph node cells (Chew and Lawrence 1937), and in rats injected with antiserum prepared in rabbits with rat lymph node cells (Cruickshank 1941).

The antiserum injections did not result in the depletion of lymphocytes from the lymphoid tissues. On the contrary the antiserum as well as the normal rabbit serum caused hyperplasia (Chew and Lawrence 1937). This led them to suggest a disparity in the antigenicity of the destroyed cells and those resisting destruction. Similarly no change was noted in the spleen or lymph nodes except proliferation of the reticular cells in the sinuses of these tissues in the antiserum as well as in the normal serum injected rats (Cruickshank 1941).

The production of specific antisera against lymphocytes has been attempted for a long time. Although some investigators spoke of it as antileukocytic it is evidently what we call antilymphocytic (or antilymphoid). This is shown by the fact that they prepared their antisera by immunisation with lymphoid tissue (lymph nodes, thymus or with spleen cells suspension in some cases). The antisera prepared by the latter cells will not be discussed.

Metchnikoff prepared an antiserum in guinea pigs against rabbit mesenteric lymph nodes * (contrary to what has been cited by some investigators). The antiserum termed "antileukocytic" by Metchnikoff, was effective against

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* "En injectant des ganglions lymphatiques du mésentère de lapins sous la peau de cobayes, j'ai obtenu un autre échantillon de serum anti-leucocytaire." (Metchnikoff 1899, P 762).
rabbit lymph node cells (mononuclear cells) as well as the polymorphonuclear leukocytes. The antiserum was also species specific when tested against rat leukocytes (Metchnikoff 1899).

Since that time antilymphocytic (or antilymphoid) antisera have been prepared. Besredka prepared rabbit-anti-guinea pig, and guinea pig-anti-rabbit, antiserum using the mesenteric lymph nodes as a source of the antigenic stimulus. Similarly he prepared various antisera against lymph nodes of the horse, ox, sheep, goat and dog, to study their species specificity. He described in detail the immediate effects of injecting rabbit-anti-guinea pig antilymphocytic serum on the guinea pig's general condition, and the post-mortem findings. He also studied the effect of guinea pig anti-rabbit serum on the ear vein white blood count after the subcutaneous injection of 3.5 or 5 ml. (Besredka 1900).

Flexner similarly used lymph node cells to prepare antisera in the rabbit against guinea pigs, and in the goose against the rabbit. He studied the effect of injecting such antisera on the lymph nodes and spleen. He noted hyperplasia of the lymphoid cells and enlargement of the nodes and follicles; the degenerative changes in these were minimal (Flexner 1902).

Bunting also prepared an antiserum in geese against rabbit lymph nodes. The antiserum agglutinated and was cytotoxic to lymph node cells in vitro. The control serum showed a similar reaction but of a lesser degree (Bunting 1903).

Moorhead produced an antiserum in rabbits against guinea pig thymus but it was neither agglutinating nor cytotoxic to thymus cells when tested in vitro (Moorhead 1905).
Ritchie similarly prepared an antithymic serum, but in ducks against guinea pigs thymus. The antiserum was complement fixing and lymphoid tissue specific \textit{in vitro} (when tested with the thymus, lymph node, spleen and also bone marrow); it did not fix complement when tested with other tissues as liver, kidney, thyroid and suprarenal. Similarly he showed that \textit{in vitro} the antiserum was species specific when tested with kittens lymphoid tissue (Ritchie 1908).

Pappenheimer prepared rabbit anti-rat thymus and rabbit anti-human tonsil immune sera to study their properties mainly by \textit{in vitro} as well as by some \textit{in vivo} experiments (Pappenheimer 1917 a, b). By these experiments he demonstrated that the antithymic serum besides affecting the thymus and lymph node cells \textit{in vitro}, severely damaged the rat thymus \textit{in vivo} (Pappenheimer 1917 b).

With the introduction of antilymphocytic serum as an immunosuppressive agent in experimental transplantation by Woodruff and Woodruff, they showed that antilymphocytic serum is not well tolerated by adrenalectomized rats (cited Woodruff and Forman 1951; Woodruff 1960). Woodruff and Forman studied some \textit{in vitro} properties of antilymphocytic serum. They found that the antiserum (rabbit-anti-rat lymph node) is neither cytotoxic nor agglutinating to rat lymph node cells, despite causing marked lymphopenia when injected \textit{in vivo}. They thus suggested that the mode of action of the antiserum \textit{in vivo} is not by its direct destructive effect on the lymphocytes (Woodruff and Forman 1951), also that it is not adreno-
cortical in origin (Woodruff, Forman and Fraser 1951).

The effect of antilymphocytic serum on the tuberculin reaction was studied during the investigation of the role of lymphocytes in such a delayed type of hypersensitivity (Inderbitzin 1959; Humphrey 1960). Inderbitzin showed that the histamine content of the skin in the tuberculin reaction is increased to reach its maximum after 72 hours coinciding with the cellular infiltration (mononuclears), while the administration of "rabbit anti-lymphocyte guinea pig immune serum" (antilymphocytic serum), suppressed both the histamine content in the skin and the intensity of the reaction in the tuberculin sensitive animals (Inderbitzin 1959); some antilymphocytic sera, though effective in producing lymphopenia as those inhibiting the tuberculin reaction, did not exert such an effect (Inderbitzin and Humphrey, see Humphrey 1960). Similarly the effect of antilymphocytic serum (rabbit-anti-guinea pig lymph node cells) on various reactions of the delayed type were studied (Waksman & Arboys 1960; Waksman, Arboys & Arnason 1961). They confirmed the results of Humphrey and of Inderbitzin by suppressing the tuberculin reaction, and studied the effect of the antiserum on the first and second sets skin homografts in guinea pigs. Although the survival of the first set skin homograft was not appreciably prolonged they noted that the onset and degree of cellular infiltration were relatively delayed and suppressed in some grafts.

Prolongation of skin homograft survival in rats across a strong antigenic barrier to a mean value of 28 days by antilymphocytic serum (rabbit-anti-rat thoracic duct lymphocytes) administration was obtained for the first time
(Woodruff and Anderson 1963). They also showed that thoracic duct drainage prior to grafting and antiserum administration was more effective (mean survival 35 days). By the long-continued antiserum administration they prolonged the mean homograft survival to 48 days (20 and 75 days) when the antiserum was administered alone, and to 100 days* (19 - >168 days) when preceded by thoracic duct drainage (Woodruff and Anderson 1964).

The use of rabbit-anti-mouse lymph node cells immune serum prolonged skin homograft survival in mice for more than 30 days, when it was administered daily (Gray, Monaco and Russell 1964). The antiserum also delayed the onset of rejection to 20 days when it was administered for a short period (6 injections in two weeks). Similarly the antiserum depressed the primary (but not the secondary) response to Salmonella II antigen. The antiserum, though effective in producing lymphopenia, did not maintain this state and by the fourth week the count was back to the pre-treatment level; continued antiserum administration however resulted in the return to the lymphopenic state in some cases (Gray et al 1964).

By such an antiserum but of a higher titer administered to thymectomized mice, the skin homograft survival across a strong histocompatibility barrier was appreciably prolonged to beyond 50 days in about one third of the treated mice. Thymectomy was performed prior to one week of antiserum administration. Similarly this combined procedure of thymectomy and antiserum administration resulted in depression of the primary immunological response to sheep erythrocytes and in some cases the secondary response as well (Monaco, Wood and

* Reported rat rejected skin homograft on day 219. Accordingly mean survival = 110 days.
Russell 1965). It has also been shown in rats thymectomized in adult life that antiserum administration (rabbit-anti-rat lymph node cells) for 8 days after the lapse of 180-240 days from thymectomy prolonged the skin homograft survival to a mean value of 43 and 62 days respectively (Jeejeebhoy 1965). These last studies by Monaco et al, and by Jeejeebhoy showed that the combined effect of antiserum and thymectomy is more effective than either alone. It was also shown (Hintz and Webber 1965) that purified labelled antithymic globulin was specific to the thymus but also localised in the lymphatic tissues (except the spleen). Recently an antilymphoid antiserum (rabbit-anti-mice thymocytes) was shown to be effective in prolonging skin homograft survival when administered in few doses, and was more effective when administered after grafting. The antiserum was capable of appreciably prolonging the survival of second set homografts performed 11 days after the antiserum administration (Levey and Medawar 1966).

This review of the antisera produced since the time of Metchnikoff shows that antilymphocytic (or antilymphoid) antisera were produced since the turn of the century; they were used for different investigations. The first investigators produced sera which, though active in vitro were generally toxic when administered in vivo. Later investigators, Chew and Lawrence and Cruickshank, were mainly interested in studying its in vivo effect on both the circulating lymphocytes and the lymphoid tissue. It was not until the introduction of antilymphocytic serum to tissue transplantation by Woodruff and Coworkers, and the demonstration of its effect in prolonging skin homograft's survival in rats by Woodruff and Anderson in 1963 and 1964, that interest in this immune serum started to take place.
It has been established that renal homograft rejection is an immunological reaction. Despite the fact that a satisfactory technique for vascular anastomosis has been established for a long time, most attempts to prolong canine renal homografts survival were generally unsuccessful. The clinical application of the successful experimental methods was either unsuccessful or remote. These latter were by irradiation and bone marrow infusions (Mannick, Lochte, Ashley, Thomas and Ferrebee 1959), and neonatal exanguination transfusion (Gomboš, Tischler and Jacina 1960; Gomboš, Jacina and Tischler 1961).

It was shown that 6-Mercaptopurine, a purine analogue, suppresses to a marked degree the immunological responsiveness of rabbits to bovine serum albumin when both were administered simultaneously (Schwartz, Stack and Dameshek 1958). Similarly Schwartz and Dameshek produced with 6-Mercaptopurine a state of drug-induced tolerance to human serum albumin. They showed this by studying the $^{131}$I labelled human serum albumin disappearance curve (Schwartz and Dameshek 1959). Consequently it was shown that 6-Mercaptopurine prolongs skin homograft survival in rabbits when administered in high doses for two weeks to a mean value of 24 days (Meeker, Condie, Weiner, Varco and Good 1959) but had no effect on second set homografts (Meeker, Condie, Good and Varco 1960).

By the introduction of 6-Mercaptopurine to renal homografting by Calne in 1959 and by Zukoski, Lee and Hume, prolongation of the first set homograft survival was achieved (Calne 1960, 1961a; Zukoski, Lee and Hume 1960) and of second set homograft (Calne 1961a). The toxic side-effects comprising mainly marrow depression, biliary stasis and lowered resistance to
infection, led to the trial of its Imidazole derivative (Calne 1961; Calne and Murray 1961).

These experiments showed that the marrow depression and inconsistency of the immunosuppressive effect on first set renal homografts encountered with 6-Mercaptopurine are less pronounced with its Imidazole derivative B.W.57-322 (6(1-Methyl-4-nitro-5-Imidazolyl)thiopurine) "Imuran" (Calne 1961 b). The mean survival of 35 days was attained when Imuran was used alone, and 58 days (with one dog living at time of report) when used in combination with Actinomycin C (Calne, Alexandre and Murray 1962). Although discontinued administration of Imuran after six months treatment resulted in the renal homograft rejection (Calne et al 1962), it was possible in some cases after 14 months (Murray, Sheil, Moseley, Knight, McGavic and Dammin 1964). Withdrawal of 6-Mercaptopurine was possible in three dogs reported by Pierce and Varco after 6-8 months treatment without rejection for a further 10-14 months (Pierce and Varco 1963), and of 6-Methyl Mercaptopurine after 6.5 months treatment (Zukoski and Callaway 1963).

Since encouraging results were obtained by immunosuppressive drugs screening of various drugs and their combinations to achieve the best results with the least toxic effects were carried out in dogs (Calne et al 1962; Zukoski, Lee and Hume 1962; Alexandre, Murray, Dammin and Nolan 1963). These immunosuppressive drugs were similarly tested for their activity against adenocarcinoma (Hitchings and Elion 1959) in mice challenged with TAB vaccine (Berenbaum 1960), sheep red cells (Nathan, Bieber, Elion and Hitchings 1961), or in neonatal rabbits challenged with spleen cells that have been previously mixed in vitro with Brucella suis (Sterzl 1961).
The encouraging results of the renal homograft experiments in dogs led to the use of these immunosuppressive drugs to abrogate the immunological responsiveness to renal homotransplants in the human (Murray, Merrill, Dammin, Dealy, Alexandre and Harrison 1962; Murray, Merrill, Harrison, Wilson and Dammin 1963; Woodruff, Robson, Nolan, Lambie, Wilson and Clark 1963; Calne, Loughridge, MacGillivray, Silva and Levi 1963; Hume, Magee, Kauffman, Rittenbury and Prout 1963; Starzl, Marchioro and Waddell 1963; Transplantation 1964 a & b).

In this thesis a study was made on the effect of antilymphocytic antiserum as an immunosuppressive agent on canine renal homografts performed by vascular anastomosis. Thoracic duct lymphocytes of the dog have been used as the antigenic source for raising such antisera in all the experiments described.

Antilymphocytic antiserum was prepared in three species, the rabbit, the sheep and the horse. The methods of preparing and assaying such antisera are described.

The effect of antisera prepared in the rabbit on the white blood cells and those prepared in the sheep on the white blood cells and renal homografts will be dealt with briefly.

The body of the thesis is devoted to describing the effect of antilymphocytic antiserum prepared in the horse on canine renal homograft function together with the effect of such an approach on the recipient's well-being, blood cells and chemistry, and lymphoid tissues as well as other organs - also will describe in detail the in vitro methods for assaying the antilymphocytic
potency of the antiserum.

The thoracic duct has and is still used as the passage of choice for lymphocytes collection and depletion both experimentally and clinically. The lymphocytes were and are still the target of immunologists and transplantation surgeons. Since little is generally known about the history of the discovery of these cells and lymphatic passages I have devoted a part on such historical discoveries to remember the efforts of the keen workers who cannot witness the application of their discoveries in modern surgical research. Without their work, as without the work of the researchers in the field of medicine, I would not be making this contribution regarding the homograft problem.
PART II
"... in the history of science as in that of any expression of human intelligence and emotion the past is never past but continues and is very active in every manifestation of the present."

Arturo Castiglioni (1941)

The history of the scientific evolution of knowledge, the observations and the discoveries regarding the lymphatic system will be divided into three phases:

1. The first phase is the phase of mere observations, without a real knowledge of their significance or its misinterpretation. This was in the period before the seventeenth century.

2. The second phase is the phase of proper anatomical and physiological discoveries. This includes the seventeenth and eighteenth centuries.

3. The third phase is the phase of real scientific experimentation, and that includes the nineteenth century.
The first phase or the phase of observations:

This started as early as 300 B.C. by the observations of the two eminent physicians and researchers of Alexandria, Herophilus (c. 335-280 B.C.) of Chalcedon (fl.c. 300 B.C.) the physician and the father of anatomy, and Erasistratus of Chios (c. 310-250 B.C.) his contemporary and the father of physiology. Their original writings are not available but according to Dobson, Galen's writings mention their works -

"Nature made veins specially belonging to the whole mesentery, which are dedicated to the nourishment of the intestines themselves and do not pass to the liver; for as Herophilus said, 'These veins end in glandular bodies, while all others are carried to the portae; ....'" (Dobson 1925).

Herophilus, using the term 'veins' for both blood and lymph vessels (though he had a reputation for his invention of scientific terms, some of which are still in use), observed the lacteals and their passage to the mesenteric lymph nodes.

Erasistratus, the physician and physiologist, worked in Alexandria at the same time as Herophilus, observed the 'vasa chylifera' of the mesentery (the lacteals) in suckling newborn kids (Bartholin 1653 a). He believed them to be arteries transporting the chylos (food ground by the action of the stomach) from the intestine to the liver to be transformed into blood (Dobson 1927). It also seems, according to A.V. Haller (Haller 1757 a) that Aristotle (384-322 B.C.) referred in his "History of Animals" to fibres between the arteries and veins, some of which contained fluid. These latter he mentioned are probably what we now know as the lymphatics.

The first to differentiate between the lacteals and mesenteric vessels
was Jacopo Berengario da Capri, (1470-1530). He was also the first to describe the vermiform appendix and to give a good description of the thymus gland. This was later also described together with the mesenteric lymph nodes by the French anatomist Charles Estienne (1503-1564) in his book "De dissectione partium corporis humani", Paris, 1545, (Singer and Underwood 1962).

Still during that century the Italian anatomist Gabriele Fallopio (Fallopia = Fallopius, 1523-1563) described the portal lymphatics. He "saw passages full of yellow fluid stretching from the liver to the pancreas" (Bartholin 1653 b.) and Nicolas Massa discovered the renal lymphatics as described in his book in 1532.

The honour for discovering the thoracic duct must go to the Italian anatomist, Bartolomeo Eustachio, (1520-1574) who was its first discoverer in 1553 and described it as he saw it in a horse. This is the first record ever made on this duct. He gave his description of the duct from its termination downwards to its beginning as he traced this unknown, accidentally discovered vessel (Fig. 1). He described its lower part after piercing the diaphragm and reaching the middle of the lumbar region where he noticed the enlargement (the cysterna chyli) that is continuous with the thoracic duct and embracing the aorta. Beyond this point he found that the mode of termination of this enlargement was obscure (Eustachio 1564).

"There is a certain vein in horses which I once believed contributed to this natural function (of nourishing the thorax). Although this is certainly not its purpose it is worthwhile to expound it since it is astoundingly ingenious and not devoid of interest and significance. So then in these animals, actually from the conspicuous left trunk of the jugular vein opposite the posterior bed of the root of the internal
jugular vein, there sprouts a kind of large stem, which has at its beginning a small semicircular opening and is, further, white and full of watery fluid. Not far from its beginning it divides into two parts, coming together again soon afterwards into one. This puts forth no branches and near the left side of the vertebrae it penetrates the septum transversum and goes right down into the middle of the lumbar region. There it broadens out, winds round the aorta and comes to a very obscure end which I have not yet properly seen."

By these words, translated from the Latin (Fig. 1), Bartolommeo Eustachio described his discovery of the thoracic duct in a horse (Eustachio 1564).

Eustachio considered the beginning of the discovered passage at the left jugular vein, and its end in the abdomen, which is not the real state of affairs. It seems from his description that he either misjudged the direction of flow of the white fluid in the duct, or he just named the beginning and the end according to his tracing of the vessel which he accidentally saw at what is now known as its termination. He undoubtedly noticed the semi-circular valve guarding the orifice of that duct in the left jugular vein when he described the duct orifice as "a small semi-circular opening". He described the mode of the thoracic duct branching near the venous confluence and traced the duct down till the cysterna chyli. He is thus to be considered the first discoverer of the thoracic duct and the cysterna chyli.

* Translated from the Latin by David A. West, Lecturer, Humanity Department, University of Edinburgh.
Fig. 1: The first written evidence on the discovery of the thoracic duct by Bartolommeo Eustachio in "Opuscula Anatomica", Venice 1564.
Quero ita magna non est, ut hoc per se præclare queat; sed alterius auxilio egeat; ad septimum vel octauum, atque adeo nonum, spondylum descendent; ibique uno, ant duobus, uena sine pari ramis ei currentibus, con functa definit: tuncque, hieuti etiam paulo ante dixi, contrecto illa manere ut uinique fungitur. Quandoque etiam uena sine pari, e regione sex. thoracis ucrtebrib, ramum ad finitiam partem mittat; qui siuolum elatus, ad quintam eiudem, cumalio, a truncu sinistro juguli orto, coniungitur: indeque uinque ad tertium internum, alimentum praebens, confendit. Ad hanc naturæ prouidentiam quandam equorum uenam alias pertinere credidi: qua, quum artifici & admirationis plena sit; nec delectatione ac fructu carcat; quamuis minime sit ad thoracem alendum instituta; operæ precium est, ut exponatur. Itaque in illis animantibus, ab hoc ipso insigni truncu sinistro juguli, qua posterior sedes radi- cis uenæ internæ jugularis spectat; magna quædam pro pago germinat; qua præterquam quod in eius origine hostiolum semicirculare habet; est etiam alba, & aquei humoris plena; nec longe ab ortu in duas partes seindi tur; paulo post rursus coeuntes in unam; quæ nullo ramos diffundés, iuxta finitrum uertebrarum latus, penetrato septo transfuerlo, deorsum ad medium usque lum borum fertur: quo loco latior effecta, magnamque ar- teriam circumplex, obscurissimum finem, nihilque ad- hue non bene perceptum, obtinet. Ita uigitur ex hoc, & precedent, non animamate uinique patere arbitror, utrumque thoracis latus ab una uena in hominibus perpetuo non mutat: sed alteram in sinistro lateris, eis manere non semel fungitur, quæ uel a primum demone uenæ causæ in jugulo, uel partum mina ab ipfam et cauæ, uel
The second phase, or the phase of proper anatomical and physiological discoveries:

During the seventeenth and eighteenth centuries the anatomy and physiology of the lymphatic system were properly investigated. Many discoveries were made by mere chance but further investigation and verification of the findings and observations accidentally encountered was undertaken with meticulous planning and work.

Gaspare Aselli (1581-1626) of Cremona (Fig. 2) the Italian anatomist and surgeon at Pavia on June 23rd, 1622, while dissecting a dog, saw whitish vessels in the mesentery which he then punctured and saw milky liquid coming out. He named these vessels 'venae albae et lacteae' and described their course. He also noticed their valves and attributed to them their proper function (Foster 1901 a.). He also saw the portal lymphatics and illustrated them, but mistakenly thought that these lymphatics were connected with the lacteals (Haller 1757 a.) and that they transfer the lacteal's contents to the liver. He showed and illustrated the lacteals going to the mesenteric lymph nodes which he termed 'the pancreas'. (To this day these nodes are sometimes referred to as the 'Pancreas of Aselli' particularly when describing them in the dog, the animal on which Aselli's discovery was made).

After his discovery of the lacteals in the dog Aselli dissected many species of the animal kingdom, including besides the dog, cats, lambs, cows, pigs and horses (Poirier and Cunéo 1901 a.), thus confirming his discovery and proving that this new system of vessels
is shared by all the species he dissected. He then assumed that these vessels must also exist in man, basing his assumption on discovering these vessels in all the species he dissected.

Aselli's rediscovery (after Herophilus, Erasistratus and da Capri) of the lacteals was a great asset for science at that time. He demonstrated publicly, and by illustrations, the lacteals and their confluence at the mesenteric lymph nodes, showed their valves and assigned to them their proper function, showed that the lacteals exist in many species of the animal kingdom and based on this his assumption that they also existed in man, though he never proved it. He saw the portal lymphatics, but he made the error of thinking that the lacteals, after reaching the mesenteric lymph nodes, joined the portal lymphatics to empty their contents in the liver.

Aselli's discovery brought to light what has been previously observed by Herophilus and Erasistratus in Alexandria nineteen centuries before, but by rediscovering the lacteals and by proving their existence in many animals, he stimulated research in this field, which rapidly gained a momentum and continued as an important phase in anatomy. For this is the real glory for which he must not be forgotten.

His work was published in 1627, one year after his death, under the title of "De lactibus sive lacteis venis quarto
vasorum mesarsicorum genere novo invento dissertatio", Milan 1627.

It was the first to contain coloured anatomical illustration. The protrait of Aselli (Fig. 2) was done when he was forty-two years old, i.e., about the same time he made his discovery.

In 1628 Fabri de Peiresc, who made the publication of Aselli's work possible by his financial aid, demonstrated the lacteals in a human (Pecquet 1653 a.).

The rediscovery of the thoracic duct was made in 1629 by Jacques Meutel (1599-1670): (Mettler 1947 a.), and in 1634 the human lacteals and thoracic duct were shown in a cadaver by Johann Vesling (Wesling) (1598-1649) (Pecquet 1653 a.). He showed many passages like the lymphatic vessels in the lesser omentum region and where the stomach and pancreas lie in relation to the spleen. He also found lymphatic vessels over the diaphragmatic surface of the liver (10) (Haller 1757 b.).

The discovery of Asellius was proved in man by other workers besides Fabri de Peiresc. It was shown by Veslingius in Padua, by Folius in Venice, and Tulpius (Nicholas Tulp, 1593-1674) in Amsterdam (Pecquet 1653 a.).

In 1637 Nathaniel Highmore (1613-1684) differentiated between the lacteals and mesenteric veins, thus repeating da Capri's and Aselli's findings. He also concluded, as
Asellius, that the lacteals terminated in the liver (Bartholin 1653 c.).

It was not until later when the French student Jean Pecquet of Dieppe (1622-1674) (Fig. 3), while experimenting on a living dog to examine the motion of the heart, saw whitish fluid in the superior vena cava which he found coming from small openings at the venous confluence, and which were guarded by valves (Pecquet 1653 b.). This led him to investigate its source, which at first he could not find, but noticed that as he pressed on the abdomen the flow of the white fluid increased from its opening in the venous confluence. Like Eustachius he first encountered the distal part of the thoracic duct which he then followed downwards to its origin. On the right side he saw a whitish vessel which he thought might be either a nerve or the passage that transported the chyle from the abdomen to the neck. He thus applied a ligature to differentiate and noticed that above the ligature it became flaccid, while below it it became turgid and apparent. He then proceeded to examine the left side of the chest and found a duct similar to that on the right side. On examination of their termination he found that they break up into branches which open into the junction of the jugular and subclavian veins. He then followed downwards the two passages he discovered, to find that they were joined at the level of the fourth thoracic vertebra and separate from that level downwards
Fig. 3.

JEAN PECQUET
PECQUET'S FIGURE OF THE THORACIC DUCT IN THE DOG.

Fig. 4.
to the level of the tenth vertebra:

"The fourth vertebra did uphold their joyning together, the rest of the space to the tenth vertebra upheld them parted in two and divided with winding turnings like to rivers. They did flow with like fulness, not seldome with transverse trenches, as it were for mutual help obliquely tyed together. At length in a common chanel, and again in divided streams by little and little growing into embossed billows, do swell up at the center of the diaphragm; not a slender token of the near fountain from whence the chyle through the breast floweth into the subclavian veins". (Fig. 4); (Pecquet 1653 c.).

He thus made the discovery of the right lymphatic duct and the thoracic duct in the dog. He went on to discover the cysterna chyli which he termed the "receptacle", and the vessels connecting the mesenteric lymph nodes with the receptacle. He established the fact that these efferent lymphatics as well as the thoracic duct possessed valves to prevent the back-flow of lymph to the nodes and cysterna chyli respectively. He was also the first to apply ligatures to make the thoracic duct more prominent. Pecquet made these discoveries in hounds; then he proceeded to confirm and investigate them in other animal species including amongst others the cow, pig, horse and sheep. In this latter he found only one duct transporting the lymph upwards, but divides into two at the fourth thoracic vertebra until its termination.

Pecquet assumed and was convinced that man also had a receptacle
for the lymph similar to that he found in the animals he examined. Pecquet's contribution was undoubtedly great, as he discovered the right thoracic duct, re-discovered after Eustachius the left thoracic duct and cysterna chyli; he discovered the valves in the thoracic ducts and the connection of the lacteals after traversing the mesenteric lymph nodes with the cysterna by the efferent mesenteric lymphatic vessels, thus proving that the liver does not receive the chyle from the lacteals. He showed that the direction of lymph is from the cysterna upwards to the venous confluence in the neck. He published his new experimental findings in a book entitled "Experiments nova anatomica..." Paris, 1651, to which he added a dissertation on the movement of the chyle in which he attributed to the lacteals openings on the mucosal surface of the intestine and concluded that the forces responsible for the movement of the chyle are respiratory movements, peristalsis and compression of the intestine by abdominal muscular contraction.

The discoveries made by Aselli and Pecquet did not convince William Harvey (1578-1657) who expressed his views in a letter to a friend in April 1652: "With regard to the lacteal veins discovered by Aselli and by the further diligence of Pecquet, who discovered the receptacle or reservoir of the chyle and traced the canals thence to the subclavian veins, I shall tell you freely, since you ask me what I think of them. I had already in
the course of my dissections, I venture to say even Aselli had published his book, observed these white canals, ..... but for various reasons, and led by several experiments, I could never be brought to believe that that milky fluid was chyle conducted hither from the intestine and distributed to all parts of the body for their nourishment; but that it was rather met with occasionally and by accident, and proceeded from too ample a supply of nourishment and a peculiar vigour of concoction". (Foster 1901 b.).

During this time one of the most bitter controversies arose between two eminent scientists, Olaf Rudbeck and Thomas Bartholin. The controversy was not a scientific one but was concerned with priority and credit for the discoveries they made.

The Swedish student at Upsala, Olaf Rudbeck (1630-1702) (Fig. 5), during his studies and investigation of the lacteals in 1650-1651 discovered the liver lymphatics which he named 'Ductus hepatici aquosi' (aqueous hepatic ducts) because they originated in the liver and contained aqueous fluid. He followed these vessels to their termination in the chyle vesicle (cysterna chyli) and noted valves directing the flow of their contents not to the liver but away from it to the cysterna chyli (Rudbeck 1653).

In 1650, while he was examining the motion of the heart in
Fig. 5.

OLAF RUDBECK
a calf in a slaughter house, he noticed that when the intestines were manipulated a milky fluid oozed from the severed veins of the neck. On thorough examination of the carcass he discovered the thoracic duct and the upper part of the cysterna chyli and their contained fluid which was identical with that he first noticed. Then in 1651, in a cat, he saw the complete lymphatic passages: the lacteals, the cysterna chyli, the thoracic duct and its termination at the jugulo-subclavian venous confluence, by applying ligatures to demonstrate them and to control their filling and in April 1652 he demonstrated the "chyle vesicle" to the Queen, at which time he was informed of Pecquet's identical discoveries which he read in June 1652; later he knew of Johann van Horne's publication "Novus ductus chyliferous", Leyden, 1652 and came across Thomas Bartholin's work on the same subjects "Dubia anatomica de lacteis thoracici ... Publice proposita", Copenhagen 1653.

During his work between 1651 and 1653 he discovered the lymphatics and their contained valves in various parts of the body in the dog, calf, sheep and cat. He termed these vessels "vasa glandularum serosa" because they all originated from glands (lymph nodes) and contained serous fluid. He was undoubtedly describing the efferent lymphatics to which he attributed the function of carrying the serous fluid from the glands to the cysterna chyli and the thoracic duct. He mentions in his book
that the arrangement of the 'vasa glandularum serosa' is not only variable in different species but also between the members of the same species. In 1654 he noticed that lymph is salty in taste and is a coagulable fluid. Besides Rudbeck the anatomist, he was a botanist, an engineer, an architect and a scholar – the Swedish Leonardo. (Fulton 1938).

He published his discoveries on the lymphatic system in 1653, "Nova exercitatio anatomica ..." and as tabulae in Hemsterhuis' "Messis aurea", 1659.

Thomas Bartholinus (1616-1680), the Danish anatomist at Copenhagen (Fig. 6), after receiving the news of Pecquet's discoveries of the thoracic ducts and cisterna chyli in dogs, set himself with his German assistant, Michael Lyser, to confirm these discoveries and to look for them in man by dissecting the bodies of two hanged criminals who had a good meal before they were condemned. He published his findings in 1652 in "De lacteis thoraciciis in homine brutisique nuperrime observatis". Though he saw the lacteals, the thoracic duct and its insertion by three branches into the subclavian vein, he did not see a receptacle as Pecquet described in the dog, "... we shew'd the three lumares glandulas full, in the place of Pecquet's receptacle ..." (Fig. 7), (Bartholin 1653 d.).

As it was given many names (the store house of the chyle, the bladder of the chyle, the receptaculum chyli, by Pecquet
Fig. 6.

THOMAS BARTHOLINUS
Fig. 7.

Bartholin's figure of
the human thoracic duct & 'lumbares glandulae'.
and the milky bag, by van Horn), Bartholin decided "... we by one common name will call them the milkie lumber glandules, because those which in men and beasts we have seen are much of this nature". (Bartholin 1653 e.).

He gave the full description of the cysterna chyli (milkie lumber glandules) in dogs as being only one, and is membranous with a cavity that admite one to two fingers and is full of chyle; and in man, "In men the substance is quite different for it's glandulous and solid like the other glandules of the mesentery, watered as it were with the venae lacteae ..." (Bartholin 1653 f.).

It is evident that he only found the cysterna chyli in the dog but failed to do so in man; instead he found the aortic lymph nodes (pre- and para-aortic) which he considered to be the counterpart, in man, of Pecquet's receptacle. He also saw the aortic and the forelimb lymphatics in a dog and described the characteristics of the thoracic duct in the chest as being: very thin walled and is thus easily injured and becomes unrecognisable when opened. Bartholin practised cannulation of this duct and to demonstrate it he blew in the cannula. He observed that the duct is small in the chest but wider towards the diaphragm. All these characteristics described by Bartholin in 1652 are made use of till this day (see later). Unlike Pecquet he found no thoracic duct on the right side in dogs or man.
In Chapter 13 'On the use of the milkie veins of the thorax' Bartholin attributed to the thoracic duct the miracle seen on the beheading of Saint Paul under the empire of Nero, "... that a stream of milk flow'd out of his neck."

Bartholin performed the first cannulation of the thoracic duct I could find evidence of. "We made another experiment often, at Hafniae with bellowes and a hollow pipe, which making a little hole, we put into the milkie vein of the thorax beneath the clavicles, and sent in wind and water by blowing in't". (Bartholin 1653 g.). He did his cannulations in the reverse direction to what is done to-day and for a different purpose.

Until 1652 Bartholin was of the opinion that the lacteals partly emptied their contents to the heart via the oysterna chyli and the thoracic duct and partly to the liver. He quoted on this point Fallopius, Asellius, Walleus, van Horn, Nathaniel Highmore and Conringius. He hesitated to dismiss this belief which was held by such illustrious men and thus disapproved of what Pecquet had said, that all lacteals go to the receptacle of the chyle, then to the thoracic duct to reach the heart, though Bartholin himself in his book "De lacteis thoracicis ..." 1652, described that the lacteals going to the liver in dogs were not full of fluid as milky as chyle but watery. He argued that the receptaculum and the thoracic duct also sometimes
contain watery fluid. He demonstrated the liver lymphatics in man but took them for lacteals going to the liver, not away from it.

In the orbe fish he found milky veins going to the liver, one of which was large and destined to the third lobe.

All these findings left Bartholin hesitant to discard the belief of many eminent men as those he quoted above. Then in 1653 in his "Vasa lymphatica nuper Hafniae in animalibus inventa et hepatis exsequiae" he published the discovery of the lymphatics, assisted by M. Lyser, in the dog, and demonstrated that the liver lymphatics (by applying ligatures) leave, not go to, the liver, taking back all he had said before, regarding this point and wrote a famous epitaph to the liver (Skavlem 1921). Bartholin named the new vessels the "vasa lymphatica", a term that is retained up till this day.

In 1654, assisted by Henric v. Minichen (Garboe 1950), he discovered the lymphatics in man, "Vasa lymphatica in humane nuper inventa".

A famous controversy broke in 1654, when Sibold Hemsterhuis published a book "Messis aurea ..." containing the works of Pecquet, Bartholin and Rudbeck. The controversy started by Rudbeck concerning his priority to the discoveries before Bartholin. As it was a bitter controversy the conclusion given by J.H. Skavlem seems to be quite justi-

"We cannot be mistaken in saying that the discovery brought
great honour to both Bartholin and Rudbeck. When we state that Rudbeck was the one who first began the investigation and at least from an anatomical viewpoint deserves the advantage, but that Bartholin was the one who first pushed forward and advanced the investigation, taking upon himself the responsibility for it; that Rudbeck later added more than Bartholin to the anatomical knowledge of the lymph system, chiefly in his table contained in his publication "Messis aurea" but that Bartholin added more than Rudbeck to establish the truth of the discovery and its physiological significance, then satisfaction is established on both sides without detracting from the glory of either." (Skavlem 1921).

It seems that Rudbeck was unaware of neither Pacquet's nor Bartholin's work. Also J. van Horn at Leyden was working on the same subject and published his work under the title of "Novus ductus chyliferous", Leyden, 1652, (Rudbeck 1653). He called the cysterna chylis the "saccus lacteus" (milkie bag) before he even saw Pacquet. He also saw the lymphatics at the bifurcation of the aorta.

Some writers attribute the credit of the discovery of the lymphatics to George Joyliffe (1621-1658) while preparing for his degree at Cambridge, though he left no evidence of his discovery concerning this very important (at that time) set of vessels in books, manuscripts nor published thesis (Fulton 1938).
Francis Glisson in his book "De hepate", London, 1654 (Metler 1947 b.) attributed the discovery of this fourth set of vessels in distinction to arteries, veins and nerves, to Joyliffe in 1652, who informed him of their watery content and their distribution to most parts of the body (Fulton 1938).

As regards the function of the lymphatics Francis Glisson (1597-1677) in 1654 maintained that they are absorbent (Bailey 1923).

During the remaining part of the seventeenth century the work on the anatomy and physiology of the newly discovered lymphatic system rapidly increased and amongst the many who contributed to its demonstration and clarification were: the Italian anatomist Giovanni Guglielmo Riva (1627-1677) who made diagrammatic representation of the whole lymphatic system (Castiglioni 1941); the Italian anatomist and histologist Marcello Malpighi (1628-1694) discovered the lymphoid follicles in the lymph nodes and spleen; Nicolaus Steno (1638-1686), who discovered the right thoracic duct in quadrupeds (Cruikshank 1790) (rediscovered it after Pecquet who, in 1651, described two thoracic ducts, one on either side, in the dog); Fredrick Ruysch (1638-1731) cannulated the lymphatics by fine glass cannulae (Haller 1757 c.) during his studies on these vessels and their valves which he described in his
"Delucidatio valvularum ..." and attributed to the thymus lymphatics the function of 'excretory ducts' of that gland (Cruikshank 1790); Anton Nuak (1650-1692) did extensive studies on the lymphatics and the lymph nodes which he injected with mercury after cannulating the lymphatics and gave their full description in his "Adenographia" (Haller 1757 b.). The Swiss Johann Conrad Peyer (1653-1712) discovered the intestinal lymphoid follicles in the cat and described them in his "Exercitatio anatomica medica de glandulis intestinorum", Schaffhausen, 1677. Johann Gottfried von Berger (1659-1736) in 1682 defended his thesis "De circulatione lymphae et catarrhis" in his physiology, 1701, he deals with lymph and lymphatics in various parts and that lymph has to pass by one or more lymph nodes before entering the thoracic duct to reach the blood (Underwood 1953). Freidrich Hoffman (1660-1742) established the concept that the lymphatics are absorbent in function.

Alexander Monro Secundus (1733-1817), the Professor of Anatomy at Edinburgh, maintained that the lymphatics originate from the connective tissue spaces and not from the vascular system (arteries) as was believed by many, thus regarded the lymphatics as a separate system and attributed to it the function of absorption (Comrie 1932). It also seems that he injected the lacteals in the turtle and made a demonstration of these in his lectures (Hewson 1772 a.).
Monro aroused two bitter controversies. The first was his dispute with Hunter (William), in which Monro claimed his priority over Hunter regarding that the function of the lymphatic system, being a complete system by itself and is absorbent, is his idea and not that of Hunter (Hewson 1774). The second controversy he aroused was with William Hewson, claiming that he and not Hewson discovered the lymphatics in birds, fishes and the turtle (as a matter of fact Thomas Bartholin was the first discoverer of the liver lymphatics in the orbe fish "Anatomia reformata" Hag. Com. 1666, Fig. page 419).

In 1769 the English student of the Hunters, William Hewson (1739-1774) of Hexham (Fig. 8) was awarded the Copley Medal and in 1770 became a Fellow of the Royal Society for his work on the lymphatics and the lymphoid tissues. He gave an account on the discoveries he made concerning the lacteals and lymphatics in birds, fish and amphibia to the Royal Society on December 8th, 1768 (Hewson 1772 b.).

Hewson did extensive work on the lymphatic system in various species including man. He divided the lymphatics into superficial and deep, and proved that these sets exist in organs (liver, kidney and spleen) and that they communicate together. He discovered the lymphatic system in birds (geese), showing that they have two thoracic ducts which

Fig. 8.
arise from a lymphatic network in the root of the mesentery and found that birds (geese) do not have lymph nodes except on either side of the neck interrupting the cervical lymphatic (Experimental inquiries ... Part the second, Chapter 4), while amphibia (turtle) has none at all, also that it differs from birds in having a large receptaculum chyli and three thoracic ducts, one on the right side and two on the left. He dissected only one turtle and made his discovery of its lymphatic system in 1763, (the same work, chapter 5). The third species he worked on were fish and found a large receptaculum chyli and one to two thoracic ducts. He found, as in the turtle, that they have no lymph nodes (the same work, chapter 6). He gave a description of the lymphatic system in the fish in "Philosophical transactions", volume 59.

In his "Experimental inquiries ... Part the second" he gave a chapter on the properties of lymph, showing that on exposure to air it coagulates, a property the duration of which varies between species and according to age. From his work and discoveries in the animal species he concluded about the receptaculum chyli that it exists in quadrupeds, the turtle and the fish and is of a considerable size, and agreed with many anatomists that man does not possess what deserves the term receptaculum and that it is rare for man to have two thoracic ducts.
William Hewson made for the first time the discovery of the lymphocytes, though he named them "solid particles" and attributed (correctly) their origin to be from the lymph nodes: "On cutting into a fresh lymphatic gland, we find it contains a thickish, white, milky fluid. Then if we carefully wipe, or wash this fluid from any part of it and examine it attentively in the microscope, we observe an almost infinite number of small cells not such as have been before described or that have been supposed to exist in the lymphatic glands, but others too small to become visible to the naked eye". (Falconer 1777 a.).

He examined the blood of the frog, fish and the toad and noticed that the red blood cells had a central "solid particle", and on addition of water the red blood cells became spherical and the "solid particle" rolled inside from side to side like a "pea in a bladder". Then on examination of the fluid from the cut surface of a lymph node and diluting it with salt solution, found that "numberless small, white, solid particles resembling in size and shape those central particles found in the vesicles of the blood are to be seen ..." (Falconer 1777 b.). He attributed to the lymph node the function of making these "solid

* Hewson used single microscope made of a single lens (1/23 of an inch focus) between the eye and the object supported on a scroll and enlarged the object 184 times.
particles" and excreting them by way of the efferent lymphatics (lactea secundi generis) which he took to be the secretory duct of the lymph nodes, to be later surrounded by the vesicular portion in the spleen to form the red particles of the blood. He maintained that it is difficult to see these "central particles = solid particles" in the human red cells but he saw them and was convinced that they existed.

Hewson examined besides the lymph nodes, the thymus, on which he made an account; and saw its lymphatics and examined its lymph under the microscope (x 184 magnification) after diluting it with human serum and he commented "... the same appearance was exhibited as was observed on examining the fluid found in the lymphatic glands, (viz) a great number of small, white solid particles exactly resembling in size and shape the central particles in the vesicles of the blood, or such as are found in the fluid of the lymphatic glands." (Falconer 1777 c.). On cutting the thymus and examining the fluid it contained he saw the same appearance as that observed on examining the fluid from the lymph nodes and the thymus lymphatics, thus reaching the conclusion that one of the functions of the thymus is to secrete these "solid particles" and that the lymphatics convey them via the thoracic duct to the blood. He also considered the thymus to be an appendage to the lymph nodes.
since their structure and function were similar and because in early life the thymus existed when these "solid particles" were most needed, and concluded that the thymus is a large lymph node.

He attributed the origin of the "solid particles" of the red cells to be from the lymphatic system and its appendages (lymph nodes, thymus, spleen and lymphatic vessels).

Though Hewson discovered the lymphocytes he confused them with the nuclei of the red cells, which are seen in the fish, toad and frog, and the central depression of mammalian red blood cells. He named them the "solid or central particles" and thought that their function is to be coated with a vesicular portion usually in the spleen and to leave the spleen via its efferent lymphatics to reach the circulation as the red globules (red blood corpuscles) via the thoracic duct.

After Hewson's death his work on the lymphatic system was continued by his friend and co-worker, Magnus Falconer who, after repeating the experiments and adding to them, published the results under Hewson's name as "Experimental inquiries ... Part the third", thus finishing the inquiries Hewson started ("Experimental inquiries ... Part the first and part the second").

William C. Cruikshank (1745-1800), another student of
the Hunters, worked on the lymphatic system and wrote "The anatomy of the absorbing vessels of the human body", London, 1786, in which he dealt with the lymphatics of the whole human body (Cruikshank 1790). He injected the arteries on the coats of the lymphatics and saw them ramifying through their substance and concluded that the lymphatics possess *vasa vassorum* like other vessels. This was thought by Hewson to be a probability, which Cruikshank proved. On the properties of lymph, though Haller believed that lymph is acid, Cruikshank showed to the contrary that lymph is alkaline in reaction.

He also disagreed with Hewson's concept that the red cells have a central particle. About the lymph nodes supplying these particles he said (p. 106); "... that they serve to form the central particles of the globules of blood, as Mr. Hewson and Falconar contended, is as improbable since these central particles have not been seen by the first microscopic observers in the world; I never have seen them ..." He also confirmed the observations of previous anatomists that man rarely has a *cysterna chyli*, though he saw human cysternae on occasions.

Paolo Mascagni (1752–1815), the Italian anatomist, made extensive researches on the lymphatic system. He demonstrated that they are found all over the body and ultimately end in
the venous system. He disagreed that the peripheral lymphatics communicate with blood vessels, except maybe at the connective tissue spaces from which he believed the lymphatics originate (Poirier and Cunéo 1901 a.). He published "Vasorum lymphaticorum corporis humani historia et iohnographia", Senis, 1787, containing extremely fine and precise illustrations of the body lymphatics, many of which till this date are still referred to in anatomy textbooks.

The third phase or the phase of real scientific experimentation:

This phase in the history of the development of knowledge about the lymphatic system started in the nineteenth century and is the richest in its literature. This is the phase at which great scientific controversies arose and investigation of the significance of the previous discoveries was carried out. The problems studied in the first half of that period were on the origin of the lymphatics, movement of lymph and its functional significance, the white cells in the blood and their classification and the lymphocyte problem. It is beyond this general account on the history of the lymphocytes and lymphatic passages to even try and enumerate all the contributions made during that phase. A detailed account of the work carried
out in the nineteenth century is reviewed in "Recent works on the lymphatic system" 1867, and in "Système lymphatique" (see Poirier and Cunéo 1901). Amongst the many great workers at that time were Bichat who published his work on the origin of the lymphatics in 1816; Lippi, who in 1822, and Lauth in 1824 discovered lymphatico-venous communications, and Müller in 1838 showed that the villi are not perforated by injecting the lacteals with mercury that did not show on the mucous surface of the intestine, thus disproving Lieberkühn's teaching that the villi are perforated by the openings of the lacteals on the mucous surface. In 1847 Sappey started his work on the lymphatic system, the results of which he included in a large atlas in 1876 and is still referred to in the modern textbooks of anatomy.

Jakob Friedrich Gustav Henle made studies on the lacteals, Wilhelm His studied the thymus and lymph nodes' structure and so also did Teichmann. François Magendie worked on the composition of lymph; also his student Claude Bernard made many investigations on the lymphatic system. Friedrich Leopold Goltz studied lymph movement. Florence Sabin contributed to the lymphatic system by his studies on its origin and development, and Carl Ludwig studied the mechanism of lymph formation and the cause of lymph movement. He cannulated various body lymphatics and postulated that lymph is a blood filtrate (Yoffey and
Rudolph Peter Heinrich Heidenhain did not approve of Ludwig's hypothesis on lymph formation and maintained that lymph is a cellular secretion product from the cells of the capillary wall. Friedrich Wilhelm Noll in 1849 put the hypothesis concerning lymph formation by fluid diffusion from the blood vessels to tissues, and in 1894 Ernest Henry Starling showed influence of the mechanical factors on lymph production. Hinhorn (Poirier and Cuneo, 1901 b) in 1884 was the first to coin the term "lymphocyte" to the cells first observed by Hewson in the lymph node fluid, a term that is in use till this date.

"No great man lives in vain. The history of the world is but the biography of great men."

Thomas Carlyle
PART III
MATERIAL AND METHODS

"Gallen gave to medicine that method of putting questions to nature and of arranging matters so that nature may answer them, which we call experiment."

Fielding H. Garrison 1913

MATERIAL

A. Animals used

1. Dogs:

(i) Mongrel adult dogs of varying age range, of both sexes and weighing between 10-18 Kg., actively immunized against distemper and hepatitis (Epivax-plus).

(ii) Beagles, mostly males of different hair colour mixture, weighing between 11-18 Kg., 1.5 - 2.5 years old, immunized 1-12 months before admission against distemper, hepatitis, leptospira cani coli and leptospira icterohaemorrhagica by a single immunizing dose (Kavac or Maxivax).

The mongrel dogs were used as donors of thoracic duct lymphocytes for the immunization of rabbits and sheep; as donors and recipients of renal homografts in the experiments dealing with rabbit-versus-dog antilymphocytic antiserum, and in the experiments with sheep-versus-dog antilymphocytic antiserum; as

*** Hoechst, Germany.
lymphocytes donors for the in vitro assay of the antilymphocytic antisera; as blood donors for the absorption of the antilymphocytic antisera; and as normal tissue donors for histological examination as a control for the antiserum treated mongrel dogs.

The beagles were used as thoracic duct lymphocytes donors for the immunization of the horse; as donors and recipients of renal homografts in the experiment dealing with horse-versus-dog antilymphocytic antiserum; as blood donors for transfusion; occasionally as blood donors for the absorption of the antilymphocytic antiserum, and as normal tissue donors for histological examination as a control for the antiserum treated beagles.

2. **Rabbits:**
Mixed breed of both sexes, weighing about 2-3 Kg., were used for immunization with hooded rats' or mongrel dogs' thoracic duct lymphocytes, and for the production of rabbit-versus-rat antilymphocytic antiserum and rabbit-versus-dog antilymphocytic antiserum.

3. **Sheep:**
Suffolk and cross bred sheep, 1½ - 2½ years old (4 ewes and one wedder), weighing between 50-80 Kg., were used for immunization with mongrel dog's thoracic duct lymphocytes for the production of
sheep-versus-dog antilymphocytic antiserum.

4. Horse:
"Phigamas", bay, gelding, weighing about 572 Kg. 11 years old. Used for immunization with male beagles thoracic duct lymphocytes for the production of horse-versus-dog antilymphocytic antiserum.

5. Guinea Pigs:
Of both sexes, were bled for the collection of serum which was used as a complement source for the in vitro assays of the antilymphocytic antisera.

6. Rats:
Inbred hooded males, weighing about 300 g., were used as thoracic duct lymphocytes donors for the production of rabbit-versus-rat antilymphocytic antiserum, and for the in vitro and in vivo assay of the corresponding antilymphocytic antiserum.

B. Instruments and Products used are described with the part dealing with the methods.
METHODS

1. Anaesthesia:
   (i) Dogs: Anaesthetized by an intravenous injection of "Nembutal" (Pentobarbitone sodium B.P.) veterinary 1 ml/5 pounds body weight. Intravenous injections were made in the cephalic vein in the forearm after cleaning the skin with alcohol.
   (ii) Horse: 1-2 ml. (Ravocaine - Vet) local infiltration of the skin and subcutaneous tissue overlying the external jugular vein at the site selected; for both immunization and bleeding.
   (iii) Guinea Pigs: Open ether anaesthesia for bleeding by cardiac puncture.
   (iv) Rats: Open ether anaesthesia for both cannulation of the thoracic duct and tail (peripheral) blood counts.

2. Thoracic duct cannulation in the dog:
   Special instruments -
   (i) Nylon cannula (No.*3) with an internal diameter of 1.0 mm. and an external of 1.35 mm., 21 inches long.
   (ii) Polythene cannula (No. 53A = PP**205) with an internal diameter of 1.57 mm. and an external of 2.08 mm., 10 inches long, used as a cuff for the nylon cannula.

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* Nembutal (veterinary) - Abbot Laboratories Ltd., Queenborough, Kent.
(iii) Glass rods of appropriate sizes and curves.

(iv) Ligature introducer, modified from Anderson's ligature introducer (Anderson 1961).

(v) Cannula forceps.

(vi) Three inch long stainless steel needle of a bore to allow the cannula and cuff through.

All routine surgical instruments, gowns, towels, gloves and swabs were sterilized by autoclaving at a pressure of 15 lb/sq. inch for half-an-hour. All the special instruments were sterilized by boiling for 20 minutes.

Dog preparation: The dogs to be cannulated received as a routine a cup of milk or cream two hours before operation. Under Nembutal intravenous anaesthesia the hair on the left side of the chest was clipped from the shoulder region to the subcostal area and from the midline ventrally to beyond the midline by 1\(\frac{1}{2}\) inches dorsally. An endotracheal tube was inserted and artificial respiration by an electrically driven pump commenced.

Position: The dog was laid on its right side with a sandbag placed longitudinally under the ventral part of the chest so as to direct the sternum slightly upwards.
Skin cleansing: The clipped area was thoroughly cleaned with Iodofors.

Operation: An 8 inch long incision was made along the left 8th intercostal space, incising the skin and the subcutaneous fatty layers. Care was taken to secure strict haemostasis. The pectoralis and latissimus dorsi muscles were then cut along the same intercostal space. The intercostal muscles and pleura were incised. A self-retaining rib retractor was put in place. The left lung was then retracted ventrally and cephalad, while the left oopula of the diaphragm was retracted ventrally and caudal, thus exposing the descending thoracic aorta with the intercostal vessels. Three or four of these latter vessels were then cut between two ligatures after incising the pleura medial to the thoracic sympathetic chain. After retracting the descending thoracic aorta ventrally the thoracic duct lying posterior to it was dissected from the surrounding fatty tissue and a ligature was placed around it by Anderson's ligature introducer, and ligated (first ligature), thus allowing the duct to distend with the accumulating lymph. One inch caudal to the first ligature another ligature was introduced.

* Iodofor: Iodine (Resob) 20 gm; Isopropyl alcohol 240 ml; Texofor I.S. 200 gm; Dilute HCl B.P. 10 ml; Aqua dest. up to 2,000 ml. For skin dilute 250 ml stock solution and make up to 2,000 with aqua dest.
around the duct and left untied (second ligature) for securing the cannula in the duct. A third ligature was then applied an inch caudal to, the second ligature and left untied and held by an artery forceps. An appropriate site in the now distended thoracic duct was selected between the first two ligatures for the introduction of the nylon cannula. This part of the duct was then dissected from the surrounding connective and fatty tissue along half its circumference. The wide bored stainless steel needle was then passed through the chest wall above the incision at an appropriate site that will allow the cannula to lie in line with the thoracic duct. The nylon cannula with the surrounding polythene cannula cuff were then threaded through the needle extrathoracic opening till they appeared at the inner side of the chest wall. The needle was then removed. After bevelling the intrathoracic end of the nylon cannula with scissors, the cannula was filled with a mixture of Hanks' buffered salt solution and heparin (5 ml. Hanks' solution and 500 I.U. heparin) using a 5 ml. capacity glass syringe and a 19 G needle. The syringe is left connected to the cannula. Turning now to the thoracic duct, an incision was made in the selected area with fine scissors. As the lymph flooded the area, the third ligature was put on tension to prevent further flooding. The tip of the nylon cannula held by the cannula forceps was
then introduced into the opening made in the thoracic duct and pushed caudally very gently, after relaxing the third ligature, to lie for an appropriate distance within the duct. When feasible the cannula tip was made to pass beyond the third ligature, which was then tied to hold the cannula in situ. Otherwise, the ligature was cut and the second ligature was tied firmly around the duct with the contained cannula to secure it in position. The syringe with the attached needle at the extrathoracic extremity of the nylon cannula was now removed to allow the lymph to drip or was left in situ till the termination of the operation.

One of the free ends of the first ligature was made to pass around the extraductal part of the cannula and ligated around it to further secure the cannula in position, thus preventing the transmission of movements of the cannula to its tip. The free ends of the ligatures were then cut. A thoracic tube was passed through the 10th intercostal space, Polybactrin sprayed, and the wound closed in layers, under a water seal, by applying 4–6 interrupted linen stitches passing from around the rib above to the rib below. The muscle layer was then closed by interrupted linen sutures (No. 30). After closing the subcutaneous fatty layer by fine linen (No. 90)

inverting interrupted stitches, the skin was closed by a continuous linen suture (No. 30). The thoracic tube was always removed after the inflation of the lungs at the end of the operation and the tube site closed by a figure 8 stitch which was tightened as the tube was withdrawn. The thoracic duct cannula was then fixed to the chest wall from the point of exit to a distance of one inch by three interrupted linen stitches.

The extrathoracic free end of the nylon cannula was made to drip in a conical glass flask containing a mixture of heparin and Hanks' solution (see later).

All the cannulations carried out during this work were performed by this technique which is slightly modified from that described by Grindlay, Cain, Bollman and Mann (1950). The operation takes about 35-45 minutes depending on the fatness of the dog and the thoracic cage size.

**Thoracic duct cannulation in the rat**

**Special instruments:**

(i) Flexible nylon cannula (No. 1) with an internal diameter of 0.75 mm. and an external diameter of 0.94 mm.

(ii) Cannula moulder.

(iii) Ligature introducer; a modification of the


** Designed and kindly made by Dr. N.F. Anderson.
original Anderson's introducer (Anderson 1961).

(iv) Cannula forceps.

(v) Needle of a bore that would admit the passage of the nylon cannula through.

The nylon cannula was put in the cannula moulder which has a U-shaped groove with one of the limbs longer than the other. The moulder was allowed to boil for ten minutes, after which time the cannula acquired its shape.

Rat preparation: During the night preceding the cannulation the rats were given milk ad libitum. Under open ether anaesthesia the thoracic duct was exposed and cannulated sub-diaphragmatically by the method of Bollman, Cain and Grindlay (1948) as modified by J.L. Gowans and by N.F. Anderson (Anderson 1962).

Technique: The rat was fixed on its back on a felt board covered with a sterile towel. Its left arm was turned so as to lie near the right arm, thus keeping the rat in an oblique position. By a left subcostal incision two inches long, the skin and abdominal muscles were cut with scissors. The abdomen was opened and the left costal margin retracted upwards and the intestine packed with a small piece of gauze (2" x 2") and retracted ventrally after cutting the peritoneum along the lateral border of the left kidney. The retroperitoneal tissue along the medial border of the left psoas muscle was
then cut along that border using a finely tapered glass rod the distal part of which was slightly curved. The abdominal part of the thoracic duct then came into view lying between the abdominal aorta and the medial border of the left psoas muscle. A ligature was then introduced by the ligature introducer near the left diaphragmatic crura, and tied but left uncut and held on an artery forceps (first ligature). This allowed the thoracic duct to distend. Half a centimeter lower down another ligature was introduced and left untied (second ligature). The needle that adapts the nylon cannula was then passed through the layers constituting the abdominal wall below the incision, and the moulded nylon cannula long limb was passed through its intra-abdominal opening. The tip of the short limb of the cannula was then bevelled by scissors and the cannula was filled with heparin solution by means of a tuberculin syringe and a 12C needle (both are left connected to the cannula). The cannula was held by the cannula forceps. Using a sharp lancet, the distal part of which is curved slightly, an opening was made in the anterior wall of the thoracic duct between the first and second ligatures. While the lancet was still in situ the cannula was gently passed posterior to it and at the same time the lancet was withdrawn. The cannula
was then fixed in the duct by tying the second ligature and was further secured in its position by the first ligature which was made to pass around it and then knotted. The abdomen was closed in layers by a continuous black silk suture (3/0). The rat was then put in a restraining cage as described by Bollman (1948) and modified by Anderson (Anderson 1962).

3. **Lymph collection**

   **In dogs:** The free end of the thoracic duct cannula was made to drip in a sterile conical glass flask containing 10 ml. Hanks' buffered salt solution and 1,000 I.U. of heparin. The flask was immersed in a container with cold water and three cubes of ice. The cannula was adjusted so as to drip in the middle of the flask (using an M.R.C. bottle rubber stopper through which is passed a 2" long glass tube through which the cannula is inserted). This ensures against slowing of the lymph flow which potentiates lymph coagulation at the tip of the cannula. Lymph was collected until the required number of lymphocytes was obtained. The sterile lymph was either used immediately or after storage at 8°C overnight. The contained lymphocytes were used for immunization for the antiserum production or for *in vitro* assays.

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In rats: The lymph was collected as described by Woodruff and Anderson (1964) using a sterile tube of 100 ml. capacity, the tube containing heparin-Hanks' solution mixture (3 ml. Hanks' and 300 I.U. heparin). The tube was placed in a thermos flask containing ice chips.

4. Lymphocyte preparation

For immunization: The collected thoracic duct lymph was diluted with Hanks' solution, in equal volumes, in 100 ml. capacity centrifuge tubes and thoroughly mixed by inverting a few times, then centrifuged at 800 G at 16°C for 10 minutes in a Major refrigeration MSE centrifuge. The supernatant was then aspirated and discarded, leaving the cell button at the bottom of the tubes. The cells were then washed by suspending in Hanks' solution and recentrifuged at 800 G for 10 minutes. The supernatant was then aspirated and discarded leaving the cell button which was mixed with some Hanks' solution to make a homogenous suspension on which a lymphocyte and viability counts were performed. The suspension was then made to the required volume and cell number needed.

All procedures described above were carried out using sterile instruments and glassware, and under sterile precautions.

For in vitro tests: As described above but with the omission of washing.
5. **Lymphocyte viability test**

The trypan blue dye exclusion method was employed for testing the lymphocytes' viability. A cell count was done using a white cell counting pipette and freshly prepared trypan blue (0.05% dilution in Hanks' solution) as diluent. The cell dilution was 1/20. The pipette was then gently shaken for precisely one minute, then after a further two minutes (to allow for filling the haemocytometer chamber, mounting on the microscope and settling of the cells), a cell count was performed. This timing was strictly adhered to in order to have comparable results in all the experiments done.

Both viable and non-viable cells were counted in 2 mm$^2$ and the percentage of each was calculated from the total count. Any cell that was stained blue or a shade of blue was counted as non-viable, and the non-stained cells were considered viable.

6. **Immunization**

(i) **Rabbits:** These were immunized as described by Woodruff and Anderson (1964) with rat lymphocytes. The intraperitoneal injections of mongrel dogs' thoracic duct lymphocytes suspended in Hanks' solution were administered by a sterile 5 ml. capacity syringe and a 23 gauge needle. Each rabbit received at weekly intervals 200 x 10$^6$
lymphocytes suspended in 2-4 ml. of Hanks' solution for a total of three weeks. One week after the last immunizing dose the rabbits were bled to harvest the immune serum. They received a booster dose of 100-150 x 10^6 cells one week before each further bleeding.

(ii) Sheep: These were immunized by a weekly intraperitoneal injection of 0.75 - 1.0 x 10^9 mongrel dogs' thoracic duct lymphocytes suspension for a total of three weeks. The lymphocytes were suspended in 10-15 ml. of Hanks' solution. Using a two inch long 19 gauge needle the injections were done on the right side midway between the right 12th rib and the iliac crest lateral to the sacrospinalis group of muscles, then bled one week after the last immunizing injection. The sheep received an intraperitoneal booster dose of 0.5 - 0.75 x 10^9 lymphocytes one week prior to each further bleeding.

(iii) Horse: The horse was immunized with beagles' thoracic duct lymphocytes suspended in 10-15 ml. Hanks' solution, by a single weekly intravenous injection for a total of four weeks. The first immunizing dose was 0.9 x 10^9 lymphocytes and the
subsequent weekly doses were 0.8; 2.7 and $1.7 \times 10^9$ lymphocytes respectively. Bleeding was performed one week after the last immunizing injection. One week before each further bleeding the horse received an intravenous booster dose of $1.0 \times 10^9$ lymphocytes suspended in 10 ml. Hanks' solution. All the injections were made in the external jugular vein, under local infiltration anaesthesia. All the lymphocytes suspensions were freshly prepared thoracic duct lymphocytes obtained from male beagles. Each dose of lymphocytes injected was obtained from one male beagle (i.e. four male beagles contributed to the immunization of the horse).

7. Bleeding

(1) Rabbits: Were bled from the marginal vein of the ear, during which time they were kept in a restraining device to minimize their movements. The ear to be bled was warmed by approximating a desk lamp. The hair at the selected bleeding site was shaved and the area cleaned, then smeared with sterile vaseline. The marginal vein was slit open lengthwise for $\frac{1}{2}$ cm. The blood was received in a sterile paraffin coated 100 ml. capacity glass tube, held in a stand at about two inches below the ear, at room temperature. The
rabbits were bled in complete silence. The tube containing the blood was then covered and kept at room temperature overnight. The ear incision was then covered with sterile gauze and moderate pressure applied to secure haemostasis. The area was then sprayed with sulphonamide powder and the rabbit was returned to its cage and given water *ad libitum*.

(ii) Sheep: The sheep were held in a vertical position steadied by an assistant to keep the animal resting on its hind limbs. The hair overlying the external jugular vein on both sides was clipped. Pressure was applied with the left thumb to the distal part of one vein to allow its filling. A sterile bleeding set with a wide bore needle (13 B.W.G.), two inches long to which is attached a nylon tube two feet in length, was used. The needle was introduced into the external jugular vein at an angle with the skin of 45°. The blood issuing from the free end of the nylon tube was received in 300 ml. capacity paraffin coated tubes. At the end of the bleeding the needle was withdrawn and gentle pressure was applied to the needle site till haemostasis was secured. The tubes were covered and kept overnight at room temperature.

* Arnold and Sons, Edinburgh.
Horse: The horse was bled from the external jugular vein under local infiltration anaesthesia, after shaving the hair overlying the selected site. A skin incision (½") was made overlying the external jugular vein. A sterile trocar and cannula was then introduced into the external jugular vein and connected with the sterile receiving set consisting of nylon tubing attached to M.R.C. bottles by "Y" shaped plastic connections. The trocar was then withdrawn and the blood was allowed to fill the sterile, silicone coated bottles. At the end of the bleeding (not exceeding 9 L.) the trocar was replaced and the cannula was withdrawn. Moderate pressure was then applied to secure haemostasis. The blood bottles were left overnight at room temperature.

Guinea pigs: Under open ether anaesthesia bleeding was performed by cardiac puncture using a sterile 19 G needle and a 10 ml. glass syringe. After clipping the hair on the left side of the thorax and cleaning the area with Cetavlon, the needle was introduced through the intercostal space below that of the apex of the heart, first in a perpendicular direction to the chest wall, then as the thoracic cavity was entered it was directed upwards medially
and slightly backwards till it punctured the heart (the heart beats are felt transmitted through the needle as the needle tip touches the pericardium). The blood filled the syringe by its own force. Very gentle aspiration was applied at intervals. The syringe contents were emptied in a sterile paraffin coated glass tube and left overnight.

Preparation of paraffin coated tubes:

Blocks of Paraffin with a melting point of $48^\circ$C. were melted and poured in bottles, and was then sterilized by auto-claving.

Before use the paraffin was melted (by putting the bottle in a water bath over a strong flame), then poured into the sterile glass tube to be coated. These were kept immersed in cold water or surrounded with ice. After filling the tube with paraffin, which solidifies in contact with the cold glass forming a thin coating layer, the excess was poured into another tube for coating and so on. The tubes were immediately covered and used on the same day.

8. **Sera preparation**

**Immune sera:**

(i) **Immune rabbit serum:** The collected blood was allowed to coagulate at room temperature for 18-20 hours. The separated serum was then aspirated and centrifuged at 800 g. for ten minutes to sediment
the contaminating red blood corpuscles. The clear serum was then sterilised by filtration and was put in sterile 20 ml. capacity containers. To inactivate the contained complement the serum was heated at 56°C for half-an-hour in a water bath, during which time the containers were shaken twice. After cooling to room temperature the serum was stored at -20°C. for further use.

(ii) **Immune sheep serum**: Was prepared by the same procedure described above except that the antibodies directed against dog red cells were absorbed.

(iii) **Immune horse serum**: The siliconized M.R.C. blood bottles containing the collected blood were allowed to coagulate at room temperature for 18-20 hours. The bottles were centrifuged at 800 g. for 1-1½ hours in a Major refrigeration MSE centrifuge at 16°C. to separate the serum from the clot. The collected serum was then recentrifuged at 800 g. for ten minutes to sediment any contaminating red cells. The clear serum was then bottled in sterile medical flats (4 oz. capacity) with metal caps and silicone rubber liners. To inactivate the contained complement the serum was heated for 30 minutes at 56°C. in a water bath, during

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* Esco (Rubber) Ltd., Seal Works, Steal Street, London E.8.*
which time the contents of the bottles were mixed twice to allow a uniform temperature of the contained serum. The serum was then cooled to room temperature before storage at -20°C. until its further use.

Normal sera

(i) Normal guinea pig serum: The collected blood was allowed to coagulate at room temperature for 18-20 hours. The serum was then aspirated and centrifuged at 800 g. for 10 minutes to remove contaminating red cells. The clear serum was then put in ampoules and stored at -20°C. with no preservative and was used within days from preparation.

(ii) Other normal sera: Normal rabbit, sheep and horse sera were obtained prior to immunization of the animals, and were prepared by a similar procedure as the immune sera (vide supra), but were not absorbed with red cells

9. Preparation of dog R.B.C.s. for absorption:

(i) Dog bleeding: All blood donors were healthy mongrel dogs or grey hounds of both sexes. Bleeding was done by femoral artery puncture under Nembutal anaesthesia. The artery was either exposed or punctured directly through the skin after meticulous cleaning of the area. Using standard sterile Acid-Citrate-Dextrose (A.C.D.) blood bottles and taking sets, one pint was withdrawn. For
exsanguination both femoral arteries were used for bleeding simultaneously. The contents of the bottles were gently mixed all through the bleeding procedure. If about one pint was only to be taken, the needle was withdrawn and pressure was applied for at least three minutes.

(ii) Blood storage: The blood was either used immediately after withdrawal, or stored at 8°C. for not more than seven days.

(iii) R.B.Cs. washing: For absorbing small volumes of antisera or antisera with low haemoagglutination titers, small volumes of packed red cells were prepared in centrifuge tubes (10-100 ml. capacity). For the preparation of larger volumes of washed packed R.B.Cs. larger containers were made from M.R.C. blood bottles by cutting away the bottle neck and adjoining curved part, thus leaving a 500 ml. capacity container with a wide opening to facilitate the proper aspiration of the buffy coat. These will be termed 'centrifuge bottles'.

The aim of R.B.Cs. washing was mainly to remove the lymphocytes by repeated centrifugation and aspiration of the supernatant fluid together with the buffy coat layer, and secondly to wash off the blood proteins.
Procedure: The blood in the centrifuge bottles was centrifuged for 15 minutes at 800 g. The supernatant plasma layer and buffy coat were aspirated and discarded and to the remaining packed red cells layer 0.9% saline solution was added in an equal volume. The contents of the centrifuge bottles were thoroughly mixed by inverting the covered bottles a few times then recentrifuged at 800 g. for 10 minutes. The supernatant saline layer and the white cell layer were aspirated and discarded. This was considered to be the first wash. The process was repeated for a total of 4-5 washes, i.e., until no white cell layer was seen overlying the packed red cells. If meticulous aspiration of the white cell layer was done after each centrifugation and the mixing of the packed R.B.Cs. with the saline was thoroughly carried out, no white layer appears after the fourth or the fifth washing. To confirm the absence of white cell layer 2 ml. of washed cells were mixed with 0.9% saline in a Khan tube and centrifuged at 800 g. for 5 minutes. If no white layer was seen overlying the packed red cells, the washed R.B.Cs. were considered to be satisfactory for absorption. In the first samples of antiserum that were absorbed further confirmation was by making a blood film on a slide, the Leishman stained film was then examined for the presence of lymphocytes; also a sample from the supernatant fluid was taken and tested for the presence of proteins by "Albustix"
(64)

reagent strips. After the fourth or fifth wash, the protein test, the blood smear and the sample centrifugation were all negative and the packed red cells were then considered satisfactory for the antiserum absorption. The smear test and protein tests were later stopped and the Khan tube test was adopted as a routine. The packed red cell layer was then gently mixed to form a homogenous suspension.

10. **Antiserum absorption:** Certain investigations were undertaken beforehand, to estimate - (a) Haemolysis and haemagglutination titers of the non-absorbed antiserum; (b) Volume of packed red cells needed to clear that given sample of antiserum from antibodies directed against the red cells; (c) Haemolysis and haemagglutination titers after absorption, and (d) Effect of absorption with packed red cells on the antilymphocytic potency of the antiserum as tested by the lymphoagglutination titer.

(i) **The volume of packed red cells needed to absorb R.B.Cs. antibodies**

1. 10 ml. of the inactivated antiserum (heated at 56°C. for half-an-hour) were placed in each of four 20 ml. capacity bottles.

2. To each bottle a measured volume of packed washed red cells, in a red cells : antiserum ratio of 25%, 50%, 75% and 100% in that order, were to be

added (4/5ths before warm incubation and 1/5th before cold incubation). The red cells were added and the bottles were gently shaken and incubated at 37°C. for 1½ hours during which time they were shaken gently every ½ hour to allow admixture of their contents.

4. After the incubation period the remaining volume of red cells was added and the bottles were placed at 8°C. and were gently shaken ¼-hourly for one hour then left overnight.

5. The antiserum was then separated from the agglutinated red cells and centrifuged at 800 g. for 10 minutes.

6. The haemolysis/hemagglutination titers of the absorbed antiserum samples together with a non-absorbed control (that underwent the same procedure but without red cells addition) were then estimated.

(ii) **Effect of antiserum absorption on the antilymphocytic titer**

The antiserum samples' titers were tested by the lymphagglutination method (see *in vitro* studies).

1. **Antiserum produced in sheep**: was absorbed with packed red cells in the ratio of 4:1 (4 parts of antiserum to 1 part packed red cells) and absorption
carried out as described below but in smaller containers and sterilized either immediately after absorption or 24 hours later.

2. **Antiserum produced in the horse**: was absorbed in sterile M.R.C. blood bottles with metal caps and silicone rubber liners. Packed red cells were added to the antiserum in the ratio of 3:4 or in an equal volume. The red cells were slowly added to the antiserum by means of a 30 ml. capacity syringe with a wide bore needle to which was attached a 7 inch long polythene cannula. During this procedure the bottles were continuously shaken to allow the thorough mixing of the antiserum with the red cells. The bottles were then incubated in a water bath at 37°C for two hours during which time they were gently shaken and inverted a few times. This was carried out every 15 minutes to allow the red cells to be in contact with the antiserum during the whole period of incubation. At the end of the incubation period the bottles were inverted a few times, then put in the ice box (8°C.), and for the next 1-2 hours the bottles were gently shaken and inverted every 15 minutes then left thereafter overnight, to be shaken again 1-2 hours before removal from the ice box.
At the end of the cold agglutination period (the period in the ice box) the contents were poured in 100 ml. capacity centrifuge tubes and centrifuged at 300 g. for 10 minutes to sediment the agglutinated red cells and the supernatant antiserum was aspirated and recentrifuged at 800 g. for 15 minutes to clear the antiserum of contaminating agglutinated red cells. The clear antiserum was then bottled in medical flats of 4 oz. capacity and stored at -20°C. until further use.

11. Antiserum sterilization

All the procedures previously described were carried out with sterile equipment and glassware. To ensure that no bacterial contamination takes place during the serum preparation the horse antiserum was tested bacteriologically for contaminating micro-organisms both aerobically and anaerobically, which proved its sterility before as well as after sterilization.

Sterilization was carried out in a Millipore pyrex filter holder using Millipore filters, 47 mm. in diameter, with a filtering area of about 9.6 cm.²; pores size of 0.45 µ and 150 µ in thickness. Filtration was by the creation of vacuum. Occasionally prefiltration with microfibreglass prefilters

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* Cat.No. XX10 047 00. Millipore Filter Corporation, Bedford, Massachusetts, 01730, U.S.A.

** MF Type H.A.
(35 mm, diameter) were used with horse serum. Antisera were stored till further use.

12. Antiserum storage
All the antisera were stored at a temperature of -20°C until use. Antiserum produced in rabbits was stored in glass ampoules (5-20 ml. capacity); antiserum produced in sheep was stored in 24 ml. capacity bottles, and antiserum produced in the horse was stored in 100 ml. capacity medical flats. No antiserum batch used was stored for longer than two months.

13. Antiserum assay
(See in vitro studies).

14. Sera injection
The frozen stored antiserum was thawed in water, temperature not exceeding 40°C. The contents of the bottles were thoroughly mixed by inverting a few times. All injections were given intravenously in the cephalic vein in the forearm, using sterile 19 gauge needles after clipping the hair and swabbing the skin thoroughly with absolute alcohol.

15. Kidney grafting
Donor kidney preparation: Under Nembutal anaesthesia the operation area was thoroughly cleaned with Iodofores. The kidney was exposed through a midline abdominal incision from the xiphisternum to one inch below the umbilical scar, or by a
lumbar incision. The parietal peritoneal layer was incised along the whole length of the medial border of the kidney, which was then enucleated from its bed. The renal artery was then examined to ensure the absence of double arterial supply at its origin. Three inches of the ureter with its vascular supply was then freed from the surrounding tissue. The renal vein was first cleared from the surrounding fatty tissue from below the renal hilum till its termination, (and in the case of preparation of the left kidney the gonadal vein was ligated and cut). The artery was then cleared in the same manner from the sympathetic nervous bundle and surrounding fatty tissue till its origin. When the renal vessels were cleared, the kidney was ready for transplantation. The kidney was replaced in its original position and covered with an abdominal towel wetted with warmed sterile saline. As soon as the recipient site was prepared to receive the transplant the renal artery, then the vein, were ligated and cut. The ureter was then tied and cut three inches below the pelvi-ureteric junction. The ischaemia time was recorded from the time the arterial ligature was tightened. By the tip of a non-toothed forceps introduced into the opening of the renal artery, the opening was widened by allowing the forceps to open by its spring action. The kidney was then flushed with heparin saline mixture (15-30 I.U./ml) through its artery, using gentle
pressure and the kidney was then ready for anastomosis to the recipient's vessels.

Recipient preparation: The skin of the anaesthetised recipient dog was cleaned as previously described. Through a midline abdominal incision commencing 2 inches above the umbilical scar to the pubic symphysis, the abdominal contents were exposed, and the intestines were packed upwards by an abdominal towel moistened with sterile warm saline solution. The colon was retracted to the left side if the kidney was to be transplanted to the right iliac fossa (or to the right side if the transplantation was to be performed in the left iliac fossa). The trifurcation of the abdominal aorta was then identified and the parietal peritoneum covering the iliac vessels was cut medial to the gonadal vessels, and along the line of the iliac vessels. A bed was made for the kidney in the iliac fossa retroperitoneally. The adventitia of the iliac artery was cleared and the artery was clamped with two bulldog arterial clamps, one above (at its origin) and the other $1\frac{1}{2}$ inches distally. Next, the adventitia of the common and external iliac veins were cleared and the small venous tributaries were ligated. Using venous bulldog clamps, the flow in the iliac veins was obstructed by placing one clamp as low as possible on the external iliac vein and a second on the internal iliac vein (sometimes resort to its ligation was found to be necessary). A third clamp was
placed on the termination of the common iliac vein close to the inferior vena cava origin.

With fine scissors a longitudinal opening was made in the anterior wall of the common iliac vein, near the lateral border, and of a size equal to that of the transplant vein. Heparin saline mixture was used to flush the interior of the common iliac vein, and the excess was dried.

Anastomosis technique: The donor kidney was turned along its long axis so that the posterior surface faced anteriorly, thus bringing the renal pelvis anterior to the renal vessels. An end-to-side anastomosis of the renal to the iliac vessels was performed using sterilized, braided, silicon-treated, non-capillary, black silk sutures, number 6/0, with a 12 mm. atraumatic needle, 3/8ths of a circle, which were smeared with sterile liquid paraffin before use. An anchoring suture was passed through the wall of the common iliac vein and the renal vein, 1 mm. away from the free edge at the lower extremity of the openings of these veins. A similar suture was inserted at the upper extremity. As both ligatures were tightened and knotted, the kidney was approximated to the suture site to minimise the tension on the suture points. Commencing on the operator's side, and using the needle on the lower anchoring suture, the walls of both veins were anastomosed by a continuous

over and over suture passing through all the layers of the vessel's wall (care being taken not to include the opposite wall in the suture). The opposite side was done using the needle of the upper anchoring suture. With the completion of the venous anastomosis, a longitudinal opening of the dimensions of the renal artery was made in the anterior wall of the iliac artery near its lateral border, and the interior of the artery was flushed with heparin saline mixture. An everting, anchoring suture was then passed at the lower extremity of the opening in the iliac and renal arteries; a similar one was passed at the upper extremity. Each was then knotted thus anchoring the renal to the iliac artery.

Commencing on the operator's side, the walls of both arteries were anastomosed by a continuous mattress suture (everting) passing through all the layers of the vessel's wall, (taking care not to include the opposite wall in the suture). With the completion of the anastomosis, "Gel-Foam" was patched on the suture line and the distal venous; then the distal arterial clamps were removed, allowing retrograde filling of the vascular segments bearing the anastomoses. Gauze was then applied on both sides of the anastomoses and gentle pressure applied, while the proximal venous, then the proximal arterial, 

* Sterispon absorbable gelatine sponge: Allen & Hanbury's, London.
clamps were removed. The kidney was then seen to regain its colour and swell with the restoration of its arterial supply. The ischaemia time was then calculated from the moment of ligating the donor renal artery till the proximal arterial clamp was released. The ischaemia time varied between 35-47 minutes in the renal homografts reported in this thesis (except in one dog it was 75 minutes).

The distal end of the ureter was then slit open along its long axis for a distance of \( \frac{1}{2} \) cm. and a site was selected in the bladder for the ureterovesical anastomosis. The mucosa of the bladder was slit open after splitting the muscle layers along the axis of their fibres and forming a submuscular tunnel for the ureter. Three stay sutures were then placed to anchor the ureteric flap at its corners to the vesical opening, using plain 5/0 B.P. catgut sutures on a curved R/B 16 mm. atraumatic needle, and the anastomosis was completed with interrupted sutures. During the anastomosis attention was made not to intrude on the lumen of the ureter, and to ensure this, a probe was introduced in the ureter and only removed after placing the last suture in situ.

16. Nephrectomy: When nephrectomy was performed at the same time as the transplantation, it was carried out through the same incision, while if the lapse of days was allowed to pass

* Ethicon mersuture: Ethicon Ltd., Edinburgh, Scotland.
between the transplantation and the nephrectomy, it was performed through an oblique lumbar incision from the angle between the last rib and the iliocostalis muscle (a component of the "sacrospinalis" group of muscles) dorsally, extending downwards and ventrally for four inches. The external and internal oblique, and the transversus abdominis muscles, were split along the long axis of their fibres, and the peritoneum opened in line with the skin incision. The kidney was freed from its bed as previously described to expose its vascular supply. The renal artery was ligated by double ligatures, then the renal vein was treated in a similar manner after the kidney had emptied its blood content. Both vessels were cut. The kidney was then removed after ligating and cutting the ureter as distal as possible to its origin.

17. **Preparation and sterilization of instruments and equipment**

**Glassware:** was cleaned with detergent, then thoroughly rinsed with hot, cold, then distilled water. The medical flats (for the antiserum) were occasionally cleaned with chromic acid, then thoroughly rinsed with water before the use of detergent. All glassware was dried in the hot air oven, then:

1. Syringes were wrapped in kraft paper;
2. Tubes were covered with aluminium foil;
3. Bottles were covered with silicone rubber-lined metal caps;
4. Needles were packed in petri dishes (or were sterile
disposable);

5. Flasks and blood 'centrifuge bottles' (see before) were covered with a double layer of aluminium foil and a layer of kraft paper.

6. Pasteur pipettes were packed in a glass tube and covered with aluminium foil or non-absorbent cotton wool.

Sterilization was carried out at 160°C. for one hour in an electric oven with a fan (to ensure a homogenous temperature), or 165°C. for 1½ hours without a fan.

Nylon and polythene tubing were sterilized by boiling in water for 20 minutes.

Surgical instruments, gown, towels and gloves were sterilized by autoclaving for 30 minutes at a pressure of 15 lb./square inch.

Saline solution was sterilized by autoclaving for 30 minutes at a pressure of 15 lb/square inch.

In vitro glass tubes were cleaned with acid, then detergent tap water and finally distilled water, dried, but were left non-sterile and packed in nylon bags.

Millipore Filters and apparatus - The filters and the Pyrex filter holder were sterilized by autoclaving for 30 minutes at a pressure of 15 lb/square inch. The flasks were sterilized in hot air oven at 160°C. for one hour.
Animal Care

(i) Dogs: All dogs were previously vaccinated at least 2-3 weeks before use and housed separately in kennels carpeted with sawdust, and received "Dog Diet". Each dog received \( \frac{3}{4} \) lb. of that diet once a day at 3.30 p.m. either mixed with a \( \frac{1}{4} \) pint of milk or each given separately. Drinking water was supplied ad libitum. The dogs were exercised daily for half-an-hour.

A. Pre-operative management

Cannulation dogs: Two hours before cannulation the dogs were given a cup of milk, having had normal diet the preceding day.

Renal homograft recipients: were given \( \frac{1}{4} \) portion of diet but milk and water as usual, on the day preceding the operation. This semi-fasting was to lower the incidence of vomiting during and after the operation, especially with the antiserum therapy.

B. Post-operative care. The dogs were put on their side in their kennels on a sack after wiping the floor clear of sawdust. The endotracheal tube was removed as the anaesthesia started to wear off, to ensure a free air passage. Nothing was given by mouth till the dog was

* Spillers Ltd., Technological Research Station, Station Road, Cambridge.
well on its feet. A teacupful of water was first given in the morning if the dog had fully recovered, which was repeated at noon, and for dinner half a plate full of water (200 ml.) and a quarter portion of "Dog Diet" and milk.

On the second post-operative day normal feeding and drinking were commenced and the dogs were exercised for a short time.

(ii) Rats: Cannulated rats were housed in a restraining cage, which accommodates one rat, at room temperature and darkened surroundings and given rat cake and 10% glucose in 0.9% saline solution for drinking ad libitum, as described by Woodruff and Anderson (1964).

19. Experimental designs in dogs.

Experiment 1: Effect of rabbit-versus-dog antilymphocytic antiserum on the general condition and white blood cells.

Experiment 2: Effect of sheep-versus-dog antilymphocytic antiserum on the general condition, white blood cells and renal homograft survival.

Experiment 3: Effect of horse-versus-dog antilymphocytic antiserum on the general condition, white blood cells and renal homograft survival.
Experiment 1: Effect of rabbit-versus-dog antiserum on the general condition and white blood cells.

Four male mongrel dogs, weighing between 16 and 20 Kg. received small daily doses of 5-10 ml. rabbit-versus-dog antilymphocytic antiserum (R/DALAS) intravenously over the period of 2-3 months. A blood sample was withdrawn before each injection, for a white cell count (total and differential), haemoglobin percentage and haematocrit value estimation. The antiserum used had an antilymphocytic titer of 1/384 as estimated by the lymphoagglutination technique (see in vitro studies) and was sterilized by filtration, then heated at 56°C, for half-an-hour but not absorbed with dog red cells. In this group the following studies were carried out:—

(i) Study of the effect of small daily doses given over a prolonged period on the general condition of the dog;

(ii) Study of the effect of the small daily doses on the peripheral venous blood white cell counts;

(iii) Study of the effect of the antiserum on the red cells in vivo.

Animals Used

Rabbits: Immunized and boosted by the intraperitoneal injection of mongrel dog thoracic duct lymphocytes (see before) and the antiserum harvested
one week after the last immunizing or booster injection.

**Dogs:** Lymphocytes donors for the antiserum production were mongrel dogs of both sexes picked at random. The antiserum recipients were four male mongrel dogs (Nos. 534; 542; 555 and 557).

**Experiment 2:** Effect of sheep-versus-dog antilymphocytic antiserum on the general condition, white blood cells and renal homograft survival.

Sixteen mongrel dogs of both sexes, weighing 10.6 - 17.6 Kg and picked at random received intravenous injections of sheep-versus-dog antilymphocytic antiserum (S/DALAS) in a daily dosage of 3 ml/Kg body weight, till the day of death.

All the dogs were weighed before commencing the treatment and the dose calculated accordingly. This dosage/dog was maintained throughout the treatment period regardless of any gain or loss in weight. The daily dosage administered in this series ranged from 31-52 ml/day.

The antiserum used was heated at 56°C for half-an-hour, and was absorbed with dog red cells, then sterilized by filtration and stored at -20°C. It had an antilymphocytic titer of 1/192-1/384 as estimated by the lymphoagglutination technique (see in vitro studies).

The treatment was commenced at various intervals before,
or on the same day of renal homotransplantation. A blood sample was withdrawn before the antiserum injection for a white cell count (total and differential), haemoglobin percentage, haematocrit value and blood urea estimations.

In this group the following studies were carried out:

(i) Study of the effect of the antiserum when administered in large single daily doses on the general condition of the dogs;
(ii) Study of the effect of the antiserum on the peripheral venous blood white cell counts;
(iii) Study of the effect of the antiserum on the red cells in vivo;
(iv) Study of the effect of the antiserum on the renal homograft survival.

Protocol:

Renal homografting and bilateral nephrectomy were performed on day 0. Antiserum therapy was commenced on day -7 in nine dogs; on day -6 in 1 dog; on day -2 in 3 dogs; on day -1 in 3 dogs, and on day 0 in 1 dog. The antiserum was administered as a single daily injection of 3 ml/Kg. body weight till the day of death.

Animals used:

Sheep: These were immunized and boosted by the intra-peritoneal injection of thoracic duct lymphocytes as previously described and the antiserum harvested one
week after the last immunizing or booster injection.

**Dogs:** All the dogs used in this experiment were mongrel dogs of both sexes picked out at random.

**Experiment 3:** Effect of horse-versus-dog antilymphocytic antiserum on the general condition, white blood cells and renal homograft survival.

Eighteen beagles (17 males and one female) weighing between 11.5 and 17 Kg. were used in this series. Eight received horse-versus-dog antilymphocytic antiserum, five received normal horse serum and five received no treatment.

Eight male beagles weighing between 11.5 and 15.5 Kg. received intravenous injections of horse-versus-dog antilymphocytic antiserum (H/DALAS) in a single daily dose of 3 ml/Kg. body weight for 60 days, then half the initial calculated dose for a further 20 days. All the dogs were weighed before the commencement of the treatment and the daily dose was calculated accordingly. This dosage was maintained regardless of any gain or loss in weight.

The antiserum used was heated (at 56°C. for half-an-hour), absorbed with dog red cells, and stored at -20°C. The sterile, heated and absorbed antiserum injected was of a standard antilymphocytic titer of 1/768 as estimated by the lymphoagglutination technique (see in vitro) and had a haemoagglutination
titer of not more than 1/3. Each batch of antilymphocytic antiserum, absorbed and non-absorbed, was assayed for its antilymphocytic titer using mongrel dog thoracic duct lymphocytes by the lymphoagglutination technique. The absorbed antiserum batches were also assayed for their haemagglutination titers before their use for therapy. The absorbed and non-absorbed antiserum batches occasionally underwent full bacteriological examination (aerobic and anaerobic culture) to ensure their sterility.

Protocol:

A. Group receiving antiserum: The day of renal homotransplantation was taken as day 0; the antiserum treatment was commenced on day -1 (Fig. 9).

Day -1:

(a) The dog was accurately weighed.

(b) A blood sample was withdrawn for the following analyses - a white cell count (total and differential); haemoglobin percentage; blood urea; plasma uric acid, creatinine, sodium, potassium.

(c) The antiserum was injected at noon in the dose calculated from the weight.

(d) At 4 p.m. a blood sample was withdrawn for a white cell count (total and differential).
Day 0:

(a) A blood sample was withdrawn at 9 a.m. for a white cell count (total and differential) and haemoglobin percentage estimation.

(b) Renal homotransplantation and a right nephrectomy were performed.

(c) The antiserum was injected after the effect of anaesthesia started to wear off whilst the endotracheal tube was still in situ.

Day +21:

(a) A blood sample was withdrawn for complete blood analysis (as described on day -1).

(b) Left nephrectomy was performed.

(c) The antiserum was injected after the effect of anaesthesia started to wear off with the endotracheal tube still in situ.

Day +59: The antiserum dosage was halved (half the initial dose) and given daily for a further 20 days.

Day +79: No more injections.

During the whole course of the antiserum treatment and afterwards the investigations carried out on day -1 were repeated at intervals.
EXPERIMENTAL PROTOCOL

H-antiserum therapy full dose for 60 days.

- Renal transplant
- Left nephrectomy
- Antiserum therapy
- Full dose for 60 days

No injections

Fig. 9.
In this group the following studies were carried out:—

(i) Study of the effect of the horse-versus-dog anti-lymphocytic antiserum given in large single daily doses on the general condition of the dogs.

(ii) Study of the effect of the antiserum on the white cell counts performed on the peripheral venous blood.

(iii) Study of the effect of the antiserum on the red cells in vivo.

(iv) Study of the effect of the antiserum on the renal homograft survival performed one day after commencing the antiserum therapy.

(v) Histological studies on the effect of the antiserum on renal homotransplantation.

(vi) Study of the effect of the antiserum on the lymphoid organs.

(vii) Histological studies on the effect of the antiserum given for three weeks on the normal kidney.

(viii) Histological studies on the effect of the antiserum on the body tissues.

B. Control groups

1. Normal horse serum group: Five beagles (all males)
received sterile horse serum (heated at 56°C for half-an-hour) according to the protocol described for the antiserum treated dogs. On day 0 a renal homotransplant and a left nephrectomy were performed and on day +21 a right nephrectomy was carried out. The investigations were carried out as for the antiserum group.

2. No treatment group: Five beagles (four males and one female) each had a renal homograft performed on day 0 together with a left nephrectomy, and on day +21 a right nephrectomy was carried out. The investigations were carried out as for the antiserum group. Two dogs from each of the control groups died before the last nephrectomy.

Animals used

Horse: Immunized and boosted by the intravenous injection of beagle thoracic duct lymphocytes as previously described. Bled one week after the last immunizing or booster injection.

Dogs: All dogs used in this series were beagles of which 9 males contributed their thoracic duct lymphocytes for the antiserum production. The recipients of renal homotransplants and their kidney donors were picked at random from the beagle stock and were of non-identical hair colour except on one occasion.
20. **Investigations**

(i) **Total white cells counts:** performed on samples of venous blood taken in a Lithium heparin tubes from the cephalic vein in the forearm.

(ii) **Differential white blood cells counting:** was carried out on a Leishman stained blood film by the method of Woodruff and Forman (1950). The absolute granular and lymphocyte counts/cmm were calculated and recorded.

(iii) **Haemoglobin percentage** was estimated by the Sahli Haemoglobinometer.

(iv) **Blood urea** was estimated by the sodium hypobromide method as described by van Slyke (Peters and van Slyke 1932).

(v) **Serum sodium and potassium** were measured photometrically using an E.E.L. flame photometer.

(vi) **Plasma creatinine** was estimated on the autoanalyser.

(vii) **Plasma uric acid** was estimated by a modification to the method of Eichorn et al (1961).

(viii) **Urine:** A mid-stream sample was routinely tested for the presence of proteins.

(ix) **Histological specimens** were taken mostly at post-

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* Stayne Laboratories Limited.
mortem and fixed in formal saline (with the exception of lymph nodes which were fixed in Bouin's fluid) and in formal alcohol; sections were then stained with haematoxylin and eosin, and by the Unna Pappenheim stain.

**Tissues examined**

**In the chest:** Thymus, heart, both lungs, mediastinal lymph nodes.

**In the abdomen:** The renal transplant, stomach, duodenum, intestine with Peyer's patches, appendix, colon, mesenteric lymph nodes, spleen, liver, pancreas, bladder, lymph node near the renal transplant (the right iliac group).

(x) **Post-mortem examination** was performed as soon as it was feasible, with complete examination of the chest and abdomen. The skull, joints and marrow were not examined.
PART IV
RESULTS

"As it is the ultimate end of philosophy to investigate the truth, so it must afford the greatest pleasure to generous minds to embrace it, in whatever form they find it."

Magnus Falconar
1777

Experiment 1: Effect of rabbit versus dog antilymphocytic antiserum on the general condition and white blood cells.

(i) Effect on the general condition

The four dogs used did not seem to mind receiving the antiserum, were lively during the experiment period, and gained slightly in weight (about 1 Kg.). The injections were followed by vomiting 1-3 times and a bowel motion. While the former manifestation subsides in one week, a bowel motion was the rule after each injection. No other reactions were observed.

(ii) Effect on the peripheral blood lymphocytes

To study the effect of the daily antiserum injections (titer 1/384) on the peripheral blood lymphocytes and granular cells, a blood sample was collected before each injection for
total and differential counts.

Twenty-four hours after the first intravenous injection of 5, 10 ml., or 10 ml. in two divided doses, the absolute lymphocyte count dropped to a varying degree ranging from 15.9 – 35.8% of the pre-injection value. The injection of 5 ml. resulted in a drop in the absolute lymphocyte counts to 35.8% and 20.1% of the pre-injection value in two dogs (Nos. 542 and 557). With 10 ml. administered in two divided doses, 5 ml. at 9 a.m. and 5 ml. at 5 p.m. (dog No. 534), the count dropped to 29.8% of the pre-injection value, while with 10 ml. in a single dose (dog No. 555), the count dropped to 15.9% of the pre-injection absolute lymphocyte count. The average drop for the two dogs receiving 5 ml. was to 29.4%, while that for the two dogs receiving the 10 ml. was to 20.8% of the average pre-injection value.

After the initial drop, the absolute lymphocyte count rose gradually over the period of 2-3 days to reach the normal pre-injection level, in three dogs, then dropped again and remained below the normal value till day 11. One dog (No. 534) that received 5 ml. twice daily maintained its lymphopenia during this period. After this initial lymphopenia the count of three dogs rose on day 12 to show a peak of a variable intensity on about day 14, reaching 3-4 times the original normal pre-injection value. During the next week the count remained above the normal level, registering peaks of varying intensities. One dog (No. 555) showed a count that was maintained below the
pre-injection normal value during the first 76 days of the experiment. Figure 10 represents the whole course of the lymphocyte counts and also shows that doubling or quadrupling the antiserum dose had no marked effect on the count.

From these results it is seen that although an initial lymphopenia followed the daily antiserum administration this state was not maintained despite the continued injections and doubling or quadrupling the dosage. The rise of the count to high levels during the third week of antiserum administration manifesting in three of the four dogs is most striking.

(iii) **Effect on the granular leukocytes in the peripheral blood**

Twenty-four hours after the first antiserum injection the reaction of the granular leukocytes was variable. One of the two dogs that received 5 ml. of antiserum, showed a drop of 25% and the other a rise of more than 100%. The dog that received 10 ml. in two divided doses did not show a change in the count. The dog that received 10 ml. in a single dose showed a rise in the granular cells count of about 50%.

The further course of the granular cells (Fig. 11) was variable. On the whole the count showed no striking abnormality except in one dog (No. 557) which showed a marked rise commencing after nine injections.
THE EFFECT OF SMALL DAILY INTRAVENOUS INJECTIONS OF RABBIT X DOG ANTILYMPHOCYTIC ANTISERUM (R/DALAS) ON THE PERIPHERAL BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.

Fig. 10.
THE EFFECT OF SMALL DAILY INTRAVENOUS INJECTIONS OF RABBIT V DOG ANTILYMPHOCYCTIC ANTISERUM (R.DALAS) ON THE PERIPHERAL BLOOD ABSOLUTE GRANULAR LEUCOCYTES IN THE DOG.

Fig. II.
Experiment 2: Effect of sheep versus dog antilymphocytic antiserum on the general condition, white blood cells and renal homograft survival.

The antiserum was injected intravenously in a daily dose of 3 ml/kg body weight. The first blood counts were performed twenty-four hours after the injection as a routine. Counts were performed also after four hours in five of the sixteen dogs used.

(i) Effect on the general condition: All the dogs receiving this large daily dosage manifested the same signs as those that received small doses of antiserum prepared in rabbits, i.e., vomiting and a bowel motion. Vomiting 1-3 times immediately followed the first antiserum injection. This reaction recurred after each of the injections then subsided. A bowel motion following each injection was manifested throughout the therapy course. Besides these signs the dogs were all lively and did not seem to bother about the injections. Regarding the body weight most dogs tended to lose rather than gain in weight. One dog (No. 712) manifested gastro-intestinal bleeding after six antiserum injections, but survived the experiment, and another (No. 690A) manifested a severe form of such bleeding which resulted in its death after six doses of antiserum (one day prior to homografting). The source of the bleeding could not be identified on post-mortem examination.

(ii) Effect on the peripheral blood lymphocytes: Four hours after the antiserum injection (titer 1/192 - 1/384) the count of five dogs dropped to a varying degree ranging from 6.7% - 34% of
the pre-injection value, with an average drop to 21.2%.

Twenty-four hours after the injection the count recorded in fourteen dogs showed a wide range of variation (Fig. 12). The range was 16-94.7% with an average of 62.9% of the pre-injection value. Five of the fourteen dogs showed, twenty-four hours after the injection, a maintained lymphocyte suppression to below 50%; of these only one dog showed a maintained drop to 16% of the pre-injection value.

With the daily injections the count after an initial period of suppression of a varying degree reached the pre-injection level within 2-6 days, to rise above that level for a variable period of time (Fig. 13). The rise was most prominent after 8-18 injections, to fluctuate thereafter till the day of death. From Fig. 13 it is seen that there is no relation between the degree of the initial depression in the absolute lymphocytes count and the renal homograft survival.

Figure 14 shows the course of the lymphocyte count of the eight dogs that received the antiserum for one week before grafting and daily thereafter. It is seen that only a temporary moderate depression of the count was achieved during the first few days, after which the average count rose to remain at or above the pre-injection value.

(iii) Effect on granular leukocytes in the peripheral blood:

Four of the five dogs examined four hours after the injection, showed a drop in the count, the degree of which ranged from 40.6 - 86.2% of the pre-injection level, while the
THE EFFECT OF SHEEP V DOG ANTILYMPHOCYTIC ANTISERUM (S/DALAS) 3ml/Kg BODY WEIGHT ON THE ABSOLUTE LYMPHOCYTE AND GRANULAR CELL COUNTS IN THE PERIPHERAL VENOUS BLOOD 24 HOURS AFTER THE INTRAVENOUS INJECTION IN DOGS.

Fig. 12.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF SHEEP V. DOG ANTILYMPHOCYTE ANTISERUM (S/DALAS) 3%/KG BW BEFORE AND AFTER RENAL HOMOGRAFTING ON THE PERIPHERAL BLOOD ABSOLUTE LYMHPHOCYTE COUNT IN DOGS.

FIRST ARROW INDICATES TIME OF COMMENCING THERAPY.
SECOND ARROW INDICATES TIME OF DEATH.
O IS DAY OF RENAL HOMOGRAFTING AND BILATERAL NEPHRECTOMY.

Fig. 13.
THE EFFECT OF DAILY INTRAVENOUS INJECTION OF SHEEP V DOG ANTILYMPHOCYTIC ANTISERUM (S/DALAS) 3ml/kg B.W. ON THE ABSOLUTE LYMPHOCYTE COUNT IN THE PERIPHERAL VENOUS BLOOD BEFORE AND AFTER RENAL HOMOTRANSPLANTATION IN DOGS.

Fig. 14.
remaining dog showed a rise to almost double the original value.

After twenty-four hours eleven of the fourteen dogs manifested a rise in the granular cells of a varying degree, the remaining three showed a slight drop (Fig. 12).

Figure 15 shows the whole course of the granular leukocytes on daily antiserum injections commencing on day -7 till the day of death. All the dogs showed a rise in their counts with the exception of two dogs showing a transient drop during the fourth and fifth injection.

(iv) Effect on red cells: The haemoglobin percentage estimated at intervals was taken to reflect the effect of the antiserum on the red cells. Despite the absorption of the antiserum with packed red cells to lower its haemoagglutinating titer from 1/512 to 1/8, there was a drop in the haemoglobin percentage of a variable degree throughout the therapy period. The haemoglobin percentage lowest values in these homografted dogs reached 4.0%.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF SHEEP V DOG ANTILYMPHOCYTIC ANTISERUM (S/DALAS) 3 ml/Kg BW BEFORE AND AFTER RENAL HOMOGRAFTING ON THE PERIPHERAL BLOOD GRANULAR CELLS IN THE DOG.

ABSOLUTE GRANULAR CELL COUNT
x 1000/c.mm

Fig. 15.
TABLE I: Effect of antilymphocytic antiserum (sheep-versus-dog) on renal homografts.

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Injection commenced</th>
<th>Sex</th>
<th>Survival (days) after transplantation</th>
<th>B.U. mg/100 ml.</th>
<th>Cause of death</th>
<th>P.M. appearance of transplant</th>
<th>macroscopic</th>
<th>microscopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>718</td>
<td>-1</td>
<td>F M</td>
<td>7</td>
<td>640</td>
<td>Uraemia.</td>
<td>Normal.</td>
<td>Round cells ++, Red cells ++</td>
<td></td>
</tr>
<tr>
<td>752</td>
<td>-2</td>
<td>M M</td>
<td>18</td>
<td>292</td>
<td>Enlarged ++, Patent anastomoses.</td>
<td>As 732 but plasma cells +++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>673</td>
<td>-7</td>
<td>M M</td>
<td>10</td>
<td>197**</td>
<td>Normal partial arterial thrombus.</td>
<td>Necrosis +++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>679</td>
<td>-7</td>
<td>F F</td>
<td>10</td>
<td>440**</td>
<td>Uraemia.</td>
<td>Normal.</td>
<td>As 760.</td>
<td></td>
</tr>
<tr>
<td>690A</td>
<td>-7</td>
<td>F F</td>
<td>10</td>
<td>G.I.Hge.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>701</td>
<td>-7</td>
<td>M M</td>
<td>4</td>
<td>570</td>
<td>Uraemia.</td>
<td>Normal.</td>
<td>As 760.</td>
<td></td>
</tr>
<tr>
<td>712</td>
<td>-7</td>
<td>F F</td>
<td>13</td>
<td>830</td>
<td>Uraemia.</td>
<td>Normal.</td>
<td>As 832.</td>
<td></td>
</tr>
<tr>
<td>761</td>
<td>-7</td>
<td>F F</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Normal.</td>
<td>As 760.</td>
<td></td>
</tr>
</tbody>
</table>

* 3 days before death
** 6 days before death

R = recipient; D = donor; F = female; M = male;
G.I.Hge. = gastrointestinal haemorrhage;
R.B.Cs. = red cell extravasation; B.U. = blood urea;
+, ++, +++ = slight, moderate, extensive.
(v) **Effect on renal homograft survival:**

Table I summarises the results of the homograft survival, and Fig. 16 shows the blood urea level of the three long surviving dogs. The table shows that in the group that received the antiserum on day -1 the survival period was 6, 7 and 8 days with an average of 7 days; those receiving the antiserum on day -2 it was 3, 4 and 18 days with an average of 8 days, while those receiving antiserum on day -7 the survival period was 2, 4, 4, 10, 10, 13 and 13 days with an average of 8 days. The dog that received the antiserum on day 0 survived for 3 days and the dog that received the therapy on day -6 died one day after grafting. The longest survival (dog No. 752) in the 15 grafted dogs was for 18 days. It commenced the antiserum treatment on day -2.

In this series of 16 dogs one dog died of severe bleeding per anum after 6 antiserum injections and one day prior to grafting; of the remaining 15 dogs that received a renal homograft the longest survival was 18 days (dog No. 752). This dog commenced the antiserum treatment on day -2 and daily thereafter. Another dog died with a macroscopically normal kidney due to slipping of the posterior anchoring ligature of the uretero-vesical anastomosis, resulting in urine leakage into the peritoneal cavity and thus uraemia.

(vi) **Histological picture:** The homograft reaction varied over a wide range from slight to extensive cellular infiltration, red cell extravasation, tubular necrosis and pyroninophilic cells accumulation. Table I summarizes the salient features. No correlation between the degree of the homograft reaction and the survival period of these dogs is markedly apparent. Fig. 17 shows the histological picture of the renal homograft of dog No. 752 (18 days survival).
THE EFFECT OF DAILY INTRAVENOUS INJECTION OF 3ml/kg B.W. SHEEP V DOG ANTILYMPHOCYTIC ANTISERUM (S/DALAS) ON THE FUNCTION OF CANINE RENAL HOMOGRAFTS TAKING THE BLOOD UREA AS THE INDEX OF RENAL FUNCTION.

Fig. I6.
Fig. 17.
Post-mortem section of the renal homograft of dog No. 752, which received sheep v dog anti-lymphocytic antiserum, and died 18 days after transplantation. Section shows a marked homograft reaction. H. and E. x 130.
Experiment 3: Effect of horse versus dog antilymphocytic antiserum on the general condition, white blood cells and renal homograft survival.

Eighteen dogs were used and were divided into three groups; eight received horse versus dog antilymphocytic antiserum (H/DALAS); five received normal horse serum and five received no treatment. The sera were injected intravenously in a dose of 3ml/Kg. body weight. All eighteen dogs received a renal homotransplant on day 0. Two from each of the control groups (normal horse serum and no treatment groups) died before the completion of the investigation but will be mentioned for their interesting findings.

Five more dogs received antiserum; three were a technical failure, one was an anaesthetic death on day 0 and one had severe ear infection that was missed before the antiserum therapy. These dogs will be excluded from the analysis of the results except for the lymphocyte count of the first four, which is included in Figure 19.

(1) Effect on the general condition
   (a) Effect of the antiserum (H/DALAS):

   All the dogs (100%) showed a reaction immediately after the first injection. I will refer to it (though without implying any particular view of its nature) as the "primary serum reaction." This manifested as follows - drowsiness and staggering gait with eventual lying down within five minutes from the end of the injection (which was given very slowly). This was followed within a further five minutes by salivation, retching, vomiting (once or twice), emptying of the bladder (with some), and a bowel
motion. Thereafter all the dogs lay very quietly in their kennels and vomited 4-6 times during the next six to eight hours. By the next morning they had completely recovered. Within 15 minutes from the end of the injection in one dog examined (No. 784), the rectal temperature showed a slight rise 1°F over the pre-injection value, then after returning back to normal within a further 15 minutes it dropped gradually to reach its maximum drop of 8.5°F in another ½ hour. Four hours after the injection the temperature was back to its normal pre-injection level. Regarding the bowel motion, this, in association with salivation and water drinking, recurred throughout the whole course of the experiment, manifesting after each injection.

The next set of manifestations were shown by 25% of the dogs (dog Nos. 799 and 801), and will be referred to (again without implying any particular view of its nature) as the "Secondary serum reaction". These manifestations appeared after the eleventh and thirteenth injections respectively (day +9 and day +11 in the protocol outlined previously - see material and methods). They appeared immediately after the antiserum injection and were similar to the "primary" manifestations but were associated with dyspnea. Within half-an-hour the dogs gradually regained their normal activity but were somewhat calmer than usual. This "secondary serum reaction" manifested from the time of its onset, only after the injection, for six days (dog No. 799), and for two days (dog No. 801).
Therafter it subsided and never recurred. All the dogs that did not develop this reaction during the first two weeks of the antiserum therapy maintained this state up to the day of their death.

At a variable time interval which coincided with the day prior to, or the same day, of death, three dogs showed macroscopically bright blood in the faeces, two of these (dogs Nos. 784 and 804) died from sudden massive gastro-intestinal bleeding, while the third (No. 799) died from uraemia. Only one dog (No. 784) showed haematuria with blood clots in the urine after 60 injections (59 days after transplantation) and lasted for one day (blood urea 209 mg/100 ml.); it died from gastro-intestinal bleeding (after 74 injections).

All the dogs showed a tendency to lose weight during the treatment period. Three weeks after commencing the antiserum therapy the weight loss ranged from 0.5 - 3.2 Kg. and by the end of the sixth week the loss ranged from 3 - 3.6 Kg. (according to the original weight before treatment). After this period of time the tendency was either to remain at the same weight or to lose a further 0.5 - 1 Kg.

Apart from the "primary" and "secondary serum reactions" all the dogs were active (except dog No. 784 which became quieter and more timid) and enjoyed normal health until a few days before death, when they were taken ill (see later - cause of death).
Effect of normal horse serum:

One of the five dogs injected (20%) showed a very mild form of the "primary serum reaction" by retching and vomiting and within half-an-hour returned to its normal state. A bowel motion and water drinking were manifested by all the dogs after the injection and were as active as normal during the next twenty-four hours. The bowel motion and water drinking, together with salivation, were constantly observed after each injection.

All the injected dogs (100%) showed the "secondary serum reaction" after 7-8 injections (day +5 to day +6 in the protocol). This recurred after each injection for 3-5 days. It later re-appeared in two of the three surviving dogs after a total of 15-16 injections to persist only for a day, after which time it never recurred.

Initially five dogs received normal horse serum; two of these died within twenty-four hours from manifesting the "secondary serum reaction"; one after developing severe gastrointestinal bleeding and the other from no apparent cause. On post-mortem examination both dogs had an intra-peritoneal haemorrhage. One of the remaining three dogs developed gastrointestinal bleeding on two occasions, once after the tenth injection (the fourth and last day of the "secondary serum reaction"), and again after the fifteenth injection on the recurrence of this reaction. All the dogs showed a tendency to lose weight which ranged from 1.2 - 3.9 Kg. in three weeks.
(c) **Effect of no treatment:**

None of these dogs manifested any bleeding tendency or symptoms identical to the "serum reactions". All showed a tendency to lose weight, which ranged from 2-5 Kg. in three weeks (during which time they had, besides the renal homograft, one of their own kidneys still in situ).

(ii) **Effect on the peripheral blood lymphocytes**

(a) **Effect of the antiserum:**

Figures 18 and 19 show the effect (after four and twenty-four hours) of a single intravenous injection of the antiserum on the peripheral venous blood absolute lymphocyte count. Fig. 18 also shows that no relationship exists between the degree of initial drop in the count and the survival of the renal homograft.

Four hours after the antiserum injection (titer 1/768) the absolute lymphocyte count dropped to a varying degree ranging from 3.2% - 40.3% of the pre-injection value with an average drop to 13.2%. Five of the twelve dogs initially injected showed a drop in their count to lower than 10% (of these only two showed a drop to below 5% of their pre-injection value).

Twenty-four hours after the injection the absolute lymphocyte count showed a wider range of variation, with a drop to 6.9% - 70.9% with an average drop to 34.1% of the pre-injection value.

The further effect of the daily antiserum injections on the absolute lymphocyte count after renal homotransplantation is shown in Figures 20-27 which show the response of each dog.
THE EFFECT OF HORSE V. DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg BW ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT 4 AND 24 HOURS AFTER A SINGLE INTRAVENOUS INJECTION, AND ITS RELATION TO RENAL HOMOGRAFT SURVIVAL TIME IN DOGS. (TITER 1/768)

**Fig. 1B.**
THE IMMEDIATE EFFECT OF A SINGLE INTRAVENOUS INJECTION OF HORSE V. DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg B.W. ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN DOGS.

Fig. 19.
Since the dogs used were not isogeneic it is best to consider each dog separately to start with.

**Dog No. 784**: Four hours after the first injection the absolute lymphocyte count dropped to 35.7% of the pre-injection value, to continue dropping slightly for a further 48 hours to 27.6%. The count rose to the pre-injection value after nine antiserum injections (day +3), then dropped to rise again with a peak after 16 injections (day +15). The further course of the count is shown in Figure 20. It is noticed that after 69 injections (of which the last nine were half the initial dose), i.e., day +68, the lymphocyte count was still capable of reaching the pre-injection value and slightly exceed it.

The suppression of the peripheral blood lymphocytes was only of a slight degree and was not maintained.

**Dog No. 799**: Four hours after the first injection the absolute lymphocyte count dropped to 40.3% of the pre-injection value, to rise after 24 hours and re-drop after the second injection, to fluctuate at about 30% of the original value to show a peak after 14 injections (day +13), then it dropped again and remained, after 28 injections (day +27), at or below 30% of the original count and was maintained at such a level for a further 19 days (Figure 21) till the day of death on day +45.

**Dog No. 801**: Four hours after the first injection the lymphocytes dropped to 16.8% of the pre-injection level to rise and re-drop after the second injection and continue at a low level
till after 16 injections (day +15) when a peak showed and lasted for four days (day +15 to +18) (Figure 22), after which the count dropped over seven days; the dog died from uraemia on day +27 with a non-functioning homograft.

**Dog No. 804:** Four hours after the antiserum injection the lymphocytes dropped to 8.9% of their pre-injection value to rise by 24 hours, re-drop on the second injection, then re-rise to 50% of the normal level. Thereafter the count remained at a low level (Figure 23) till the day of death (+25) with a functioning homograft.

**Dog No. 807:** Four hours after the first injection the absolute lymphocyte count dropped to 11% of the pre-injection value to rise again, then re-drop after the second injection. After 21 injections (day +20) the count was above the normal level and continued rising to a peak of three times the pre-injection level (day +23), to drop below the pre-treatment value on day +27 and onwards (Figure 24). The dog died on day +39 with a functioning homograft.

**Dog No. 813:** Showed a drop to 4.8% of pre-treatment count four hours after the first injection with a slight rise afterwards, then a drop maintained at a low level till after 18 injections (day +17) a small peak was apparent which soon disappeared within a further two days. The count was maintained thereafter at a very low level (Fig. 25) till the day of death (day +45) with a normally functioning renal homograft.
Dog No. 615: Four hours after the first antiserum injection the lymphocyte count dropped to 19.2% of the pre-injection level, then rose over a period of 48 hours. The count showed two small peaks, one after 9 injections (day +8), and one after 22 injections (day +21); both were of short duration and below the original count. The count remained at a suppressed level throughout the treatment course and especially so after 62 injections, to continue at such low level for a further 19 days (Fig. 26) till the day of death (day +79) with a normally functioning homograft.

Dog No. 818: The absolute lymphocyte count four hours after the first antiserum injection dropped to 16% of the pre-injection value, after which a rise was recorded. The count remained at a suppressed level during the remaining period with signs of attempted recovery at variable intervals, after which it dropped to very low levels. After 60 injections (day +59), and after 67 injections (day +66), the absolute lymphocyte counts/cmm. were 92 and 73 cells, i.e., 3.4 and 2.7% of the pre-injection value respectively (Fig. 27). The dog died on day +69 with a normally functioning homograft.

Figs. 28 and 29 show the lymphocyte count of the whole group.

From these results it is seen that only two dogs (Nos. 764 and 807) showed an absolute lymphocyte count that exceeded their pre-injection value at any time during the antiserum administration. The first dog (No. 784) showed a rise of
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE V. DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg BW BEFORE AND AFTER RENAL HOMOGRAFTING ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.

Fig. 20.

THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE V. DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg BW BEFORE AND AFTER RENAL HOMOGRAFTING ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.

Fig. 21.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE V. DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg B.W. BEFORE AND AFTER RENAL HOMOGRAFTING ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.

Fig. 22.

Fig. 23.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE V. DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg B.W. BEFORE AND AFTER RENAL HOMOGRAFTING, ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE V DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg B.W. BEFORE AND AFTER RENAL HOMOGRAFTING, ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.

Fig. 26.

THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE V DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg B.W. BEFORE AND AFTER RENAL HOMOGRAFTING, ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.

Fig. 27.
Fig. 28: The effect of daily intravenous injection of horse-versus-dog antilymphocytic antiserum (3 ml/Kg. B.W./d.) before and after renal homografting, on the peripheral venous blood absolute lymphocyte count of 8 dogs. The figure represents figures 20-27 inclusive, for comparison.
Fig. 28.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE V. DOG ANTILYMPHO_CYC'T ANTISERUM (H/DALAS) 3ml/Kg B.W. BEFORE AND AFTER RENAL HOMOGRAFTING ON THE PERIPHERAL VENOUS BLOOD ABSOLUTE LYMPHOCYTE COUNT IN THE DOG.

Fig. 29.
lymphocytes/cmm. on days +14 and +15 (with the peak on day +15), after which period it dropped to, below the pre-injection value but still remained at an appreciably high level. The lymphocyte count of dog No. 807 showed a significant rise above the pre-injection value on days +19 to day +26, with a peak on day +23 of 9360 cells/cmm., i.e., about three times the pre-treatment value. After that period the count fell to below the original level till the time of death.

Almost all the remaining dogs showed a rise in the count of a varying intensity and duration during the period between +14 and +23 days, but not exceeding the pre-injection level or even the pre-injection mean value (of 4921 cells/cmm.) for the whole group.

(b) Effect of the normal horse serum:

Fig. 30 shows the effect of normal horse serum on the absolute lymphocyte count. Four hours after the injection the count was at, or slightly above, the pre-injection value. One dog showed a slight drop after four hours, and another showed a similar drop after 48 hours. During the remaining period the count remained at about the original value, to drop before death from uraemia.

(c) Effect of no treatment:

The absolute lymphocyte count in dogs that received a renal homotransplant without any form of therapy remained above or within the pre-grafting value.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF NORMAL HORSE SERUM (N.H.S.) 3ml/Kg. B.W. ON THE PERIPHERAL BLOOD ABSOLUTE LYMPHOCYTE COUNT AND THE BLOOD UREA BEFORE AND AFTER RENAL HOMOTRANSPLANTATION IN DOGS.

Fig. 30.
It is seen that normal serum has no effect on the lymphocyte count as compared with the antiserum. The drop in the count is genuinely due to the antilymphocytic property of the antiserum.

3. **Effect on granular leukocytes**

(a) **Effect of the antiserum:**

Four hours after the injection the granular leukocytes tended to drop to 59.2% - 90.1% of the original value in six of the twelve dogs initially injected; the remaining showed a rise of a variable degree above the pre-injection value.

Twenty-four hours after the injection the tendency for the granular cells was to rise, in seven of the nine dogs examined, for a variable degree. Of the remaining two dogs one showed a drop to 34.7% and the other to 81.6% of the pre-injection value.

The later course of the granular cells is shown in Fig. 31.

(b) **Effect of normal horse serum:**

Four hours after the injection the granular cells count rose to a variable degree in all the dogs. The further course of the granular cells during the course of the experiment period is shown in Fig. 32.

(c) **Effect of no treatment:**

The granular cells were above the original level till the day of death.

It is seen that the antilymphocytic antiserum did not affect the granular cells to any marked degree as it did with
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF HORSE Y DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg BW. BEFORE AND AFTER RENAL HOMOGRAFTING, ON THE PERIPHERAL BLOOD GRANULAR CELLS IN THE DOG.

Fig. 31.
THE EFFECT OF DAILY INTRAVENOUS INJECTIONS OF NORMAL HORSE SERUM (N.H.S.) 3 ml/Kg. B.W. BEFORE AND AFTER RENAL HOMOGRAFTING ON THE PERIPHERAL BLOOD GRANULAR CELLS IN THE DOG.

Fig. 32.
lymphocytes. After the post-operative rise the granular
cells were on the whole within the normal range with occasional
exceptions.

4. **Effect on the red cells**
   (a) **Effect of the antiserum:**
   The effect of absorbed antiserum on the red cells was
only tested by the haemoglobin percentage. After a primary drop
following the transplantation, there was a tendency towards a
rise but throughout the experiment period the haemoglobin
percentage ranged between 40 and 80% (normal pre-injection
range 80-100%). Though the antiserum was absorbed to a
haemoagglutinating titer of 1/8 the drop in the haemoglobin
percentage was quite noticeable.

(b) **Effect of normal serum:**
   All the dogs showed a drop in the haemoglobin
percentage to 50-60% (normal pre-injection range 90-100%)
during the experiment period though the injected serum showed no
demonstrable haemoagglutinins *in vitro*.

(c) **Effect of no treatment:**
   Showed similar findings to the preceding group. One
of the dogs showed severe spontaneous agglutination of all the
blood samples taken during the third post-operative week, at the
end of which time the dog died one day prior to the second
nephrectomy.

5. **Effect on renal homograft survival**
   The protocol used (see material and method) was a renal homo-
graft and a right nephrectomy on day 0 and on day +21 a left nephrectomy. Serum administration was commenced on day -1 (24 hours prior to grafting). The transplant was not considered to be functioning unless the dog was seen passing urine after the last nephrectomy.

(a) **Effect of the antiserum:**

Seven of the eight dogs used in this series showed a renal homograft survival of over 21 days. Table IIa summarizes the results and shows that the survival period ranged from 25-79 days (mean 50 days), after which six dogs died with a functioning homotransplant, of which one was failing, and one dog (No. 799) died after 45 days from uraemia. The eighth dog (No. 801) was not seen to be urinating after the last nephrectomy, on day +21, and died from uraemia on day +27.

**Cause of death**

Two dogs (Nos. 784 and 804) died with a functioning homograft 72 and 25 days respectively after transplantation from sudden massive gastro-intestinal bleeding (manifesting by haematemesis and bleeding per anum).

Two dogs (Nos. 799 and 801) died of uraemia 45 and 27 days after homografting respectively. The first dog urinated normally after the nephrectomy on day +21, while the other (No. 801) was not seen to pass urine after this procedure and died six days later.

One dog (No. 807) died 39 days after transplantation
### TABLE II: Antilymphocytic antiserum: Effect on renal homotransplants survival & behaviour

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Survival (days) after transplantation</th>
<th>Second nephrectomy (days)</th>
<th>Transplant function (days)</th>
<th>Cause of death</th>
<th>Urination After 2nd nephrectomy</th>
<th>Day of death</th>
<th>B.U. mg/100 ml.</th>
<th>P.M. appearance of transplant macroscopic</th>
<th>microscopic</th>
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</thead>
<tbody>
<tr>
<td>804</td>
<td>25</td>
<td>4</td>
<td>25</td>
<td>G.I.Hge.</td>
<td>Yes</td>
<td>Yes</td>
<td>246</td>
<td>Normal.</td>
<td>As 799 but no R.B.C.</td>
</tr>
<tr>
<td>807</td>
<td>39</td>
<td>18</td>
<td>39</td>
<td>Hepatitis</td>
<td>Yes</td>
<td>Yes</td>
<td>110</td>
<td>Enlarged + Otherwise normal. Plasma cells + Round cells +</td>
<td></td>
</tr>
<tr>
<td>813</td>
<td>45</td>
<td>24</td>
<td>45</td>
<td>Pneumonia</td>
<td>Yes</td>
<td>Yes</td>
<td>31</td>
<td>Normal.</td>
<td>As 807.</td>
</tr>
</tbody>
</table>

* Died with a functioning transplant.  
G.I.Hge. = gastro-intestinal haemorrhage; B.U. = blood urea; P.M. = post-mortem; R.B.C. = red cells extravasation; +, ++, +++ = slight, moderate, extensive.
### TABLE IIb  Normal Serum : Effect on renal homotransplants survival and behaviour.

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Transplantation</th>
<th>Second nephrectomy</th>
<th>Transplant function (days)</th>
<th>Cause of death</th>
<th>Urination After 2nd nephrectomy</th>
<th>Day of death</th>
<th>B.U. mg/100 ml.</th>
<th>P.M. appearances of transplant macroscopic</th>
<th>P.M. appearances of transplant microscopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>839</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>G.I.Hge.</td>
<td>Yes</td>
<td>Not known</td>
<td></td>
<td>Normal size, less firm than normal.</td>
<td>Round cells ++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I.P.Hge.</td>
<td></td>
<td></td>
<td></td>
<td>Plasmacellular ++</td>
<td>Haemorrhagic areas.</td>
</tr>
<tr>
<td>840</td>
<td>25</td>
<td>4</td>
<td>&lt; 21</td>
<td>Uraemia.</td>
<td>No</td>
<td>No</td>
<td>524</td>
<td>Very large thick capsule which stripped easily.</td>
<td>Massive necrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Matted vessels, ureter, bladder &amp; gut to transplant.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vessels thrombosed. Haemorrhagic areas.</td>
<td></td>
</tr>
<tr>
<td>842</td>
<td>25</td>
<td>4</td>
<td>&lt; 21</td>
<td>Uraemia.</td>
<td>No</td>
<td>No</td>
<td>478</td>
<td>As 840</td>
<td>As 840</td>
</tr>
<tr>
<td>843</td>
<td>26</td>
<td>5</td>
<td>&lt; 21</td>
<td>Uraemia.</td>
<td>No</td>
<td>No</td>
<td>528</td>
<td>As 840 + evidence of disintegration.</td>
<td>As 840</td>
</tr>
<tr>
<td>845</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>I.P.Hge.</td>
<td>Yes</td>
<td>Not known</td>
<td></td>
<td>Enlarged ++ Haemorrhagic areas.</td>
<td>Patent vessels</td>
</tr>
</tbody>
</table>

B.U. = Blood urea; G.I.Hge. = gastrointestinal haemorrhage; I.P.Hge. = intraperitoneal haemorrhage; P.M. = post-mortem; +, ++, +++ = slight, moderate, extensive. R.B.C. = red cells extravasation.
<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Survival (days) after Transplantation</th>
<th>Second nephrectomy</th>
<th>Transplant function (days)</th>
<th>Cause of death</th>
<th>Urination After 2nd nephrectomy</th>
<th>Day of death</th>
<th>B.U. mg/100 ml</th>
<th>P.M. appearance of transplant macroscopic</th>
<th>microscopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>834</td>
<td>26</td>
<td>5</td>
<td>&lt;21</td>
<td>Uraemia</td>
<td>No</td>
<td>No</td>
<td>656</td>
<td>Very large thick capsule which stripped easily.</td>
<td>Massive necrosis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Matted vessels, ureter, bladder &amp; gut to transplant.</td>
<td>Round cells ++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vessels thrombosed. Haemorrhagic areas.</td>
<td>Plasma cells +++</td>
</tr>
<tr>
<td>835</td>
<td>5 *</td>
<td>-</td>
<td>5</td>
<td>Uraemia</td>
<td>-</td>
<td>Not known</td>
<td>378</td>
<td>Enlarged ++ Haemorrhagic areas.</td>
<td>Round cells ++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vessels patent.</td>
<td>Plasma cells +++</td>
</tr>
<tr>
<td>836</td>
<td>26</td>
<td>5</td>
<td>&lt;21</td>
<td>Uraemia</td>
<td>No</td>
<td>No</td>
<td>542</td>
<td>As 834</td>
<td>As 834</td>
</tr>
<tr>
<td>838</td>
<td>25</td>
<td>4</td>
<td>&lt;21</td>
<td>Uraemia</td>
<td>No</td>
<td>No</td>
<td>578</td>
<td>As 834 + evidence of disintegration.</td>
<td>As 834</td>
</tr>
<tr>
<td>846</td>
<td>20</td>
<td>-</td>
<td>&lt;20</td>
<td></td>
<td>-</td>
<td>Not known</td>
<td>As 834</td>
<td>As 834</td>
<td>As 834</td>
</tr>
</tbody>
</table>

B.U. = Blood urea; P.M. = post-mortem; R.B.C. = red cells extravasation;
+, ++, +++ = slight, moderate, extensive.
with a functioning kidney, from hepatitis with severe jaundice of two days duration, ten days after a matched transfusion of packed R.B.Cs.

One dog (No. 813) died 45 days after homografting with a normally functioning kidney from pneumonia.

Two dogs (Nos. 815 and 818) died of distemper with a normally functioning homograft, 79 and 69 days after transplantation respectively (Table IIa).

(b) Effect of normal horse serum:

Three of the five dogs used in this group survived till the day of nephrectomy and died of uraemia (Fig. 33 and Table IIb) 4-5 days after this procedure.

The other two dogs died five days after transplantation on the first day of the "secondary serum reaction"; dog No. 839 from sudden massive gastro-intestinal bleeding in conjunction with intraperitoneal haemorrhage from the graft site; the other (No. 845) died from intraperitoneal haemorrhage, the source of which could not be identified.

(c) Effect of no treatment:

Three of the five dogs used in this group survived till the day of nephrectomy and died of uraemia (Fig. 33 and Table IIc) 4-5 days after this procedure. One of the two dogs that did not survive till the day of nephrectomy (dog No. 835) died five days after transplantation from uraemia (378 mg/100 ml) with an apparently normal, but a non-functioning, right kidney; the other (No. 846) died 20 days after homografting with a grossly
rejected kidney. The cause of death was uncertain, but it is worth mentioning that for one week before death spontaneous agglutination of the blood samples withdrawn (with difficulty) for investigation, was observed daily, thus preventing their analyses.

From these results it is seen that the antiserum, as compared to normal serum or no treatment, prolonged the survival of renal homografts considerably. Most of the dogs died with evidence of renal function from a cause other than uraemia.

6. **Effect on renal function:**

The renal homograft function was assayed by the blood urea, serum sodium and potassium, and by serum uric acid and creatinine level estimation, together with seeing the dog passing urine and estimating the urinary proteins on a midstream sample.

(a) **Effect of the antiserum:**

(i) **Urine** - All the dogs in this group were seen passing urine till the day of death, except two dogs; No. 801 which was anuric following the nephrectomy, and No. 799 two days before death.

(ii) **Blood urea** - Fig. 33 shows the changes in the blood urea levels from just prior to the antiserum injection (on day -1) till the time of death; that the normal blood urea ranged between 20-52 mg/100ml. and that just prior to the nephrectomy it was still within the normal level and ranged from
THE EFFECT IN DOGS OF DAILY INTRAVENOUS INJECTION OF HORSE V DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) ON THE RENAL HOMOGRAFT FUNCTION AS ASSAYED BY THE BLOOD UREA LEVELS.

• 3 ml/Kg BW/d FULL DOSE FOR 60 INJECTIONS THEN 20 INJECTIONS OF HALF THE ORIGINALLY CALCULATED DOSE (HALF DOSAGE)

Normal horse serum control

No treatment control

Fig. 33.
20-44 mg/100 ml. During the ten days preceding the nephrectomy there was a rise in the blood urea level of almost all the dogs, to drop thereafter to a variable degree. The highest urea value attained during the 10 day period was to 308 mg/100 ml. on day +27, after which it dropped to 75 mg/100 ml. one week later (dog No. 807).

Dog No. 784: Showed a gradual rise in its blood urea level with occasional drops (Fig. 33) till the day of death 72 days after homografting, having urinated 600 ml. the previous day (while investigating the homograft function, collecting 24 hours' urine in a metabolism cage). Seventy days after homografting the dog was put in a metabolism cage for 24 hours, during which time the urine was collected and blood samples were taken (the dog received its normal ration of food and water). Table III shows the results of such an investigation. It is evident that the renal homograft was failing. It was unable to clear the blood of urea or creatinine, and unable to concentrate filtrate normally. The urine volume, serum and urinary sodium and potassium were within the normal range.

Dog No. 799: After the nephrectomy the blood urea rose over a period of three days to 238 mg/100 ml. and continued to rise for a further five days, to reach 266 mg/100 ml., then dropped over a few days to rise again. After a further drop it rose steadily (Fig. 33) to reach 506 mg/100 ml.
TABLE III

Dog No. 784, with a 71 days renal homograft and bilateral nephrectomy.  Study of urine and blood chemistry while in a metabolism cage for 24 hours.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Urine</th>
<th>Urine normal values</th>
<th>Serum</th>
<th>Serum normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume ml.</td>
<td>600/24 h.</td>
<td>31/Kg/d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>46/Kg/d</td>
<td></td>
<td>140/L</td>
<td>150/L (135-160)</td>
</tr>
<tr>
<td>Na+ mEq.</td>
<td>37.2/d</td>
<td>32/d</td>
<td>140/L</td>
<td>150/L (135-160)</td>
</tr>
<tr>
<td></td>
<td>62.0/L</td>
<td>1-209/L</td>
<td>140/L</td>
<td>150/L (135-160)</td>
</tr>
<tr>
<td>K+ mEq.</td>
<td>15.6/d</td>
<td>31/d</td>
<td>4.8/L</td>
<td>4.4/L (3.7-5.8)</td>
</tr>
<tr>
<td></td>
<td>26.0/L</td>
<td>(3-128)</td>
<td>4.8/L</td>
<td>4.4/L (3.7-5.8)</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea mg/100ml.</td>
<td>1310</td>
<td>-</td>
<td>435</td>
<td>20-40</td>
</tr>
<tr>
<td>Urea Clearance</td>
<td>1.4 ml/M</td>
<td>56 ml/m</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(20-83)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Creatinine mg/100 ml.</td>
<td>27.4</td>
<td>-</td>
<td>6.6</td>
<td>1-1.7</td>
</tr>
<tr>
<td>Urinary osmoles mos/L</td>
<td>404</td>
<td>1700 - 3000</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

forty-five days after transplantation (the ischaemia of which was unduly long - 75 minutes).

**Dog No. 801**: Was anuric after the nephrectomy and showed a steep rise in the blood urea level to 628 mg/100 ml. six days after this procedure (Fig. 33).

**Dog No. 804**: Enjoyed normal vigour and was passing urine in normal quantities after the nephrectomy despite a rise in the blood urea to 246 mg/100 ml. three days after the operation. Twenty-four hours later, 25 days after transplantation, the dog died from sudden massive gastro-intestinal bleeding.

**Dog No. 807**: Immediately following the nephrectomy, the blood urea rose to reach 308 mg/100 ml. on day +27 and within a further week dropped to 75 mg/100 ml. The dog died 39 days after grafting with a blood urea of 110 mg/100 ml. (estimated 2 days prior to death).

**Dog No. 813**: Did not exhibit any rise in its blood urea after the nephrectomy and died 45 days after transplantation, with a urea level of 31 mg/100 ml., from pneumonia.

**Dog No. 815**: The blood urea during the 10 days that followed the nephrectomy rose to a peak of 170 mg/100 ml. five days after this procedure, to drop thereafter to reach 84 mg/100 ml. on day +40 (Fig. 33). The blood urea reached 71 mg/100 ml. before death, 79 days after homografting.

**Dog No. 818**: The blood urea rose to 96 mg/100 ml. five days after the nephrectomy, to drop thereafter and remain between
40 and 60 mg/100 ml. Before death, 69 days after transplantation, the blood urea was 36 mg/100 ml.

(iii) Serum potassium: Fig. 34 shows the serum potassium estimations of the homografted dogs. It is seen that the rise in the potassium level of dog No. 801 is comparable to that of the controls. The remainder of the dogs showed some changes but generally the potassium level was within the normal range, except dog No. 807 which showed a drop in its potassium level 27-30 days after transplantation to reach 3.2 mEq/L but regained the normal value thereafter, and dog No. 799 showed a similar drop to 2.9 mEq/L on day 44 but regained the normal value on the day of death. Though dog No. 784 had a failing homograft it showed normal serum potassium values.

(iv) Serum sodium: All the dogs, including dog No. 801, which was anuric after the nephrectomy, showed the serum sodium to be within the normal range of 135-160 mEq/L. (Fig. 34).

(v) Serum uric acid: Fig. 35 shows the uric acid estimations of the homografted dogs. During the ten days proceeding the nephrectomy the serum uric acid rose in all the dogs with the exception of one dog (No. 799) and thereafter there was a tendency towards a drop in all the antiserum treated dogs surviving beyond that period.

From Figs. 33 and 35 it is seen that there is a correlation between the rise in the blood urea and uric acid levels during the post-nephrectomy period, except in one dog (No. 799), which showed a marked
THE EFFECT IN DOGS OF DAILY INTRAVENOUS INJECTIONS OF HORSE V. DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3ml/Kg INITIAL B.W. BEFORE THERAPY/DAY FOR 60 INJECTIONS THEN HALF DOSE FOR 20 INJECTIONS ON THE RENAL HOMOGRAFT FUNCTION: EFFECT ON SERUM SODIUM AND SERUM POTASSIUM (BROKEN LINE = UPPER AND LOWER LIMITS OF NORMAL SERUM Na⁺·K⁺ LEVELS)

Fig. 34.
THE EFFECT IN DOGS OF DAILY INTRAVENOUS INJECTIONS OF HORSE V DOG ANTILYMPHOCYTIC ANTISERUM (H/DALAS) 3 ml/kg initial body weight before therapy/day for 80 injections then half the dose for 20 injections on the renal homograft function: effect on serum uric acid and creatinin.

SERUM URIC ACID

- Normal horse serum
- No treatment

SERUM CREATININ

Fig. 35.
rise in the urea but maintained its normal uric acid level.

(vi) **Serum creatinine:** Fig. 35 shows the serum creatinine estimations of the three dogs (Nos. 801, 799 and 784) that exhibited a rise in their blood urea level and died shortly after the last creatinine estimation, with a high blood urea and serum creatinine values. Dog No. 807, which had a blood urea of 308 mg/100 ml. 27 days after transplantation (day of the last recorded creatinine estimation), showed a normal serum creatinine level (0.8 mg/100 ml.). Thereafter the blood urea of this dog dropped to 125 mg/100 ml. during the next three days and to 75 mg/100 ml. after a further four days. The Figure also shows the creatinine level of one dog that had a normal blood urea (dog No. 813).

(vii) **Proteins in urine:** Four of the seven dogs that passed urine after the nephrectomy showed a rise in the urinary proteins for up to 10 days after this procedure, reaching 100 mg/100 ml. This rise is related to that of the blood urea levels during this period (Fig. 33). Thereafter the urinary proteins diminished to 30 mg/100 ml. till the time of death of three of these dogs. The fourth dog (No. 704) died 4 days after nephrectomy. Despite the gradual rise in the blood urea of two of these dogs (Nos. 784 and 799) the urinary proteins
were low (30 mg/100 ml.).

The other three dogs that died with a normally functioning homograft 45, 79 and 69 days after transplantation (dogs Nos. 813, 815 and 818 respectively) showed no, or a trace of, proteins in urine after the nephrectomy.

(b) Effect of normal horse serum
After the nephrectomy all the dogs were anuric and died 4-5 days after this procedure. The changes in blood chemistry during this period were as follows:

**Blood urea:** Rose sharply to reach 478-528 mg/100 ml. just before death (Table IIb and Fig. 33).

**Serum potassium and sodium:** The serum potassium rose steeply to reach before death 7.4 - 9 mEq/litre (normal 3.7 - 5.8 mEq/litre); the simultaneously estimated serum sodium reached 136-139 mEq/litre, i.e., about the minimum of the normal level (normal 135-160 mEq/litre) (Fig. 34).

**Serum uric acid and creatinine:** Serum uric acid level rose to 2.8 - 3.2 mg/100 ml. just prior to death (normal 0 - 0.5 mg/100 ml.), while the creatinine level rose sharply to reach 11.4 - 13.05 mg/100 ml. (normal 1 - 1.7 mg/100 ml.) (Fig. 35).

(c) Effect of no treatment
After the nephrectomy all the dogs were anuric and died 4-5 days after this procedure. The changes in the blood chemistry during this period were similar to those in the group receiving
normal horse serum. The blood urea reached 542-656 mg/100 ml. just prior to death (Table IIc and Fig. 33); the serum potassium rose sharply to 8.3 - 10.2 mEq/litre, while the simultaneously estimated serum sodium was 142 - 152 mEq/litre, i.e., within the normal range (Fig. 34); the serum uric acid rose to 1.6 - 2.3 mg/100 ml. and the simultaneously estimated serum creatinine rose to 8 - 12.15 mg/100 ml. (Fig. 35).

One of the dogs that died five days after homografting with one of its own kidneys still in situ died of uraemia (378 mg/100 ml.) with an apparently normal but a non-functioning own kidney.

7. Post-mortem findings

(a) Effect of the antiserum:

Dog No. 784 died 72 days after transplantation from sudden massive gastro-intestinal bleeding and uraemia.

Abdomen - (i) The renal homotransplant was slightly enlarged and the cut section showed a few haemorrhagic areas in the cortex, but otherwise normal. The vascular and uretero-vesical anastomoses were patent and the bladder contained urine.

(ii) Lymph nodes: (a) The right iliac nodes were enlarged, & softer than normal nodes; (b) The mesenteric lymph nodes were smaller and softer than normal; (c) The mediastinal lymph nodes were similar to the mesenteric group.

(iii) Gastro-intestinal tract: Stomach full of dark blood and the mucosal lining severely haemorrhagic (dark red all
over) and thicker than normal. No ulceration, angiomatous formation or tumour were detected. Intestine contained darkly coloured blood and the duodenal and upper intestinal mucosa showed similar findings to that of the stomach. Peyer's patches showed loss of substance (thinner than normal). Colon contained darkly coloured blood.

(iv) Liver: No abnormality detected.

(v) Other viscera and major vessels: No abnormality detected.

Chest

(i) Lungs: No abnormality detected.

(ii) Heart: No abnormality detected.

Dog No. 799 died 45 days after transplantation from uraemia

Abdomen - (i) The renal homotransplant was slightly enlarged together with a slightly thickened capsule that stripped readily in areas. The cut section showed a few haemorrhagic areas in the cortex but otherwise normal. The vascular and uretero-vesical anastomoses were patent and the bladder contained very little urine.

(ii) Lymph nodes: (a) The right iliac nodes were enlarged and softer than normal; (b) The mesenteric nodes were very small and soft; (c) The mediastinal nodes were similar to the mesenteric group.

(iii) Gastro-intestinal tract: The stomach, intestine and colon were of normal appearance except that the duodenal and upper intestinal mucosa were congested. Peyer's patches showed loss of substance.
Liver: No abnormality was detected.

Other viscera and major vessels: No abnormality detected.

Chest - (i) Lungs: Haemorrhagic pleural effusion. Though both lungs were congested the right lung was more affected than the left, with rounded edges and purulent exudation from the pulmonary ramifications of the bronchial tree.

(ii) Heart: Slight pericardial effusion but nothing abnormal was detected otherwise.

Dog No. 80I died 27 days after transplantation, being anuric for the six days following nephrectomy.

Abdomen - (i) The renal homotransplant was shrunken and showed scattered circumscribed areas of haemorrhage in the cortex. The capsule was of normal appearance and the anastomoses were patent. The bladder contained no urine.

(ii) Lymph nodes: (a) The right iliac nodes were moderately enlarged and soft; (b) The mesenteric lymph nodes were smaller and softer than normal.

(iii) Gastro-intestinal tract: Nothing abnormal was detected except loss of substance of Peyer's patches.

(iv) Liver: No abnormality was detected.

(v) Other viscera & major vessels: No abnormality detected.

Chest - (i) Lungs: No abnormality was detected.

(ii) Heart: No abnormality was detected.
Dog No. 804 died 25 days after transplantation from sudden massive gastro-intestinal bleeding.

Abdomen - (i) The renal homotransplant was of a normal size and cut section, with patent vascular and uretero-vesical anastomoses. The bladder contained urine.

(ii) Lymph nodes: (a) The right iliac nodes were enlarged and soft; (b) The mesenteric nodes were very small in size and soft.

(iii) Castro-intestinal tract: Stomach was full of dark coloured blood and the mucosa thick and severely haemorrhagic. No ulceration, angiomatous formation or tumour were detected. The duodenal mucosa was congested. Oesophagus, intestine and colon showed nothing abnormal. Peyer’s patches showed loss of substance.

(iv) Liver: No abnormality was detected.

(v) Other viscera & major vessels: No abnormality detected.

Chest - (i) Lungs: No abnormality was detected.

(ii) Heart: No abnormality was detected.

Dog No. 807 died 39 days after homografting with severe jaundice that was apparent two days before death and ten days after the transfusion of 400 ml. of compatible packed red cells.

Abdomen - (i) The renal homotransplant was slightly enlarged but otherwise normal. The vascular and uretero-vesical anastomoses were patent and the bladder contained yellow urine.
(i) **Lymph nodes**: (a) The right iliac nodes were enlarged and soft; (b) The mesenteric nodes were very small and soft.

(ii) **Gastro-intestinal tract**: Peyer's patches showed loss of substance. Nothing abnormal was otherwise detected.

(iii) **Liver** was tough in texture and gritty on sectioning. The cut surface was granular. Yellow discolouration all over.

(iv) **Other viscera and major vessels**: The spleen was dark in colour, soft, slightly enlarged and showed rounded borders. Nothing abnormal was otherwise detected.

Chest - (i) **Lungs**: No abnormality was detected.

(ii) **Heart**: No abnormality was detected.

Dog No. 813 died 45 days after homotransplantation, the last three days of which it looked ill and had a high temperature (104–105°F). It was not jaundiced.

Abdomen - (i) The renal homotransplant was of normal appearance and cut section, with patent anastomoses and the bladder contained urine.

(ii) **Lymph nodes**: (a) The right iliac nodes were enlarged and soft; (b) The mesenteric lymph nodes were very small and soft.

(iii) **Gastro-intestinal tract**: Nothing abnormal was detected except loss of substance of Peyer's patches.

(iv) **Liver** was pale brown in colour, with a few small
white patches scattered on the surface. The bile was of a yellow tinge.

(v) **Other viscera & major vessels:** No abnormality detected.

Chest - (i) **Lungs:** were congested and had rounded borders. On section, purulent fluid oozed from the bronchial ramifications. Yellow coloured pleural effusion.

(ii) **Heart:** No abnormality was detected.

Dog No. 815 died 79 days after homotransplantation after a few days' illness with a high temperature (104°F).

Abdomen - (i) The renal homotransplant was of normal appearance and cut section, and with patent anastomoses. The bladder contained urine.

(ii) **Lymph nodes:** (a) The right iliac nodes were enlarged and soft; (b) The mesenteric lymph nodes were of extremely small size and soft.

(iii) **Gastro-intestinal tract:** Nothing abnormal was detected except loss of substance of Peyer's patches.

(iv) **Liver:** No abnormality was detected.

(v) **Other viscera & major vessels:** No abnormality detected.

Chest - (i) **Lungs:** Congested with purulent fluid oozing on sectioning.

* Distemper was diagnosed and confirmed by the Veterinary College, Edinburgh University.
(121)

(ii) **Heart**: Nothing abnormal detected.

Dog No. 618 died 69 days after homotransplantation, after a few days' illness and high temperature (104°F).

**Abdomen** - (i) The renal homotransplant was of normal appearance and cut section, and with patent anastomoses. The bladder contained urine.

(ii) **Lymph nodes**: (a) The right iliac nodes were enlarged and soft; (b) The mesenteric lymph nodes were very small in size and soft.

(iii) **Gastro-intestinal tract**: Nothing abnormal was detected except loss of substance of Peyer's patches.

(iv) **Liver** was tough in texture and gritty on cutting. The cut surface was granular.

(v) **Other organs and major vessels**: No abnormality detected.

**Chest** - (i) **Lungs**: were congested and on section, purulent fluid exuded from the cut surface.

(ii) **Heart**: Nothing abnormal was detected.

From these post-mortem findings it is seen that six grafts were functioning at the time of death (the bladder contained urine), while the remaining two (dogs Nos. 799 and 801) were not. The renal homografts of four dogs showed normal appearance and cut section, slightly enlarged in three dogs (Nos. 784, 799 and 807) and in one dog it was shrunken (No. 801). The cut section showed a few scattered areas of haemorrhage in the cortex of three

* Distemper was diagnosed and confirmed by the Veterinary College, Edinburgh University.
transplants (one kidney was functioning - dog No. 784), while the other two (dogs Nos. 799 and 801) were anuric at the time of death.

The right iliac nodes (in the immediate vicinity of the transplants) were enlarged and softer than normal in all the dogs. The mesenteric lymph nodes were involuted and soft. Peyer's patches showed loss of substance in all the dogs. Sudden severe gastro-intestinal bleeding was responsible for the death of two dogs with functioning kidneys at the time of death (Nos. 784 and 804). In both, the stomach was full of dark coloured blood and the gastric mucosa was extensively haemorrhagic (dark red in colour) and thickened. The duodenal and upper intestinal mucosa were severely haemorrhagic in one dog (No. 784) and were congested in two dogs (Nos. 799 and 804).

The liver was tough and gritty on sectioning and showed a granular cut surface in two dogs (Nos. 807 and 818). One dog (No. 813) showed a change in the colour of the liver and bile to pale brown and yellow respectively; the dog was not jaundiced.

Pulmonary infection was manifest in four dogs. One died from uraemia (No. 799), one from pneumonia (No. 813) and two from distemper (Nos. 815 and 818).

(b) Effect of normal horse serum

The three dogs that survived till the nephrectomy operation (Nos. 840, 842 and 843) died 4-5 days after this procedure and were anuric from after this operation till the date of death.
Abdomen - (i) The renal homograft was greatly enlarged in all three dogs with very thick capsule that stripped readily. One kidney was partially necrotic with a cavity formation (dog No. 843). The cut section showed the broad cortex to be haemorrhagic in areas. The renal pedicle was matted firmly together with the ureter and bladder to the homograft. The vessels were thrombosed. Dog No. 842 showed extension of thrombosis to the right iliac artery above and below the anastomosis site. Loops of intestine and the greater omentum were firmly adherent to the homograft and separated only by sharp dissection.

(ii) Lymph nodes: (a) The right iliac lymph nodes were enlarged, firm and adherent to the homograft; (b) The mesenteric lymph nodes were of normal size and texture.

(iii) Gastro-intestinal tract: The ilium and greater omentum were firmly adherent to the homograft. Nothing abnormal was otherwise detected in the gastro-intestinal tract, liver, other viscera and major vessels, lungs and heart. Dog No. 842 showed thrombosis of the iliac artery but not the vein.

The two dogs (Nos. 839 and 845) that died five days after transplantation (within 24 hours from the "secondary serum reaction" attack) showed the following:

Abdomen: Both dogs showed extensive intra-peritoneal haemorrhage. One dog (No. 839) showed also blood clots around the graft (in its retroperitoneal bed) and around its vascular pedicle, an indication that the site of bleeding was from the anastomosis area. The other dog (No. 845) showed no clots in the kidney bed or around
its pedicle. The source of haemorrhage was not from the nephrectomy operation in both dogs, and in dog No. 845 the source of bleeding could not be verified.

(i) The renal homotransplant was of normal size but less firm than normal in dog No. 839, and moderately enlarged and of normal firmness in the other (No. 845). Both kidneys showed areas of haemorrhage in the cortex. The anastomoses were not examined in one dog (No. 839) and in the other (No. 845) they were patent.

(ii) Lymph nodes: (a) The right iliac nodes were slightly enlarged and firm but not adherent to the grafts; (b) the mesenteric nodes were of normal size and texture.

Nothing abnormal was detected in the gastro-intestinal tract, liver, other abdominal viscera and major vessels, lungs and heart.

(c) Effect of no treatment
All three dogs that survived till the nephrectomy operation (Nos. 834, 836 and 838) died 4-5 days after this procedure and were anuric from after this operation till the date of death. One dog (No. 846) died one day before nephrectomy and the post-mortem findings were similar to the above dogs.

Abdomen - (i) The renal homotransplant showed similar findings to that of the control group on normal serum. The transplants were markedly enlarged in all four dogs with very thick capsules that stripped readily; one kidney was partially necrotic (dog No. 838) and contained a large necrotic cavity. The cut section
showed the broad cortex to be haemorrhagic in areas. The
renal pedicle, together with the ureter, bladder, greater
omentum and loops of intestine were firmly adherent to the
transplant. The vessels were thrombosed. No urine was
seen in the bladder of the first three dogs and was not
looked for in the other.

(ii) Lymph nodes: (a) The right iliac nodes were
enlarged, firm and adherent to the graft; (b) The mesenteric
nodes were normal in size and texture.

(iii) Gastro-intestinal tract: Nothing abnormal was
detected besides the adherent loops of intestine to the homo-
graft.

Nothing abnormal was detected in the liver, other
abdominal viscera and major vessels, lungs and heart. Nothing
abnormal was detected in dog No. 846 that would account for
its death.

The remaining dog in this group (dog No. 835) died five
days after transplantation from uraemia (blood urea 378 mg/100 ml.)

Abdomen - (i) The renal homotransplant was moderately
enlarged; the cut section showed haemorrhagic areas in the
cortex. The anastomoses were patent.

(ii) Lymph nodes: (a) The right iliac nodes were
enlarged and firm; (b) The mesenteric nodes were normal in
size and texture.

Nothing abnormal was detected in the gastro-intestinal
tract, liver, other viscera and major vessels, lungs and heart.
The dog's own kidney looked normal in appearance, but was not dissected. (A blood sample was taken before death, when the dog was very ill, on the assumption that the dog may have had a congenitally absent kidney). The post-mortem examination showed the contrary, and the blood urea was 378 mg/100 ml., indicating that the dog's own remaining kidney was non-functioning.

8. Histological studies

   Renal homotransplant

   (a) Effect of antiserum:

   **Dog No. 78A:** The H and E preparation shows the capsule to be slightly thickened and the cortex (Fig. 36) extensively infiltrated with round mononuclear cells (perivascular, peritubular and periglomerular); tubular necrosis and total replacement, in areas, by the masses of infiltrating cells; hyaline casts in tubules, and scattered areas of red cell extravasation. The medulla shows interstitial oedema, areas of tubular necrosis and moderate round cell infiltration at its outer part. The majority of the infiltrating round mononuclear cells are pyroninophilic in the Unna Pappenheim (U.P.) preparation (stained with pyronin-methyl green) and are mature and immature plasma cells (Fig. 37).

   **Dog No. 799:** The H and E preparation shows the capsule to be thickened and the cortex moderately infiltrated with round mononuclear cells; scattered areas of red cells extravasation; hyaline casts in some tubules, and areas of tubular
necrosis. The medulla shows little round cell infiltration in its outer part and interstitial oedema. The majority of the infiltrating cells are mature and immature plasma cells and are pyroninophilic in the U.P. preparation.

**Dog No. 801**: The H and E preparation shows the capsule to be thickened in areas; moderate round cell infiltration in the cortex, areas of necrosis and red cell extravasation, and few hyaline casts in the tubules. The majority of the infiltrating cells are plasma cells and pyroninophilic in the U.P. preparation.

**Dog No. 804**: The H and E preparation and the U.P. preparation show the same picture as in dog No. 799 but without red cell extravasation.

**Dog No. 807**: The H and E preparation shows in the cortex some round cell infiltration; healthy tubules, and the absence of tubular casts and red cell extravasation. The U.P. preparation shows pyroninophilic plasma cells.

**Dog No. 813**: Both the H and E and the U.P. preparations show a similar picture to dog No. 807.

**Dog No. 815**: The H and E and the U.P. preparations show some interstitial oedema but otherwise a normal kidney architecture (Fig. 38).

**Dog No. 818**: Both preparations show a normal kidney architecture (Fig. 39).

(b) **Effect of normal serum**

**Dogs Nos. 839 and 845**: The H and E preparation shows the
cortex to be moderately infiltrated with round mononuclear cells, areas of red cell extravasation and some hyaline casts (Fig. 40). The outer part of the medulla is infiltrated with some round cells. Many pyroninophilic cells (plasma cells) in the U.P. preparation.

Dogs Nos. 842 and 843: Both H and E, and U.P. preparations show thick capsule and massive necrosis of both cortex and medulla (Fig. 41) with extensive red cell extravasation, some round cell infiltration in the cortex, and the absence of pyroninophilic cells.

(c) Effect of no treatment

Dog No. 835: The H and E preparation shows the cortex infiltrated with round cells (Fig. 42) and areas of red cell extravasation, hyaline and granular casts in tubules, and some round cell infiltration of the outer medulla. Many pyroninophilic cells in the U.P. preparation, (i.e., a picture similar to dog No. 839 on normal serum).

Dogs Nos. 834, 836, 838 and 846: The H and E and the U.P. preparations show a thick capsule and massive necrosis of both cortex (Fig. 43) and medulla with extensive red cell extravasation, some round cell infiltration in the cortex and the absence of pyroninophilic cells, (i.e., a picture similar to dog No. 840 on normal serum).

Normal kidney removed three weeks after transplantation

Light microscope. All the dogs in the three groups showed a normal kidney architecture except for scattered perivascular
Fig. 36: Post-mortem section of the renal homograft of dog No. 784, which received horse versus dog antilymphocytic antiserum, and died 72 days after transplantation with a functioning but failing homograft. Section shows a marked homograft reaction. H. & E. x 100.

Fig. 37: Cells infiltrating the renal homograft of dog No. 784. Post-mortem section showing numerous plasma cells (Mature and immature). Unna Pappenheim x 580.
Fig. 38: Post-mortem section of the renal homograft of dog No. 815, which received horse versus dog antilymphocytic antiserum, and died 79 days after transplantation from distemper with a normally functioning homograft. Section shows a healthy looking kidney with some interstitial oedema and fibrosis. H. & E. x 100.

Fig. 39: Post-mortem section of the renal homograft of dog No. 818, which received antilymphocytic antiserum and died 69 days after transplantation from distemper with a normally functioning homograft. Section shows a normal kidney. H. & E. x 100.
Fig. 40: Post-mortem section of the renal homograft of dog No. 839, which received normal horse serum, and died 5 days after transplantation. Section shows marked round cell infiltration (peritubular and periglomerular).
H. & E. x 100.

Fig. 41: Post-mortem section of the renal homograft of dog No. 842, which received normal horse serum, and died 25 days after transplantation, with a non-functioning homograft, from uraemia (see Fig. 9 for the experimental protocol). Section shows massive necrosis and red cell extravasation, together with some round cell infiltration.
H. & E. x 100.
Fig. 42: Post-mortem section of the renal homograft of dog No. 835, which received no treatment and died 5 days after transplantation from uraemia. Section shows marked round cell infiltration. H. & E. x 100.

Fig. 43: Post-mortem section of the renal homograft of dog No. 836, which received no treatment and died 26 days after transplantation, with a non-functioning homograft, from uraemia (see Fig. 9 for the experimental protocol). Section shows extensive necrosis and red cell extravasation with some round cell infiltration. H. & E. x 100.
and peritubular round cell aggregations, with many pyroninophilic cells.

**Electron microscope.** No abnormality was detected by electron-microscopic study of the normal kidney removed three weeks after transplantation and antiserum administration. The two kidneys examined were that of dog No. 799 which manifested the "secondary serum reaction", the other was that of dog No.804 which died of acute massive gastrointestinal haemorrhage four days after the nephrectomy.

**Mesenteric lymph nodes:** The antiserum treated dogs showed a marked diminution in the number of the lymphoid follicles, which when present were small and far apart to a varying degree, as seen in four dogs (Nos. 784, 801, 804 and 807), and were completely absent in four dogs (Nos. 799, 813, 815 and 818). The control groups showed a normal picture.

The antiserum treated group showed moderate (dogs Nos. 784, 801, 804 and 807) to marked (dogs Nos. 799, 813, 815 and 818) depletion of small lymphocytes, while the control groups showed a normal picture. There was a marked increase in the macrophages and reticular cells in the antiserum treated dogs, while the control groups showed them in normal numbers.

In the U.P. preparation, the antiserum treated group showed an excessive number of pyroninophilic cells, some at the periphery of the lymphoid follicles but the majority in the medulla, in five dogs (Nos. 784, 799, 801, 804 and 807), a moderate number in one dog (No. 815) and their absence in two
dogs (Nos. 813 and 818). The normal serum treated group showed absence of such cells in three dogs (Nos. 839, 843 and 845) and their presence in big numbers in two dogs (Nos. 840 and 842). Three dogs in the group that received no treatment (Nos. 834, 838 and 846) showed the absence of the pyroninophilic cells, and one dog (No. 836) showed them in big numbers. Normal lymph nodes from different dogs showed a wide range of variation from a slight to an excessive number.

**Fig. 44** - a photomicrograph of a mesenteric lymph node (H and E preparation) of a normal dog for comparison with that (Fig. 45) of an antiserum treated dog (No. 815).

**Regional lymph nodes:** All the antiserum treated dogs showed some diminution in the number and size of the lymphoid follicles with their complete absence in two dogs (Nos. 815 and 818). The control groups showed the normal follicular pattern. The nodes were moderately depleted of small lymphocytes in the antiserum group and markedly so in four dogs (Nos. 799, 813, 815 and 818). A moderate number of large lymphocytes were apparent in all the nodes. The control groups showed only some small lymphocytes and an excessive number of large lymphocytes all over the cortex. The antiserum treated dogs showed an increase in the macrophages and reticular cells. The controls showed them in normal numbers.

All the dogs, antiserum treated and controls, showed an excessive number of pyroninophilic cells by the U.P. preparation.
Fig. 44: Section of a mesenteric lymph node of a normal dog showing the cortex with the lymphoid follicles and part of the medulla; for comparison with Figure 45. H. & E. x 34.

Fig. 45: Post-mortem section of a mesenteric lymph node of dog No. 815. Section showed marked lymphocyte depletion after 80 antiseraum injections; for comparison with Figure 44. H. & E. x 34.
Spleen showed moderate to marked small lymphocytes depletion in the antiserum treated group. Three dogs (Nos. 784, 799 and 804) showed moderate depletion, four dogs (Nos. 807, 813, 815 and 818) showed marked depletion, and one dog (No. 801) showed only slight depletion. The spleen of the control groups showed a normal picture, except one dog (No. 845) that received normal serum and died of intra-peritoneal haemorrhage, showed a marked depletion.

The U.P. preparations of the antiserum group showed the absence of pyroninophilic cells in three dogs (Nos. 813, 815 and 818), two dogs (Nos. 801 and 807) showed these cells in a moderate number in the red pulp and three dogs (Nos. 784, 799 and 804) showed them in great number, some at the periphery of the lymphoid follicles but the majority in the red pulp.

The control group that received normal horse serum showed a great number of pyroninophilic cells in three dogs (Nos. 839, 840 and 842), one dog (No. 845) showed a moderate number, and one dog (No. 843) showed only some. One of the untreated dogs (No. 838) showed a great number and three showed a moderate number of these cells (Nos. 834, 836 and 846).

Peyer's patches showed a moderate degree of small lymphocyte depletion and loss of follicular arrangement and amount of lymphoid tissue. The control groups showed a normal picture. Appendix showed no small lymphocytes depletion in all the antiserum treated dogs.

Thymus: When present, showed no pyroninophilic cells.
Liver showed areas of necrosis in five out of the eight dogs that received the antiserum (dogs Nos. 799, 807, 813, 815 and 818), while none was seen in the control group on normal horse serum. The liver showed macrophages and round cells aggregated around the central veins in four dogs (Nos. 784, 799, 801 and 804) and some of these cells were pyroninophilic. A similar picture was observed in the liver of the control group on normal horse serum.

Pancreas: All the antiserum treated group showed no change in the pancreas from the normal pattern with the exception of one dog (No. 807) which showed a marked reduction in the islet tissue.

Heart and diaphragm showed no change from the normal pattern in all the antiserum treated dogs.

Gastro-intestinal tract: With the exception of one dog (No. 784) all the antiserum treated dogs, including dog No. 804 (that died from severe gastro-intestinal bleeding), showed a normal picture. Dog No. 784 showed massive red and white cell extravasation in the gastric submucosa and mucosa.
IN VITRO STUDIES

Antilymphocytic antiserum assay

Aim of such a procedure

1. To show whether the antilymphocytic antiserum is active against lymphocytes of the species that contributed to its production.

2. To estimate the potency of the antiserum batches under standard conditions and thus their acceptability and standardization for therapeutic use.

3. To estimate the degree of immunization of the animal that donated the antiserum and the effect of further immunological challenging on the antiserum titer.

4. To establish the components taking part in the interaction between the antiserum and the lymphocytes; also the method of such an interaction.

Antiserum assay was approached in two ways:—


2. By an in vivo assay.
In vitro assay

1. Assay of the antilymphocytic activity.
2. Assay of the antierythrocytic activity.

1. Antilymphocytic activity assay: For such a study two procedures were evaluated regarding their feasibility and fulfillment of certain requirements thought to be necessary for the adoption of a test for routine use in the antiserum assay.

Tests: (a) A cytotoxicity test employing dye exclusion as an indication of viability.
(b) A lymphocyte-agglutination test.

Requirements necessary for the test adoption:
1. Accuracy.
2. Repeatability.
3. Simplicity.
4. Gives results after a reasonable time interval.
5. Does not entail elaborate equipment for its preparation, processing or recording.
6. To be as near physiological conditions as possible.

2. Antierythrocytic activity assay: For such a study two procedures were evaluated.

(a) Haemolysis reaction.
(b) Haemoagglutination effect.

1. Antilymphocytic activity assay
(A) Cytotoxicity test:

To employ the cytotoxicity test for the routine assay of the antiserum certain tests were carried out:
1. To verify whether complement is needed by the antiserum to exercise its cytotoxic action, and if needed, to estimate the dilution necessary for carrying out a satisfactory reaction; also whether it has any cytotoxic effect on thoracic duct lymphocytes.

2. To estimate the optimal values (cell number and sera volumes) of the components taking part in the interaction.

Sera dilutions may be spoken of according to their initial state i.e., after the doubling dilution with the diluent, or according to their final state, i.e., after the addition of all the components of each tube. This latter seems to be the more accurate in expressing the serum dilution in the in vitro reactions. Accordingly all dilutions and titers will be expressed in their final dilution unless otherwise indicated.

Material and Methods:

(a) Male hooded rats' thoracic duct lymphocytes suspended in Hanks' balanced salt solution (prepared as previously described). The suspension was made to contain $25 \times 10^6$ lymphocytes/ml.

(b) Rabbit-versus-rat antilymphocytic antiserum (ALS) heated at $56^\circ C$ for half-an-hour (see before).

(c) Normal rabbit serum heated at $56^\circ C$ for half-an-hour.

(d) Guinea pig serum as a source of complement.

(e) Hanks' solution.

(f) Tyrpan blue $0.05\%$ solution, freshly prepared in Hanks' solution.
TABLE IV

Cytotoxic and agglutinative effect of 1/4 dilution of rabbit-versus-rat antilymphocytic antiserum on male hood rat thoracic duct lymphocytes using doubling dilutions of fresh guinea pig serum as a source of complement; tested by Trypan blue dye exclusion and lymphocyte-agglutination.

<table>
<thead>
<tr>
<th>Antiserum dilution</th>
<th>Complement titer</th>
<th>Non-viable cells %</th>
<th>Agglutination degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4</td>
<td>1/8</td>
<td>98.9</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.8</td>
<td>3.2</td>
</tr>
<tr>
<td>1/16</td>
<td></td>
<td>94.6</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/32</td>
<td></td>
<td>66.6</td>
<td>4.3</td>
</tr>
<tr>
<td>1/64</td>
<td></td>
<td>38.8</td>
<td>2.0</td>
</tr>
<tr>
<td>1/128</td>
<td></td>
<td>14.2</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Were stained by Trypan blue on the addition of 0.05 ml. undilute guinea pig serum (complement) and re-incubation for half-an-hour at 37°C.

Controls non-viable cells %: Normal rabbit serum 1/5, complement 1/5 = 3.9%
Hanks’ = 5.0%
Control Agglutination: Antiserum 1/3 & 1/6 = +++
The cytotoxicity tests were carried out in Khan tubes. All glassware used was meticulously cleaned.

Effect of complement dilution on the cytotoxicity of the antiserum:
Procedure: After making doubling dilutions of 0.1 ml. fresh guinea pig serum (complement source) in 0.1 ml. Hanks' solution, 0.1 ml. undiluted ALS, then $5 \times 10^6$ lymphocytes suspended in 0.2 ml. Hanks' solution, were added to all the tubes and incubated at $37^\circ C$. for 1½ hours. After the incubation period a viability count was performed on all the tubes by the trypan blue dye exclusion method and the agglutination reaction recorded.

Results: Table 4 and Figure 46 show that with a standard ALS dilution of $1/4$ and lymphocyte number of $5 \times 10^6$, as the complement becomes more dilute, the non-viable cells percentage diminishes while the strength of the agglutination reaction increases. At a complement dilution of $1/8$, $97.8 - 98.9\%$ of the cells were non-viable and no agglutination clumps were seen, while at a complement dilution of $1/16$, $94.6\%$ of the cells were non-viable and stained small clumps of agglutinated lymphocytes were apparent microscopically. At a dilution of $1/32$ the non-viable cell percentage dropped to $66.6\%$ and the agglutinated lymphocyte reaction increased, but all the clumps were non-stained. At a dilution of $1/128$ the non-viable cell percentage was $14.2\%$ and the agglutination reaction was at its height with non-stained big masses of agglutinated lymphocytes. On the addition of 0.05 ml. undiluted guinea pig serum to the last tube and its re-incubation for half-an-hour the agglutinated
THE CYTOTOXIC EFFECT OF 1/4 DILUTION OF RABBIT V. RAT ANTILYMPHOCYTIC ANTISERUM ON MALE HOODED RAT THORACIC DUCT LYMPHOCYTES, USING SERIAL DILUTIONS OF FRESH GUINEA PIG SERUM AS A SOURCE OF COMPLEMENT, AS TESTED BY THE TRYPAN BLUE DYE EXCLUSION TEST.

- Dye exclusion viability test
- Agglutination degree
- Controls (complement cytotoxicity)

Fig. 46.
masses acquired the trypan blue stain, indicating their non-viability.

Control values for complement cytotoxicity in the absence of antiserum showed a non-viable cell percentage of 1.3 - 4.3% except in a complement dilution of 1/128, in which 6% of the cells were non-viable, i.e., almost the same value as the control of cells incubated with Hanks' solution (5% non-viable cells). This may be explained by the absence of the protective effect of serum proteins on the lymphocytes, which are thus exposed, unprotected, to the salt solution.

Cytotoxicity test technique

In the light of data obtained by preliminary tests regarding cell number, sera volumes, complement dilution and diluting fluid to be used, the following standard technique for the antilymphocytic antiserum cytotoxic titer estimation was adopted:

1. 0.1 ml. diluent (Hanks' solution) was added to all the tubes, except the first and any control tube necessitating its omission.

2. 0.1 ml. antiserum was added to the first and second tubes. Doubling dilutions of the antiserum were made using an automatic syringe adjusted to aspirate and eject 0.1 ml. and to which was attached, by means of a tightly fitting rubber tube,

* Arnold R. Horwell, Ltd., Surgical & Laboratory Supplies, 2 Grangeway, Kilburn High Road, London N.W.6.
a Pasteur pipette curved to a 90° angle. The procedure was as follows: the contents of the second tube were mixed and 0.1 ml. withdrawn and added to the third tube, thoroughly mixed, and 0.1 ml. withdrawn and transferred to the fourth tube and so on till the last tube, discarding the last withdrawn 0.1 ml. This results in having doubling dilutions of the antiserum in each tube in a volume of 0.1 ml.

3. 0.1 ml. fresh undiluted guinea pig serum was added to all the tubes, except the controls necessitating its omission.

4. 0.2 ml. of the thoracic duct lymphocytes suspension (on which a viability count has been carried out) were added to all the tubes (test and control). Each tube will thus contain $5 \times 10^6$ lymphocytes.

For the antiserum assay the controls were: diluent and cells; diluent complement and cells; diluent, complement, heated normal serum and cells.

All the tubes were gently shaken from side to side to ensure a homogenous suspension of their contents and were covered and incubated at 37°C for 1½ hours.

After the incubation period a viability count employing trypan blue (0.05%) as a diluent and a viability indicator was performed (see before) on the contents of each tube after gentle but thorough mixing. All the stained cells (blue or a shade of blue) were counted as being non-viable and unstained cells as viable. The non-viable cell percentage was calculated from the
total number of cells (viable and non-viable). The end point was recorded at the antiserum dilution resulting in the death of 10% of the cells, provided the controls show a non-viable cell percentage of 5% or less. If controls show a higher non-viable cell percentage the end point is to be taken at the last tube with double the control value. If very high control values the test should be repeated.

Recording the results of the cytotoxicity test may be performed by either:

(a) counting the viable (unstained) and non-viable (stained) cells in 2 mm$^2$. After appropriate calculations the total number of cells in each tube is obtained. This figure is then subtracted from the original put in each tube, thus obtaining the number of cells affected to the degree of dis-integrating, by the action of the antiserum. This value is then added to that of the stained cells to obtain the total number of the cells affected by the antiserum (stained and destroyed). The total affected cell percentage is then calculated from the total cell number originally introduced per tube.

(b) counting the viable and non-viable cells in 2 mm$^2$ and obtaining the non-viable cell percentage from the total number of the cells counted.
If the first method is adopted, this will give information about the cells affected by the antiserum to the degree of disintegration in the high antiserum concentration tubes. But before attributing the missing cells to the action of the antiserum a cell count should ideally be performed on each tube prior to incubation disregarding how thorough the cell number in the suspension was adjusted. From this it is seen that an extra cell count should be performed on all the tubes, keeping in mind that cell counts have a certain degree of error in themselves and that in such a lengthy test recording as the cytotoxicity test an extra count on all the tubes is an appreciable disadvantage in multiple batches assay.

If the second method is adopted, it will give the proportion of non-viable and viable cells from the total number counted in $2 \text{ mm}^2$, but will not make an account of the destroyed cells. If the cytotoxicity test is to be used as a method of choice for the antiserum assay, ideally, the first procedure should be used. The second method was originally adopted for the estimation of the cytotoxic effect of the antiserum whenever this was performed, since the cytotoxicity test was not used as the routine method for assaying the antiserum in vitro.

Disadvantages of the cytotoxicity test:

1. To perform a viability count it was found from the preliminary studies that contact of the dye with the lymphocyte suspension for 3 minutes was the optimal time (one minute allowed for mixing in the white cell pipette and two minutes for filling
the counting chamber, mounting on the microscope and allowing the cells to settle. Therefore examining ten doubling dilutions of one batch will consume not less than 40 minutes. Often it is needed to test a few batches of antiserum simultaneously. The use of such a lengthy procedure as the cytotoxicity test, when compared to the quick and simple agglutination reaction, shows the superiority of the latter test (3 minutes to record 10 dilutions of one batch), over the former (at least 40 minutes for a similar 10 dilutions).

2. It entails the use of guinea pig serum as a source of complement (heterologous component).

3. Cell counts are subject to an error factor.

(B) Lymphocyte-agglutination test

1. Material and methods
(a) Thoracic duct lymphocytes suspended in Hanks' solution (see before) with a cell count of 25 x 10^6 lymphocytes/ml.

(b) Heated antilymphocytic antiserum.

(c) Heated normal serum.

(d) Hanks' solution.

The antisera tested were rabbit-anti-rat; rabbit, sheep and horse-anti-dog. The normal sera tested were of rabbit, sheep and horse. The lymphocyte-agglutination test was carried out in M.R.C. standard pattern agglutination plates which were

* R.B. Turner, Inocula House, Church Lane and Hobbs Green, East Finchley, London N.2.
meticulously cleaned or in very clean Khan tubes.

Various criteria had to be established before the routine use of the lymphocyte-agglutination test as a standard method for assaying the antilymphocytic potency of antilymphocytic antiserum. These were:

1. Whether complement is essential for demonstrating the lymphocyte agglutinative effect of the antiserum.

2. The relationship between the agglutinative and the cytotoxic effects of the antiserum on lymphocytes.

3. The method of recording the results whether macro- or microscopically.

**Effect of complement on the lymphocyte-agglutination and a comparison with the cytotoxic reaction.**

1. After making doubling dilutions of 0.1 ml. fresh guinea pig serum (complement source) in 0.1 ml. Hanks' solution, 0.1 ml undiluted ALS (rabbit-versus-rat antilymphocytic antiserum) and $5 \times 10^6$ rat thoracic duct lymphocytes suspended in 0.2 ml. Hanks' solution were added to all the tubes, and incubated at $37^\circ$C., for $1\frac{1}{2}$ hours. Both the agglutinative and cytotoxic effects of the antiserum were tested.

**Results:** Table 4 and Figure 46 show that with a standard ALS dilution of $1/4$ and $5 \times 10^6$ lymphocytes, as the complement becomes more dilute the degree of agglutination increases, while the non-viable cell percentage diminishes. On the addition of trypan blue the agglutinated masses resisted staining except
at a complement dilution of 1/16 with the weakest agglutination
reaction.

2. After making doubling dilutions of 0.2 ml. antilympho-
cytic antiserum (ALS), 15 x 10^6 rat thoracic duct lymphocytes
in 0.4 ml. Hanks' solution were added together with guinea pig
serum (complement source) to give a final complement dilution
of 1/40. A control reaction has been carried out along similar
lines on the same batch of antiserum, but with complement
exclusion. After incubation the antiserum titer was recorded
by the degree of the agglutination reaction.
Results: Complement exclusion (control) test showed a strong agglutination reaction and an end point at an antiserum titer of 1/768, while the complement inclusion test showed a weaker agglutination reaction and an end point at an antiserum titer of 1/256.

3. After making doubling dilutions of 0.2 ml. of horse-versus-dog antilymphocytic antiserum in 0.2 ml Hanks' solution, 10 x 10^6 dog thoracic duct lymphocytes suspended in 0.4 ml. Hanks' solution were added. To a similar serial dilution of the antiserum the cells were added together with 0.2 ml of fresh undiluted guinea pig serum (as a source of complement). After 1.5 hours incubation at 37°C, the lymphocyte-agglutination titer in the first set and the cytotoxic titer in the second set were estimated.

Results: Table 5 shows a lymphocyte-agglutination titer of 1/384 and a cytotoxic titer of 1/256. (In the cytotoxicity and the agglutination tests the dilutions were not identical; due to the addition of complement to the former test).

The results of the preceding tests show that:

1. Complement is not required for the agglutination reaction. When used, it weakens the reaction and lowers the titer.

2. Antilymphocytic antiserum expresses its antilymphocytic effect by either a cytotoxic reaction necessitating the presence of complement in optimal
TABLE V

Results of the cytotoxic and lymphocyte-agglutination titers estimation of horse-versus-dog antilymphocytic antiserum as tested with dog thoracic duct lymphocytes.

<table>
<thead>
<tr>
<th>Antiserum batch</th>
<th>Cells source</th>
<th>Cytotoxicity test</th>
<th>Titer</th>
<th>Controls</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/4</td>
<td>1/8</td>
</tr>
<tr>
<td>I</td>
<td>Beagle</td>
<td>Non-viable cells</td>
<td>96.7</td>
<td>95.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>95.2</td>
<td>88.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51.4</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>0.9</td>
</tr>
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<td></td>
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<td></td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>3.4</td>
<td></td>
</tr>
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<table>
<thead>
<tr>
<th>Antiserum batch</th>
<th>Cells source</th>
<th>Lymphocyte agglutination test</th>
<th>Titer</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Agglutination degree</td>
<td>1/3</td>
<td>1/6</td>
</tr>
<tr>
<td>I</td>
<td>Beagle</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Collie</td>
<td>Teck</td>
<td>Agglutination degree</td>
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<tr>
<td></td>
<td></td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Hanks' compl., Hanks'
concentrations, or in its absence, or presence in insufficient amounts, by an agglutination reaction.

3. The results of both tests are comparable.

**Lymphocyte-agglutination technique**

From the data obtained by experimental trials the following standard technique was adopted, using M.R.C. standard pattern agglutination plates:

1. 0.1 ml. Hanks' solution (as diluent) was added to all the holes with the exception of the first.

2. 0.1 ml. antilymphocytic antiserum was added to the first and second holes. Doubling dilutions of the antiserum were made using an automatic syringe adjusted to aspirate and eject 0.1 ml. (see before - the cytotoxicity test). This results in having doubling dilutions of the antiserum in each hole in a volume of 0.1 ml.

3. 0.2 ml. containing $5 \times 10^6$ thoracic duct lymphocytes suspended in Hanks' solution were added to all the holes.

The controls tested in the antiserum assay were: diluent and cells; and diluent, normal serum and cells.

The contents of the holes were mixed by gentle blowing through a Pasteur pipette. The plate was covered with a sheet of paper and another plate to prevent the evaporation of its contents, then incubated at $37^\circ$C. for 1½ hours.

At the end of the incubation period the plate was left at

* R.B. Turner, Inocula House, Church Lane & Hobbs Green, East Finchley, London N.2.
room temperature for a period of 15 minutes, then gently shaken from side to side and the contents were examined both by the naked eye and microscopically.

The results are recorded according to the microscopical examination.

**Macroscopical examination:** This preliminary reading is performed by examining the agglutination plate near a light source, after gentle shaking, cellular clumps of varying sizes are observed in the holes with a positive reaction according to its severity. This is usually seen up to a reaction designated "+" by microscopic examination, *vid infra.* This is seen as numerous very fine clumps.

**Microscopical examination:** After gentle shaking of the plate a sample from the contents of each hole is withdrawn by a fine capillary glass tube and placed on a glass slide side by side and examined immediately under the low power after lowering the condenser. The results are recorded according to the agglutination intensity as judged by the size and number of the agglutinated lymphocyte masses, and the amount of the free non-agglutinated lymphocytes in between, as follows:

- **+++** : 2-4 big clumps with a few small clumps but almost no free cells in between (Fig. 47).
- **++** : 5-8 clumps of moderate size, with some small clumps and free cells in between (Fig. 48).
- **+** : Many small clumps with many free nonagglutinated cells in between (Fig. 49).
- **±** : The end point. Few clumps formed by the agglutination of only a few cells, together with many...
free non-agglutinated cells in between (Fig. 50).

- : Negative reaction, shows a homogenous cell suspension with no agglutinated cells (Fig. 51).

The titer is recorded at the end point (reaction "+") if the controls show a negative agglutination (reaction "-"). If the controls show a slight agglutination (reaction "±"), the titer is then recorded at the last hole with a reaction intensity designated "+".

**Advantages of the lymphocyte-agglutination test**

This test fulfills all the requirements proposed (see before) for a satisfactory routine in vitro assay of antilymphocytic antiserum:

1. **It is accurate in its readings.**
2. **It gives repeatable results when performed on the same batch of antiserum.**
3. **It is simple to execute and record.** Recording the results of one antiserum batch (involving 10 doubling dilutions) is accurately carried out in less than 3 minutes. Multiple batches assay is a simple exercise consuming 30 minutes to record the results of 10 batches.
4. **It gives its results after 1½ hours incubation.**
5. **It involves no elaborate equipment or technical assistance for its execution, processing or recording.** It only requires standard pattern M.R.C. plates, (or Khan tubes), ordinary glass slides, capillary glass tubing and a light microscope.
6. **The test is physiological since the addition of complement results in the transformation of the agglutinated viable cells into non-viable agglutination clumps.**
LYMPHOCYTE-AGGLUTINATION REACTION

Fig. 47
Reaction +++
 x 135

Fig. 48
Reaction ++
 x 135

Fig. 49
Reaction +
 x 135

Fig. 50
Reaction ±
 x 135

Fig. 51
Reaction −
 x 135
7. It gives comparable results to the cytotoxicity test.

The lymphocyte-agglutination method showed its superiority over the cytotoxicity test for routine use in the antilymphocytic antiserum assay. Therefore in all the antiserum assays performed the agglutination test was the method of choice and was performed as a routine, being simple, quick, accurate and of repeatable results.

The effect of horse-versus-dog antilymphocytic antiserum on beagle and mongrel dogs' thoracic duct lymphocytes

This procedure was undertaken to test the feasibility of assaying the antiserum by the lymphocyte-agglutination and cytotoxicity tests, using mongrel dog thoracic duct lymphocytes; also to compare the results with those obtained by using beagle lymphocytes (the breed that donated the lymphocytes for the antiserum production).

The antiserum obtained after three immunizing injections (batch I) gave an agglutination titer of 1/384 and a cytotoxic titer of 1/256 when tested by either cell source.

The effect of freeze-drying on the antilymphocytic potency

A sample of horse-versus-dog antilymphocytic antiserum (batch I) was freeze-dried and its agglutination titer was tested and compared with that of a frozen sample (−20°C. for 7 days).

Both samples had a lymphocyte-agglutination titer of 1/384.
### TABLE VI

Lymphocyte-agglutination titers of various anti-lymphocytic antisera

<table>
<thead>
<tr>
<th>Type of antiserum</th>
<th>Batch No.</th>
<th>Lymphocyte agglutination titer</th>
<th>Normal serum titer</th>
<th>Heterospecific effect on dog thoracic duct lymphocytes</th>
<th>Heterospecific effect on rat thoracic duct lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit versus Rat</td>
<td>I</td>
<td>1/384</td>
<td>&lt;1/5</td>
<td>1/6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1/768</td>
<td>&lt;1/5</td>
<td>-</td>
<td>1/3 &amp; 1/6</td>
</tr>
<tr>
<td>Rabbit versus Dog</td>
<td>I</td>
<td>1/384</td>
<td>&lt;1/5</td>
<td>-</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1/768</td>
<td>&lt;1/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sheep versus Dog</td>
<td>I</td>
<td>1/192</td>
<td>&lt;1/5</td>
<td>-</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1/384</td>
<td>&lt;1/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Horse versus Dog</td>
<td>I</td>
<td>1/384</td>
<td>&lt;1/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>II-IX</td>
<td>1/768</td>
<td>&lt;1/5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Tested with thoracic duct lymphocytes of the species that donated similar cells for raising the antiserum.
Lymphocyte-agglutination titers of antilymphocytic antisera used therapeutically and a comparison of their potency (Table 6)

1. Rabbit-versus-rat antilymphocytic antiserum (ALS) constantly showed a strong agglutination reaction up to 1/48 dilution and a titer of 1/384-1/768.

2. Rabbit-versus-dog antilymphocytic antiserum (R/DALAS) constantly showed a strong reaction up to 1/12-1/48 and a titer of 1/384-1/768.

3. Sheep-versus-dog antilymphocytic antiserum (S/DALAS) constantly showed a moderate reaction up to 1/6-1/48 and a titer of 1/192-1/384.

4. Horse-versus-dog antilymphocytic antiserum (H/DALAS) constantly showed a strong reaction up to 1/96 and a titer of 1/768 (except one batch obtained after three immunizing doses only (batch I) which had a titer of 1/384).

Anti-erythrocytic activity assay

(A) Haemolytic titer estimation:


2. Antilymphocytic antiserum (heated at 56°C. for half-an-hour).

3. Fresh guinea pig serum.

* Oxo Ltd., Thames House, Queen Street Place, London E.C.4.
4. 3% suspension of freshly collected, three times washed, dog red cells in normal saline solution.

The test was carried out in M.R.C. standard pattern agglutination plates.

Procedure: 1. 0.1 ml. oxoid was put in all the holes with the exception of the first.

2. 0.1 ml. antilymphocytic antiserum was added to the first and second holes. Doubling dilutions were then made as previously described.

3. 0.1 ml. fresh guinea pig serum (undiluted) was added to all the holes.

4. 0.1 ml. of 3% red cell suspension was added to all the holes.

The contents of each hole were thoroughly mixed by blowing through a Pasteur pipette. The plate was then covered (see lymphocyte-agglutination test) and incubated at 37°C. for 1½ hours. The results are recorded, without shaking the plates, after comparing the test with the negative control (R.B.Cs + oxoid) macroscopically regarding the haemolysis degree. The results are recorded as follows:

+++ : Complete haemolysis. No cell button.
++  : Moderate haemolysis. Small cell button.
+   : Little haemolysis with a cell button of a moderate size.
+   : The end point and shows only slight haemolysis.
   : Cell button about the same size as that of the control.
-   : Negative reaction. No haemolysis. Cell button the size of that of the control.
(B) Haemoagglutination titer estimation

**Material:**
1. Diluting fluid (oxoid or saline).
2. Antilymphocytic antiserum heated at 56°C. for half-an-hour.
3. 0.5% suspension of freshly collected, three times washed, dog red cells, in normal saline solution.

The test was carried out in M.R.C. standard pattern agglutination plates.

**Procedure:**
1. 0.1 ml. diluent was added to all the holes.
2. 0.1 ml. antilymphocytic antiserum was added to the first and second holes and doubling dilutions were made (see before).
3. 0.1 ml. of a 0.5% red cells suspension was added to all the holes.

The contents of each hole were thoroughly mixed, covered (see lymphocyte-agglutination) and incubated at 37°C. for 1 1/2 hours. The results were recorded after shaking the plates according to the degree of agglutination, macroscopically. The end point was taken at the last hole that showed agglutination which was confirmed microscopically by placing a drop on a glass slide. The strength of the reaction was graded +++ to 1 (end point). Negative reactions recorded "-" where a homogenous cell suspension with no agglutinated cells is seen.
Effect of haemolysins and haemoagglutinins absorption on the antilymphocytic potency of horse-versus-dog antilymphocytic antiserum

Red blood corpuscles contaminate the thoracic duct lymph to a varying degree. This contamination is particularly marked in the dog lymph in comparison to that of the rat. This has been constantly observed during these experiments (see also Yoffey and Courtice 1956).

The antilymphocytic antiserum prepared in the horse had a high haemolysis and haemoagglutinating antibody titers reaching 1/3072 and 1/4096 respectively. To absorb these unwanted anti-erythrocytic antibodies, and to test the effect of such a procedure on the antilymphocytic titer, the following test was performed:— Varying volumes of dog washed packed red cells were added to bottles containing a standard volume of antiserum (see material and methods - antiserum absorption), and the haemolysis, haemoagglutination and lymphocyte-agglutination titers were estimated on the absorbed sera as well as on a non-absorbed sample that had passed through the same procedure as the absorbed samples but without the addition of red cells.

Results: Table 7 and Figure 52 show that as the volume of packed red cells was increased the haemolytic and haemoagglutinative titers dropped to a minimum of 1/6 and 1/8 respectively when the antiserum was absorbed with 75% its volume, or with an equal volume of packed red cells. As the anti-erythrocytic antibody titers dropped by absorption, the antilymphocytic titer remained at the same pre-absorption value.
THE HAEMOLYSIS, HAEMOAGGLUTINATION AND LYMPHOAGGLUTINATION TITERS OF HORSE V. DOG ANTI-LYMPHOCYTIC ANTISERUM (H/DALAS) AFTER ABSORPTION WITH INCREASING VOLUMES OF DOG PACKED RED BLOOD CELLS.

**Fig. 52.**
These results indicate that the antierythrocytic antibodies could be absorbed without altering the antilymphocytic antibody titer of the antiserum, provided that the utmost care is taken to remove the blood lymphocytes from the red cells used for absorption. These results also suggest that the thoracic duct lymphocytes and red cells of the dog do not share antigenic material that is capable of producing an appreciable antibody response.

**In Vivo Assay**

Figures 18 and 19 show the *in vivo* effect in dogs of a single dose (3 ml/kg.body weight) of antilymphocytic antiserum of a standard lymphoagglutination titer (1/768) on the peripheral blood absolute lymphocyte count before, 4 hours and 24 hours after the injection. The dogs were non-isogenic but of the same sex and breed (beagles) that contributed lymphocytes for the antiserum production.

It is seen that the counts dropped 4 hours after the injection to 3.2 - 40.3% and 24 hours after the injection the drop was 6.9 - 70.9% of the pre-injection value. This individual variation in response points to the superiority of the *in vitro* rather than the *in vivo* assay of the antilymphocytic antisera.
Table VII

The haemolysis, haemoagglutination and lymphocyte-agglutination titers of horse-versus-dog antilymphocytic antiserum before and after absorption with dog packed red cells.

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Test</th>
<th>Non-absorbed</th>
<th>Absorbed with (R.B.C.: Antiserum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (H3/6)</td>
<td>Haemolysis</td>
<td>1/1536</td>
<td>1/96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/12</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>Haemoagglutination</td>
<td>1/2048</td>
<td>1/128</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte-agglutination</td>
<td>1/384</td>
<td>1/384</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/384</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/384</td>
</tr>
<tr>
<td>II (H4/7)</td>
<td>Haemolysis</td>
<td>1/3072</td>
<td>1/384</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>Haemoagglutination</td>
<td>1/4096</td>
<td>1/512</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte-agglutination</td>
<td>1/768</td>
<td>1/768</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>1/768</td>
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<td></td>
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<td>1/768</td>
</tr>
</tbody>
</table>
DISCUSSION

"To control nature we must above all understand nature."

Charles Singer 1928

Antilymphocytic antiserum is a powerful immunosuppressive agent. Its administration appreciably prolonged the renal homograft survival performed by vascular anastomosis in non-inbred dogs. The results of the preceding experiments demonstrate that a highly potent antilymphocytic antiserum with marked immunosuppressive properties was prepared in the horse.

The aim of these experiments was to study the effect of antilymphocytic antiserum on renal homografts which are believed to differ in some respects from skin homografts (Woodruff 1965). No attempt was done to treat the dogs to produce long survivors. The antiserum dosage was neither reduced nor increased during the experiment period as planned in the experimental protocol (Fig. 9). The dogs were not kept in a special environment and were not passively immunized during treatment. They received no prophylactic therapy that may ameliorate or mask the antiserum complications, nor did they receive prophylactic antibiotics.

This line of approach was adopted to experiment with, and test the potentialities of the antiserum as an immunosuppressive agent. Also to highlight the complications that may be inherent in such an approach to the renal homograft problem.
Antilymphocytic antiserum was produced in three species, the rabbit, the sheep and the horse. That produced in rabbits and administered to dogs in small daily doses (5-10 ml.) was capable of producing an initial lymphopenia, but was unable to maintain such a state. The antiserum dosage was accordingly increased (Fig. 10) but without a striking consequence. Due to the limited amount of serum that could be obtained from rabbits, the sheep were then selected.

To test the effect of the antiserum on renal homografts survival, higher (than the preceding) doses of the antiserum produced in the sheep were administered to dogs at variable intervals prior to renal homografting and continued till the time of death. The outcome of such experiments showed that this antiserum was not capable of prolonging the renal homograft survival beyond 18 days (range 1-18). The titer and strength of the reaction of this antiserum was lower than that obtained from rabbits. This was due to the different response of different sheep regarding antibody production. Accordingly the horse was selected; knowing that one horse will give an appreciable volume of serum and responds well to antigenic challenge.

An antiserum was produced which had a marked antilymphocytic potency. It showed a strong reaction and a high titer when assayed in vitro. It was administered to dogs at the same dose level as that produced in the sheep.

The results employing the horse antiserum suggest that the antilymphocytic titer is an important factor in immunosuppression.
Since the finding that the homograft rejection is by an immunological process (Gibson and Medawar 1943, Medawar 1944, Medawar 1945) attempts to abrogate such a reaction in mature recipients of homografts were not very successful until the finding that 6-Mercaptopurine suppresses the immunological response in rabbits to crystalline bovine albumin (Schwartz, Stack and Dameshek 1958) and produce tolerance in rabbits towards human serum albumin (Schwartz and Dameshek 1959).

6-Mercaptopurine was used experimentally with skin homografts in rabbits (Meeker, Condie, Weiner, Varco and Good 1959) and in renal homotransplantation in dogs (Calne 1960; Zukoski, Lee and Hume 1960). Similarly it was shown by Calne that its imidazole derivative was a potent immunosuppressive agent (Calne 1961 b, Calne and Murray 1961). Since then immunosuppressive drugs were screened for their effect on canine renal homografts in a search for the least toxic and most potent agent (Calne, Alexandre and Murray 1962; Zukoski, Lee and Hume 1962; Alexandre, Murray, Danmin and Nolan 1963; Zukoski, Callaway and Rhea 1963).

The results of the experimental trials were quite satisfactory and led to the use of immunosuppressive drugs clinically. Table 8 compares the antilymphocytic antiserum results with the results of some of such successful experimental trials.

The table shows that the antiserum compares favourably with such immunosuppressive drugs.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calne, Alexandre 1962</th>
<th>Calne, Alexandre &amp; Murray 1962</th>
<th>Forter, Calne &amp; Zukoski 1964</th>
<th>Thesis Results</th>
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</thead>
<tbody>
<tr>
<td>Total of all groups tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of dogs</td>
<td>14</td>
<td>22</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Survivors beyond 20 days</td>
<td>9</td>
<td>16</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>% Surviving beyond 20 days</td>
<td>64</td>
<td>50</td>
<td>62</td>
<td>62</td>
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<td>Survivors beyond 30 days</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>% Surviving beyond 30 days</td>
<td>64</td>
<td>50</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Survivors beyond 60 days</td>
<td>1</td>
<td>4</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>% Surviving beyond 60 days</td>
<td>64</td>
<td>50</td>
<td>62</td>
<td>62</td>
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<tr>
<td>Mean survival (days)</td>
<td>35</td>
<td>58</td>
<td>45 (23)</td>
<td>57 (32)</td>
</tr>
<tr>
<td>Mean functional survival of transplant (days)</td>
<td>21 (35)</td>
<td>35 (35)</td>
<td>31 (35)</td>
<td>35 (35)</td>
</tr>
<tr>
<td>Remarks</td>
<td>1</td>
<td>2</td>
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<td>4</td>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calne, Alexandre 1962</th>
<th>Calne, Alexandre &amp; Murray 1962</th>
<th>Forter, Calne &amp; Zukoski 1964</th>
<th>Thesis Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total of all groups tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of dogs</td>
<td>14</td>
<td>22</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Survivors beyond 20 days</td>
<td>9</td>
<td>16</td>
<td>1</td>
<td>8</td>
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<tr>
<td>% Surviving beyond 20 days</td>
<td>64</td>
<td>50</td>
<td>62</td>
<td>62</td>
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<tr>
<td>Survivors beyond 30 days</td>
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<td>4</td>
<td>7</td>
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<td>% Surviving beyond 30 days</td>
<td>64</td>
<td>50</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Survivors beyond 60 days</td>
<td>1</td>
<td>4</td>
<td>14</td>
<td>14</td>
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<tr>
<td>% Surviving beyond 60 days</td>
<td>64</td>
<td>50</td>
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<td>Mean survival (days)</td>
<td>35</td>
<td>58</td>
<td>45 (23)</td>
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<tr>
<td>Mean functional survival of transplant (days)</td>
<td>21 (35)</td>
<td>35 (35)</td>
<td>31 (35)</td>
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Table VIII shows selected data of some of the best results obtained by the use of immunosuppressive drugs to abrogate the rejection of canine renal homografts, compared with the results of antilymphocytic antiserum.

**Remarks:**

1. One dog survived for six months.

2. Three dogs survived for more than three months (one more than 8 months).

3. One dog survived for more than three months.

4. The data of this column are reported by the authors for providing a reference point for comparing new immunosuppressive agents. Nine dogs survived beyond three months.

5. Number in parenthesis reported by the authors excludes the largest single survivor (148 days).

6. At the time of the report by the authors one dog was alive 260 days after transplantation.

7. At the time of the report two dogs were alive 330 and 317 days after transplantation.

8. At the time of the report three dogs were alive 163, 265 and 217 days after transplantation.

9. Five dogs died with a functioning homotransplant from a cause other than uraemia. Another dog died with a functioning but failing homograft.

Data in this report include some results obtained by Calne et al 1962; Zukoski et al 1963; Alexandre et al 1963.

* Urinating and ** urinating beyond twenty-one days.

* Mean survival as reported by the authors excluding the dogs living till the time of the report.

One dog excluded (No.801) as it was anuric after the second nephrectomy. Accordingly the period of the homograft function is unknown.

The anuric dog after the second nephrectomy (dog No.801) is included but the period of the homograft function is considered as nil since the duration of the homograft function is unknown.

6-M.P. = 6-mercaptopurine. 6-Methyl M.P. = 6-Methyl mercaptopurine.
The results in the dogs with the use of antilymphocytic antiserum raised in the horse show that the antiserum is a powerful immunosuppressive agent.

The results show (Table IIa) that the renal homograft functioned for more than 21 days in seven of the eight dogs that received the antiserum prepared in the horse. They continued to do so for varying periods from 25-79 days (mean 50 days) after homografting. Six of these dogs died with a functioning transplant, of which only one was failing. This demonstrates that the antiserum suppressed the immunological responsiveness on the part of the recipient towards a renal homotransplant which would otherwise be rejected within 5-21 days, as shown by the control dogs receiving either no treatment or normal serum.

One of the eight antiserum treated dogs (dog No. 801) was anuric since after the second nephrectomy. It showed a sharp rise in the blood urea, serum potassium, creatinine and uric acid similar to those of the control groups. Two of the seven dogs that passed urine showed a rise in their blood urea (dogs Nos. 784 and 799) from after the second nephrectomy till the day of death, 72 and 45 days after transplantation respectively. They evidently had a gradually failing kidney which may suggest that the renal homograft rejection during the antiserum therapy is a slow gradual process; also that the rejection was not reversed despite the continued antiserum administration.

A constant feature in many dogs with some exceptions was
a blood urea higher than the normal value, during the treatment period. This was most marked during the 10 days proceeding the second nephrectomy when the homotransplant was functioning on its own. The most striking example is dog No. 807 which showed a blood urea of 308 mg/100 ml on day +27 and within a further week it dropped to 75 mg/100 ml. Since the blood urea was of a normal value prior to the nephrectomy it is undoubtedly related to the homotransplant function. Whether this is related to the accumulation of the end products of nucleic acid metabolism which may well have been markedly increased above the normal degree due to the destruction of lymphocytes by the antilymphocytic antiserum (if this latter is the mode of the antiserum action) together with the inability of the transplanted kidney to perform extra work above the normal basal level, will be the subject of future especially designed investigation.

It is evident from the histological examination of the transplants (Figs. 38, 39 & compare with 40-43) and from the renal function (Table IIabc and Figs. 33 & 34) that the antiserum succeeded to a marked degree in preventing the rejection of the homografts. The degree of cellular infiltration is by no means a criterion of the function of the homotransplant, and the absence of infiltration does not signify a well functioning kidney, yet the histological appearance of the transplants after such prolonged survival periods evaluated together with the renal function are sufficient evidence
indicating the success in immunosuppression.

Although one dog (No. 801) was anuric since after the second nephrectomy similar to the control groups and died of uraemia; nevertheless the presence of plasma cells in the transplant and the absence of extensive necrosis suggests its more prolonged function in contra-indication to the control animals.

The light and electron microscopic studies on the dogs' own kidney after antiserum administration for three weeks showed no structural abnormality. Nevertheless it would be valuable to study the effect of the antiserum administration on normal kidneys at regular intervals, by both the light and electron microscope, to exclude beyond doubt any kidney change during such treatment.

This approach using antilymphocytic antiserum to abrogate the rejection of renal homografts in dogs, being the first of its kind, puts forward many points to be inquired into.

The antiserum administration was well tolerated by all the dogs during the experiment. They were lively except at its first administration in all eight dogs and during the "secondary serum reaction" in two of these.

Although the complications encountered seem at first sight alarming they are probably to a great degree preventable. The aim in the preceding experiments was not to treat dogs and suppress the complications encountered by means of drugs or withdrawal of the antiserum therapy, on the contrary to high-
light these complications.

It is evident that more experimental work is needed to throw light on the mechanism of some of the complications encountered during sera administration particularly for prolonged periods.

Some form of infection was responsible for the death, with a functioning homograft, of 50% of the antiserum treated dogs. Though exogenous infection is not excluded, it seems reasonable to suspect also an endogenous origin when using such a potent immunosuppressive agent. Since these dogs were actively immunized by a single injection against canine diseases one month up to one year prior to admission, it seems that the antiserum weakened or even abolished not only the primary immunological responsiveness to the renal homotransplant, but also the secondary response towards the diseases they were actively immunized against. Recently Levey and Medawar obtained definite criteria regarding the suppression of the secondary immune response. They markedly prolonged the survival of second set skin homografts in mice that received antilymphocytic (anti-lymphoid) antiserum (Levey and Medawar 1966).

Thus, regarding infection and the abolition of pre-existing immunity to bacteria, it would be valuable to actively immunize an experimental group and follow their antibody titer during treatment with antilymphocytic antiserum, then test their secondary immunological response to further antigenic challenge. Similarly it is suggested to study the effect of the antiserum on second set renal homografts which will also give information on abolition of pre-existing immunity.
In the light of these experiments it may be necessary to passively immunize the antiserum recipients in future work. This has been adopted with immunosuppressive drugs experimentally (Calne, Alexandre and Murray 1962). It may prove to be quite necessary with the use of antilymphocytic antiserum.

Though the liver function was not studied, the antiserum in the dosages administered in these experiments and for such a prolonged period was feared to have a deleterious effect on this organ, especially since it is not known how dogs would eliminate or metabolise such big volumes of heterologous serum proteins. The liver in five (including the four that died of infection) of the eight dogs that received the antiserum showed lobular necrosis; all these had received the antiserum for more than five weeks. This may be explained as previously discussed by infection of either an exogenous or an endogenous origin. Another possibility that is worth considering and thoroughly investigating is that large doses of heterologous antiserum, or one or more of its fractions or a product of its interaction in vivo, may be toxic to the liver cells. It has been shown that when \(^{131}\)I labelled horse and globulin was injected intravenously into sensitized rabbits the antigen-antibody complex formed was eliminated from the circulation within one hour and was deposited mainly in the liver (Francis, Hawkins and Wormald 1957). Chew and Lawrence observed "peculiar appearance of the liver cells" but no necrosis, in guinea pigs receiving multiple injections of normal
rabbit serum as well as antilymphocytic serum (Chew and Lawrence 1937). Levey and Medawar found that antiserum prepared in rabbits against guinea pig thymus and lymph nodes was toxic to guinea pigs, and resorted to its absorption with guinea pig liver, also with kidney and lung (Levey and Medawar 1966).

These findings suggest considering the effect of heterologous sera on such a vital organ. This may be achieved by studies on the liver functions during antiserum treatment; by studies on the fashion by which the experimental animal handles heterologous (preferably horse) proteins by the blood and organ (liver) clearance of the labelled protein; also by in vitro studies. These latter may be investigated along similar lines to what has been described in the in vitro section. Although Richie showed that the incubation of thymolytic serum (duck-anti-guinea pig thymus) with liver did not fix complement (Richie 1908), nevertheless in vitro studies may prove worthy.

The administration of horse serum in the renal homograft experiments resulted in sickness reactions. These were referred to as the "primary" and "secondary" serum reactions. This was to avoid the terms "immediate" and "delayed", since the nature of the reactions in these dogs needs further investigation. However these experiments showed that all the antiserum treated dogs manifested the "primary serum reaction" while only one dog in the normal serum treated group manifested this reaction in a very mild form.

This reaction needs further immunological study as the
previous history of these dogs is not known with respect to previous sensitizations.

The "secondary serum reaction" manifested in all the normal serum treated dogs (100%) but only in two of the eight antiserum treated (25%). Also the reaction in the normal serum treated dogs manifested after 7-8 injections, while with the antiserum treated it manifested after 11 and 13 injections (i.e., somewhat delayed).

This "secondary serum reaction" is most probably what is known as "delayed serum sickness" or "serum sickness proper".

This difference in response of the two groups of dogs is of interest as it suggests that it is due to the immunosuppressive effect of the antiserum, which not only suppressed the immunological response towards the renal homograft but also that towards heterologous plasma proteins. The fact that the two dogs that received the antiserum and manifested such a reaction rejected their kidneys favours such a suggestion, one of these rejected its transplant within 21 days (dog No. 801), and the other after 45 days, having had a high and gradually rising blood urea signifying gradual rejection due to incomplete immunosuppression.

The fact that the dogs that received antiserum produced in the sheep did not manifest such a reaction and also rejected their transplants within 1-18 days, suggests that horse serum, which is known to cause serum sickness reactions, is either more powerful in that respect than sheep serum, or that it
contains a component which is responsible either directly or indirectly for such a reaction.

It has been established that the repeated injection of antigen incites antibody production; also that antigen-antibody complexes are formed during this procedure. These latter are known to be responsible for the release of pharmacologically active substances, such as histamine, 5 hydroxytryptamine and acetylene choline. These substances are believed to be the incitors of anaphylactic and serum sickness reactions (Weigle 1961; Dixon 1963; Gell 1963).

It has been shown that histamine was increased in the plasma (shift from cells to plasma) on the addition of antigen to the blood of sensitized rabbits, dogs and guinea pigs in vitro (Katz 1940). Barbaro showed in vitro that histamine is released from the platelets (Barbaro 1961). He demonstrated that when the antigen-antibody complex was formed while the antigen was in excess no histamine release was obtained. On the other hand an augmented histamine release was obtained when the complex was formed either in antigen-antibody equilibrium or in antibody excess (Barbaro 1961).

It has been shown that antihistaminic drugs suppress anaphylactic reactions incited by antigen-antibody complexes; also that these reactions occur with antigen-antibody complexes in antigen excess, but to a lesser degree than when both are in equivalence or in antibody excess (Weigle, Cochrane & Dixon 1960). Weigle et al similarly showed that pulmonary emphysema produced
during the anaphylactic reaction in guinea pigs incited by the antigen-antibody complexes could be inhibited by antihistaminics administration.

One of the most alarming symptoms in the "secondary serum reaction" met with antilymphocytic antiserum administration was respiratory distress manifesting as dyspnea. In these dogs no study was made on the histamine and antihistaminics and it is suggested that these should be investigated in the light of the above findings.

Another complication encountered in the dog experiments was massive bleeding. Two of the 16 dogs that received antilymphocytic antiserum raised in the sheep manifested such a condition. It was responsible for the death of one dog (No. 690A). The other dog (No. 712) had only one such attack, which it survived. Both dogs developed this condition manifesting as gastro-intestinal bleeding after six antiserum injections of 3 ml/Kg body weight/day. As both these dogs received the antiserum prior to transplantation, they had no renal homotransplant at the time of bleeding. Similarly, severe gastro-intestinal bleeding was partly or completely responsible for the death of two dogs receiving antiserum prepared in the horse and administered in the same dosage and by the same route (intravenously) as the preceding group. One dog (No. 784) died 72 days after homotransplantation and the other (No. 804) died 25 days after this procedure. It is also of interest to note that three of the five dogs that received normal horse serum
manifested such a bleeding tendency. Two of these died after seven injections, from intra-peritoneal haemorrhage; in one dog (No. 845) the source could not be identified at autopsy; in the other (No. 839), which also manifested gastro-intestinal bleeding, it was from the transplant vascular anastomosis. The third dog (No. 842) developed and survived the gastro-intestinal bleeding attacks after the 10th and 15th injections. Since the dogs that received no treatment did not manifest such a condition, it is evident that the serum is the responsible agent. Dogs that received normal sera manifested "secondary serum reaction" and the bleeding attacks were related in time to such a reaction.

These findings suggest a histamine action. It is known that histamine in dogs causes capillary and arteriolar dilatation but hepatic vaso-constriction and consequently engorgement of the liver and splanchnic congestion (Wilson and Schild 1961). This is favoured by the fact that no lesion could be found to attribute for the bleeding. Also the findings of Katz and of Barbaro that histamine is released in the plasma of sensitized animals (Katz 1940; Barbaro 1961) favours this suggestion. Other possibilities worth considering and investigating are the vascular lesions which have been shown to result from antigen-antibody complex formation (Cochrane and Weigle 1958); also the study of the blood clotting factors and mechanism in the serum treated dogs as has been kindly suggested to me by my supervisor, Professor Woodruff.

Accordingly the bleeding tendency may be due to an immunological reaction inciting pharmacological response or due to altered blood
coagulation mechanism.

The substitution of whole unpurified antilymphocytic antiserum by purified antibody fractions and their administration by another route other than intravenously may reduce some of the complications encountered, though these will probably have no effect on "secondary serum reaction". In the light of the findings regarding the role of histamine and the effect of anti-histaminics on this reaction, the latter may be of value whenever such reactions tend to occur. It has been seen that the antiserum suppresses this reaction to a marked degree, nevertheless it may still occur during treatment with only partial immunosuppression.

Another complication, though not met with in these experiments but should be thoroughly watched for and investigated with prolonged periods of antiserum administration, is malignant conditions of the lymphocyte and reticular cells.

The state of blood and tissue lymphocytes during antiserum administration occupied part of every paper dealing with antilymphocytic antiserum. This will continue until the mode of action of the antiserum is clarified; whether by lymphocyte destruction (consequently depletion) or otherwise. Woodruff and Anderson showed that depletion of 1-2 billion lymphocytes by thoracic duct drainage prolongs skin homograft survival for a few days. This procedure, combined with antiserum administration for two weeks after grafting, prolongs the homograft survival to a mean of 35 days. If the antiserum administration was extended to 90 days the mean survival was 110 days and a state of partial tolerance (tolerance as defined by Woodruff 1960, 1965) was achieved in two rats. Nevertheless the
lymphocyte count rose to the pre-injection level by the 4th week but the spleen showed lymphocyte depletion (Woodruff and Anderson 1964). Similar results were obtained in mice which showed recovery from the lymphopenic state by the 4th week. Lymphopenia frequently recurred by the continued antiserum administration (Gray, Monaco and Russell 1964). Similarly it was shown that a shorter period of antiserum administration (7 injections) resulted in lymphopenia and tissue lymphocytes depletion. These returned to the normal level four weeks after commencing the treatment, and so did the immunological competence of the animals which rejected the skin homografts by then (Monaco, Wood and Russell 1965). It was recently shown that very short term treatment with antilymphoid antiserum prolongs the homograft survival with only a moderate degree of tissue lymphocyte depletion and transient lymphopenia (Levey and Medawar 1966).

The fact that the mean lymphocyte count of the whole group of antiserum treated dogs (horse-versus-dog antiserum) but not that of those which received sheep-versus-dog antiserum, was lower than 50% of the mean pre-injection value; also the moderate to marked tissue lymphocytes depletion of the former group is not an indication that lymphopenia and tissue lymphocytes depletion underly the prolongation of the renal homograft survival in the horse-versus-dog antiserum treated group. This state of lymphopenia and tissue lymphocytes depletion may well have been caused by the unnecessary (if so) continued antiserum administration. Whether shorter treatment periods give similar results regarding these
data as evaluated with the homograft survival remains to be seen.

The mode of action of the antiserum *in vivo* is by no means established. From the *in vitro* results it is shown that in the presence of complement in ample amounts, the antiserum is lymphocytotoxic. Whether this effect of the antiserum is partly or wholly responsible for prolonging the survival of homografts remains to be seen by investigating the hypotheses of the mechanism of the antiserum action proposed by Levey and Medawar (1966).

If a prolonged period of treatment of the renal homograft recipients with antilymphocytic antiserum is necessary it remains to be seen how long is the duration of the "critical period" of the homograft. The "critical period" hypothesis enunciated by Woodruff in 1952 (Woodruff 1952, 1954): "homotransplants become progressively less vulnerable as time goes on, and after a certain critical period, are capable of surviving in the face of a high degree of immunity in the recipient which they would not have been able to withstand earlier in their life history" (Woodruff 1954); did we pass this period in the dog experiments? Was the antiserum administered in excess and the grafts would have survived despite cessation of the treatment? The answers will be obtained with shorter periods of therapy, as probably at least some of the complication could be avoided by such an approach.

Two dogs received half the usual dose of antiserum (prepared in the horse) from the beginning of the experiment (dogs Nos.802
and 805), otherwise the protocol was identical to the usual procedure. These two dogs were anuric after the second nephrectomy and died 4-5 days after this procedure (No. 805 sacrificed due to condition), with a blood urea of 364 and 350 mg/100 ml. Both transplants were similar to the control groups (one as that of dog No. 840 and the other as 843 - Table IIIb).

The results obtained from two dogs (not included in this thesis) are insufficient evidence of the failure of the low dosage to suppress the homograft reaction, but cast some doubt on the effect of low dosage in this respect. Shorter periods of full dosage administration may be more effective; nevertheless extension of the low dosage experiments are necessary.

Various methods have been employed to indicate cellular viability. Dyes are the most commonly used for this purpose. Amongst these are trypan blue (Evans and Schulemann 1914; Pappenheimer 1917a); eosin (Schrek 1943); safranin (Schrek 1949) and negrosin (Sacks, Filippòne and Hume 1964).

Two methods were used for assaying the antilymphocytic potency of the antiserum; one demonstrated its cytotoxic property, the other its agglutinative effect. To assay the cytotoxic potency of the antiserum, trypan blue was employed as the indicator of cellular viability.

Antilymphocytic antiserum requires the presence of complement to exert its cytotoxic action on lymphocytes. In that respect it behaves like other antibodies directed against red cells and bacteria. Though most of the cytotoxic reactions
with these latter require complement in an initial dilution of 1/10 (i.e., about 1/30 final dilution), antilymphocytic antiserum cytotoxic reaction required undiluted complement of 1/2 initial dilution (i.e., 1/4 - 1/8 final dilution respectively) to express its full antilymphocytic effect. It would be of interest to further investigate the underlying mechanism of this finding.

Contrary to the cytotoxic reaction, lymphocyte-agglutination is complement independent. The concept of agglutination, being one of the most classic reactions in serology, is by no means new. The ability of specific antisera to agglutinate lymphocytes has been previously shown (Pappenheimer 1917 a, Steinberg and Martin 1944, 1946; Amos 1946). Lymphocyte agglutination was employed in assaying the antilymphocytic potency of the antilymphocytic antisera prepared during this thesis. It was found to be a satisfactory method of assay; it also demonstrated the species specificity of the antisera. Species specificity of the antisera was also shown by earlier investigations (Metchnikoff 1899; Ritchie 1908; Pappenheimer 1917 a, b).

The feasibility of assaying rabbit-anti-mouse lymphocyte antisera by the lymphocyte-agglutination method was similarly demonstrated by Gray et al (1964); Monaco et al (1965) reported its species, but not strain specificity.

For assaying the antilymphocytic potency of antisera produced, the lymphocyte-agglutination method has been reliable, simple & accurate, which made it the method of choice in this respect. The ability of agglutinating lymphocytes implies that the antigens are at the surface or near it.
The results of the experiments presented show, for the first time, that antilymphocytic antiserum raised in the horse and administered to dogs bearing a renal homograft performed by vascular anastomosis, markedly prolonged the homograft survival. Also highlight the potential complications that may be inherent in such an approach. They demonstrate that the antiserum is a powerful immunosuppressive agent. They confirm the insight of Woodruff and Woodruff when they first introduced antilymphocytic antiserum to tissue transplantation and in some ways extend the results of Woodruff and Anderson.

These results point to the prospect and feasibility of using the antiserum in the near future, after further study of its biological properties and further screening of its results, as an immunosuppressive agent in human organ transplantation.

Nothing is more suitable to conclude my discussion than the words of Metchnikoff, the first producer of antilymphocytic antiserum:

"D'un autre côté, ces sérum anticellulaires peuvent être employés comme moyen d'étude physiologique sur le fonctionnement de certains organes, difficiles on impossibles à éliminer de l'organisme par voie chirurgicale. Quelques - uns d'entre ces sérum peuvent recevoir encore d'autres applications."

Metchnikoff 1899
PART VI
SUMMARY

"L'auteur se tue à alonger ce que le lecteur se

tue à abréger"

D'Alembert

Antilymphocytic antiserum was produced against dog thoracic
duct lymphocytes in three species, the rabbit, the sheep and the
horse.

In rabbits the antiserum was prepared from blood collected
seven days after the last of the three immunizing intra-peritoneal
injections of $2 \times 10^8$ lymphocytes administered at weekly intervals
and after the further boosting injections of $1 - 1.5 \times 10^8$ lympho-
cytes by the same route. The intravenous administration of the
antiserum to dogs in small daily doses of 5-10 ml for one to
three months was effective in producing an initial temporary
lymphopenia. The small amount of serum obtained and the vast
number of rabbits that had to be used, restricted their choice
as a source of antiserum for the renal homografting experiments;
consequently the sheep were selected.

The sheep were immunized along similar lines to the rabbit,
injecting $0.75 - 1 \times 10^9$ and $0.5 - 0.75 \times 10^9$ lymphocytes for
immunizing and boosting respectively. The antiserum administered
to dogs in a daily intravenous dose of 3 ml/Kg. produced a temporary
moderate lymphopenia. It was not effective in prolonging the
renal homograft survival in 15 bilaterally nephrectomized dogs
that received daily injections beyond 18 days (range 1-18). These died of uraemia. Accordingly the horse was selected.

A highly potent antilymphocytic antiserum was prepared by the intravenous injection of $0.8 - 2.7 \times 10^9$ beagle thoracic duct lymphocytes at weekly intervals. The antiserum was prepared from blood collected 7 days after the last immunizing injection and after the further booster injections of $1 \times 10^9$ lymphocytes at monthly intervals.

Eighteen beagles received a renal homograft and had one of their own kidneys removed on day 0, and the other, 21 days later.

Eight dogs received antilymphocytic antiserum one day prior to homografting in a single daily intravenous injection of 3 ml/kg body weight, for 60 consecutive days. The dose was halved thereafter. Five dogs received normal horse serum along a similar protocol and five dogs received no treatment.

The antiserum recipients survived for a period of 25-79 days after homografting (mean 50 days). Six dogs died with a functioning homograft. Two of these from distemper, one from hepatitis, one from pneumonia and two from massive gastrointestinal haemorrhage. The other two dogs died from uraemia. One was anuric after the second nephrectomy and died 6 days later, the other 45 days after transplantation.

None of the dogs which received normal serum or were untreated survived beyond 26 days (the first 3 weeks of which they had one of their own kidneys still in situ). All were
anuric after the second nephrectomy and died 4-5 days later from uraemia.

The homograft was completely necrotic in the control group (except those of the three which died 5 days after transplantation), in contra-distinction to the antiserum treated.

The antiserum injections were well tolerated by all the dogs except on its first administration when they all showed a reaction, "primary serum reaction". Only one dog manifested this reaction in a very mild form with normal serum administration. Two of the 8 antiserum treated, while all the normal serum treated dogs manifested the "secondary serum reaction".

A single antiserum injection resulted in a marked depression of the blood absolute lymphocyte count, but its daily administration produced a moderate lymphopenia together with moderate to marked small lymphocyte depletion of the mesenteric lymph nodes and spleen.

Two methods for assaying the antilymphocytic antiserum in vitro were used. One demonstrated its cytotoxic action and is complement dependent; the other demonstrated its agglutinative effect and is complement dependent. The latter method, the lymphocyte-agglutination test, was used as a routine for its accuracy and simplicity. Antilymphocytic antiserum produced in the rabbit showed a strong reaction and a titer of 1/384; that produced in sheep showed a weak reaction and a titer of 1/192-1/384, while that produced in the horse showed a strong
reaction and a titer of $1/768$.

Proposed experimental procedures and investigations for further work were discussed. The possible use of the antiserum in human organ transplantation after further studies of its biological properties is suggested.
CONCLUSIONS

1. Antilymphocytic antiserum is a powerful immunosuppressive agent. Its administration to dogs with renal homografts performed by vascular anastomoses appreciably prolonged the homograft survival without resort to other subsidiary means of immunosuppression.

2. Without attempting to treat the antiserum side-effects, its administration is not without complications. These include lowered resistance to infection, serum sickness reaction and bleeding tendency. Prophylactic measures to counteract these complications should be taken.

3. The antilymphocytic potency as assessed by the antiserum titer in vitro is an important factor in immunosuppression.

4. The antiserum potency could be assayed in vitro by two methods. One demonstrates its cytotoxic action (complement dependent), the other its agglutinative property (complement independent). The latter method, the lymphocyte-agglutination test, is the more simple and quick of the two procedures of assay.

5. The possibility of using antilymphocytic antiserum in human organ transplantation in the near future after further biological studies of its properties and mode of action, together with screening of its results, is suggested.
"... Nature does not always allow herself to be conquered by logic, her ways are frequently hidden, and she reveals herself only to him who has sharpened his sight for insignificant traces by persistent faithful observation."

Wilhelm His
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Transplantation (1964). (a) 2: 147; (b) 2: 561.


Some of the work reported in this thesis has been submitted for publication:

Abaza H.M., Nolan B., Watt J.G., Woodruff M.F.A.

The effect of antilymphocytic serum on the survival of renal homotransplants in dogs.

Transplantation .. in press

Abaza H.M., Woodruff M.F.A.

In vitro assay of antilymphocytic serum.

Clin. exp. Immunol .. submitted

Woodruff M.F.A., Anderson N.F., Abaza H.M.

Experiments with antilymphocytic serum.

Bristol lymphocyte symposium;
Ed. J.M. Yoffey, London,
Edward Arnold ... in press