THE ENERGY METABOLISM OF LYMPHOCYTES

A thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

by

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SECTION B

SUMMARY
(i) The Oxygen Tension of Popliteal Efferent Lymph

The $P_{O_2}$ of popliteal efferent lymph measured in vivo before antigenic challenge was in the range 41-54 mm Hg (6.3 - 8.2% $O_2$) with a mean and standard deviation of 46.6 ± 5.3 mm Hg (7.1 ± 0.8% $O_2$). The $P_{O_2}$ of the lymph changed significantly following antigenic challenge, and was found to vary inversely with the concentration of cells in the lymph. The consumption of $O_2$ by the cells in the lymph thus determined, at least in part, its $P_{O_2}$ in vivo.

(ii) The Rate of Gaseous Exchange in Lymphocytes collected from Popliteal Efferent Lymph

The rate of $O_2$ consumption by normal lymphocytes was 1.254 ± 0.204 µmoles/hr/2 x 10^8 cells as determined by "Direct" Warburg manometry, 2.476 ± 0.476 as determined by "Indirect" Warburg manometry and 1.699 ± 0.226 as determined by the Clark oxygen electrode method. The rate of $CO_2$ production was 0.973 ± 0.145 µmoles/hr/2 x 10^8 cells as determined by "Direct" Warburg manometry and 2.476 ± 0.476 as determined by "Indirect" Warburg manometry. After antigenic stimulation with Salmonella organisms, the rate of gaseous exchange in lymphocyte populations increased significantly. Maximum values were always recorded when the percentage of blast cells in the cell populations reached its peak. This phenomenon was observed with all three methods of monitoring the respiration of lymphocytes and was not related to the antigenic stimulus. Polymorphonuclear neutrophils which appeared in the lymph in large numbers on day 1 after antigenic challenge were found to respire at much higher rates than normal lymphocytes.
(iii) The Michaelis Constant and the Critical Oxygen Concentration for Lymphocyte Respiration

It was found that the rate of $O_2$ consumption by lymphocytes given a limited $O_2$ supply could be related to the $P_{O_2}$ by the Michaelis-Menten equation. The concentration of $O_2$ at which the respiration rate of the cells began to fall (the critical $O_2$ concentration, $[O_2]_{crit}$) was $1.998 \pm 0.197 \, \mu M (1.477 \pm 0.144 \, \text{mm Hg})$. No significant differences were observed in $[O_2]_{crit}$ value before or after antigenic stimulation. Since the $[O_2]_{crit}$ value was very low compared to the $P_{O_2}$ of the popliteal efferent lymph, it was concluded that lymphocytes in lymph were respiring at maximal rates all the time. The $O_2$ concentration at which the rate of $O_2$ consumption for normal lymphocytes was half maximum (the Michaelis constant, $K_m$) was $0.462 \pm 0.108 \, \mu M$ with a range of $0.342 - 0.628 \, \mu M$. This value was about one-quarter of the $[O_2]_{crit}$ value. After antigenic challenge, the $K_m$ values changed significantly and reached a maximum when the cellular response was at its peak. It was found that a linear correlation existed between the $K_m$ and the $V_{max}$ (maximum rate of $O_2$ consumption). The increased $K_m$ values observed when large numbers of blast cells were present in the lymph was thought to be due to the large cytoplasmic volume of these cells representing an increased diffusion distance for $O_2$ from the suspending medium to the mitochondria.

(iv) Glucose Uptake and Lactate Production by Lymphocytes responding to Salmonella muenchen Organisms

In normal sheep, the level of glucose in lymph was found to vary from $3.52 - 4.70 \, \text{mM}$ with a mean and standard deviation of $4.01 \pm 0.33 \, \text{mM}$. Following the injection of antigen, no
significant change occurred in the glucose level of the lymph except for the first few hours after antigen administration when an obvious fall in glucose concentration was always observed. This phenomenon was thought to be due to the high rate of glucose uptake by the large numbers of neutrophils which appeared in the lymph at this time. Normal lymphocytes had a rate of glucose uptake of $0.970 \pm 0.290 \mu$moles/hr/$2 \times 10^8$ cells and a rate of lactate production of $1.306 \pm 0.368 \mu$moles/hr/$2 \times 10^8$ cells. These rates changed significantly following antigenic challenge over a similar time course to that observed for gaseous exchanges. The maximum values occurred when the cellular response was at its peak in both primary and secondary responses. Polymorphonuclear neutrophils were found to have high rates of glycolysis.

(v) The Metabolism of C-1- and C-6-labelled Glucose by Lymphocytes

It was observed in radiorespirometric studies that sheep lymphocytes produced $^{14}CO_2$ from C-1-labelled glucose at a much higher initial rate than from C-6-labelled glucose. This suggested that the pentose phosphate pathway, as well as the glycolytic pathway plus Krebs cycle were operating actively in these cells. After antigenic challenge the rates of $^{14}CO_2$ production from C-1- and C-6-labelled glucose increased and reached maximum values when the number of blast cells in the lymph reached its peak. Polymorphonuclear neutrophils produced $^{14}CO_2$ from C-1-labelled glucose at much higher rates than did lymphocytes. These observations indicated that the pentose phosphate pathway, glycolysis and the Krebs cycle were stimulated in sheep lymphocytes following in vivo antigenic challenge.
(vi) The Effect of Oxygen Tension on the Energy Metabolism of Lymphocytes

It was found that the rates of energy metabolism of sheep lymphocytes were affected by the partial pressure of oxygen ($P_{O_2}$) in the gas phase under which the cells were metabolizing. With decreasing $P_{O_2}$ from 21% to 0%, the rates of glucose uptake and lactate production increased while the rates of $^{14}C\text{CO}_2$ production from C-1- and C-6-labelled glucose decreased. These phenomena were explicable in terms of the effect of availability of oxygen on the formation of ATP. The oxygen supply affected the relative activity of glycolysis versus the Krebs cycle and oxidative phosphorylation. The biosynthesis of ribose through the oxidative route of the pentose phosphate pathway compared with that produced through the nonoxidative route of the pathway was also affected by $P_{O_2}$. This finding indicated that most of the results on energy metabolism studies carried out by other workers under air were not applicable to the physiological activities of lymphocytes in vivo.

(vii) The Incorporation of $^3H$-thymidine, $^3H$-uridine and $^3H$-leucine by Lymphocytes

Rates of incorporation of $^3H$-thymidine, $^3H$-uridine and $^3H$-leucine by lymphocytes into DNA, RNA and protein respectively were found to be greatly enhanced in the blast cells which appeared in the lymph during an immune response to Salmonella organisms. This meant that the blast cells were actively synthesizing nucleic acids and proteins. Polymorphonuclear leucocytes did not show any significant incorporation of these isotopes. Autoradiographic studies confirmed that blast cells incorporated $^3H$-uridine and $^3H$-leucine at much higher rates than
small and medium lymphocytes. $^3$H-thymidine was found to be incorporated into nuclear DNA only by the transforming blast cells. Few small or medium cells were found to be synthesizing DNA. The $^3$H-thymidine was incorporated only into the nucleus of the cells, the majority of the $^3$H-uridine was also found in the nucleus but some was present in the cytoplasm while the $^3$H-leucine was incorporated throughout the cytoplasm.

(viii) Energy Requirements of Lymphocytes

Normal small and medium lymphocytes have relatively low energy requirements for processes such as protein and nucleic acid synthesis. Their major energy requirements would appear to be related to their migratory habits. While only 5-10% of normal lymphocytes exhibited active movement in coverslip preparations in vitro, the fact that most of these cells had arrived in the lymph following migration from the blood stream through the tissues indicated that the entire small and medium lymphocyte population is, at some stage, actively motile. Although no significant increase occurred in the number of motile small and medium lymphocytes in the in vitro assay after antigenic challenge, the tremendous increase in cell output during the "recruitment" phase of the response (the period in between "shutdown" and the peak of blast cell response) indicates the high degree of motility of these cells.

The blast cells which appeared in the lymph in large numbers were highly motile and as many as half of them could be identified moving rapidly in the coverslip assay. These cells which were migrants from the lymph node would have a significant requirement for metabolic energy for their movements and migrations. In addition to this, the large
blast cells required energy for the high rates of protein, RNA and DNA synthesis associated with their enlargement and proliferation in response to antigenic challenge. A further requirement for energy would relate to the synthesis and secretion of specific antibodies by these cells, which is their primary responsibility during the immune response.
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The work reported in this thesis was done in the Department of Immunology, The John Curtin School of Medical Research, The Australian National University. Here, I would like to record my appreciation to the University for the generous offer of an A.N.U. Scholarship. Specifically, I am grateful to Professor Beca Morris not only for supervising and assisting my research, but also for his constructive criticism during the preparation of the thesis manuscript. I am also indebted to Drs. for arguing with me on perspective of the ethics of science and for the high standards he demands of his fellow scientists.

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Other members of the Department have also rendered much assistance and advice during my stay and research in Canberra. Special thanks are due to Miss Wendy Irudia for her help with those lymphocyte calculations.

Last but not the least, my appreciation goes to my wife, who has been very patient and understanding during the past three years and to whom I would like to dedicate this thesis. My appreciation also goes to my daughter whose loveliness has always been such an inspiration.

The thesis manuscript was typed by Mrs. Jean Gillham.

To my Wife
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CHAPTER 1

INTRODUCTION

Studies on the metabolism of blood leukocytes began early this century when Lopine and his co-workers (1909) examined the capacity of these cells to oxidize glucose by glycolysis. Levere and Slayer (1912) showed that lactic acid was produced by leukocytes while Sleeper (1912) provided quantitative estimates of the extent of this reaction. Maclean and Metz (1915) compared the glycolytic capabilities of human white and red blood cells and found that glycolysis in leukocytes was 200-1000 times more active than in erythrocytes. Barron and Norred (1929) studied systematically the metabolic behaviour of human blood leukocytes and dog thoracic duct lymphocytes, and compared the metabolic activities of cells of the granulocytic series with the non-granulocytic elements, and with other cells of different stages of maturation. They also showed that the metabolic activities of human white blood cells were similar to that of other cancer cells and cells obtained from embryonic tissues. The studies on the metabolism of leukocytes up until the 1950's were mainly focussed on carbohydrate metabolism and were summarized in a comprehensive review by Beck and Valentino (1953).

Most of the early research into the metabolism of white blood cells was done on buffy-coat preparations which contained polymorphs, monocytes and lymphocytes. Attempts to assess the metabolic activity of the individual classes of leukocytes were frustrated by the difficulty of obtaining mononuclear populations of cells uncontaminated by red cells and other species of white cells. As far as the lymphocyte was concerned, this difficulty was overcome by the development of a variety of efficient methods...
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for isolating lymphocytes from different sources such as the lymph, blood and lymphoid tissues.

The preparation of "pure" populations of lymphocytes has been reviewed by Ling and Kay (1975), Natvig, Perlmann and Wigzell (1976) and Ford and Hunt (1978). The separation of lymphocyte sub-populations was reviewed by Natvig et al. (1976) and Hunt (1978). It is important to recognize that lymphocytes from various sources have a range of morphological and functional attributes which specify their organ of origin, certain aspects of their life history and their metabolic state at a particular moment of time.

Lymphocytes isolated by a variety of methods have been extensively studied with respect to their normal metabolism and the metabolic changes induced by a variety of in vivo and in vitro circumstances.

As early as 1888 Renant (quoted by Ling and Kay, 1975) suggested that some circulating lymphocytes transformed in vivo into connective tissue cells. Maximow (1902) subsequently contended that blood lymphocytes could transform morphologically and functionally under an appropriate stimulus into other types of cells. The phenomenon of in vitro leucocyte stimulation was first described by Hungerford and his co-workers (Hungerford, Donnelly, Nowell and Beck, 1959). They added phytohaemagglutinin (PHA), an extract of the red kidney bean (Phaseolus vulgaris) (Li and Osgood, 1949; Rigas and Osgood, 1955) to a culture of human blood leucocytes to induce mitosis in the white cells in order that they might study the chromosome constitution of a human phenotypic intersex. Nowell (1960) then described in some detail the lymphocyte response to PHA and the proliferation and transformation of small lymphocytes into blast cells. Carstairs
(1961, 1962) showed for the first time that the small lymphocytes were the source of the dividing cells and this was confirmed later by Brandt, Börjeson, Nordén and Olsson (1962). Lindahl-Kiessling and her co-workers (Lindahl-Kiessling, Werner and Böök, 1963) isolated populations of lymphocytes from human thoracic duct lymph and found that these cells underwent transformation in the same manner as blood lymphocytes when cultivated with PHA.

Lymphocytes can be stimulated in vitro in at least four different ways (Roos and Loos, 1970; Cooper, 1973): 1. by non-specific stimuli, such as PHA, concanavalin A, pokeweed mitogen, staphylococcal filtrate and streptolysin S, 2. by specific antigens to which the lymphocytes have been previously sensitized, 3. by allogeneic lymphocytes (mixed lymphocyte reaction), 4. by products of leucocyte metabolism (lymphokines).

There are a number of reviews of lymphocyte transformation induced by various mitogens in the literature (Robbins, 1964; Cooper and Amiel, 1965; Mellman, 1965; Gowans, 1966; Heath, 1966; Dutton, 1967; Douglas, 1972; Cooper, 1973; Ling and Kay, 1975).

(b) METABOLIC CHANGES OCCURRING IN STIMULATED LYMPHOCYTES

Studies on the phenomenon of lymphocyte stimulation have utilized two main types of approach. In the first, attempts have been made to uncover the mechanism of lymphocyte activation by characterizing the receptor sites on the lymphocytes which bind the stimulating molecules. Both direct and indirect techniques have been used to determine the fate of the stimulants after they bind to the receptors (Cooper, 1973; Ling and Kay, 1975;
Culvenor and Weidemann, 1976). The second approach has involved studies on the biochemical changes which occur in stimulated lymphocytes during the period after the cells have been stimulated with the aim of identifying the key metabolic effects produced by the stimulants. It is useful to summarize here the results of previous investigations into the metabolic changes that occur in lymphocytes stimulated \textit{in vitro} in relation to the experiments which form the basis of this thesis. The events which occur when cultivated lymphocytes are exposed to mitogenic agents \textit{in vitro} are probably related to the physiological blast cell response which occurs \textit{in vivo} in response to immunological challenge (Lindahl-Kiessling and Böök, 1964; Gowans and McGregor, 1965). It has been observed that lymphocytes from subjects who have impaired \textit{in vivo} cell-mediated immunity also show reduced mitogenic responses \textit{in vitro} (Oppenheim, 1968) and this observation suggests that some aspects at least of the \textit{in vitro} response of lymphocytes to stimulating agents may correlate with their behaviour \textit{in vivo} (Cooper, 1973).

It needs to be acknowledged from the outset that most of the \textit{in vitro} systems studied are deficient in many respects in their attempts to mimic the \textit{in vivo} circumstances that affect the outcome of a lymphocyte's response to a stimulating agent. As no formal structure can be arranged \textit{in vitro} which matches that of the fixed lymphoid tissues, \textit{in vitro} responses studied with lymphoid cells teased from lymph nodes, thymus or spleen are particularly unphysiological.

(i) \textbf{RNA Synthesis}

RNA synthesis is an important metabolic activity related to the control of cell proliferation and changes in RNA metabolism.
that accompany lymphocyte stimulation have been studied intensively.

It is well known that a significant increase in cytoplasmic basophilia occurs when lymphocytes are stimulated to transform into blast cells and this increased basophilia is related to an increase in cytoplasmic RNA (Hayhoe and Quaglino, 1965). PHA was found to cause an increase in the content of RNA in cultivated lymphocytes within 24 hr of adding the mitogen. The RNA level in the cells at 48 hr was about double the prestimulation level (Cooper and Rubin, 1965; Kay, 1966; Forsdyke, 1967; Hausen, Stein and Peters, 1969; Jagus-Smith and Kay, 1976). The increase in RNA was found to correlate closely with the increase in cellular dry mass (Sören and Biberfeld, 1973), and as a consequence there was little change in RNA concentration during activation.

The rate of RNA synthesis has usually been studied by measuring the extent of incorporation of a labelled pyrimidine nucleotide, $^3$H-uridine or $^3$H-cytidine, into RNA (Hayhoe and Quaglino, 1965; Ford and Hunt, 1978). This approach to the study of rates of RNA synthesis during lymphocyte stimulation, though widely used, has certain deficiencies and is inaccurate because the measure of RNA synthesis obtained is actually a composite of the different rates of synthesis and degradation of several classes of RNA, including a rapidly turning over, small pool of heterogeneous nuclear RNA (HnRNA), and a large pool of relatively slowly synthesized stable ribosomal RNA (rRNA). The specific
activity of the nucleoside triphosphate pools, from which new RNA molecules derive their nucleotides, is changing continually during the labelling period. This is due to the relatively slow rate at which labelled nucleosides from the culture medium are transported into the cell and phosphorylated. As a result, the rate of entry of radioactivity into RNA will be less during short labelling periods than during long ones despite the fact that the true rate of RNA synthesis is constant (Cooper, 1972, 1973; Ling and Kay, 1975). However, since RNA metabolism in lymphocytes is a complex phenomenon and since no foolproof assay for RNA synthesis has yet been presented, this method is still used as a standard assay for the estimation of rates of RNA synthesis.

The extent of incorporation of labelled pyrimidine nucleotides into acid insoluble cellular material was found to be increased within a few hours of the addition of a variety of mitogens to cultures of lymphocytes; as the cultivation period was extended RNA synthesis continued to increase (Cooper and Rubin, 1965; Pogo, Allfrey and Mirsky, 1966; MacHaffie and Wang, 1967; Polgar, Foster and Cooperband, 1968; Ling and Kay, 1975; Parkes and Howells, 1975; Wang, Marquardt and Foker, 1976; Dauphinais and Waithe, 1977; Girit, Phillips and Kay, 1978).

Mitchell (1964) investigated the RNA metabolism of lymph node cells stimulated with Salmonella flagellar antigen. He observed that blast cells were much more active in the incorporation of RNA precursors than were either mature plasma cells or small lymphocytes. There was a rapid labelling of nuclear RNA in the blast cells with a slower labelling of the
cytoplasmic RNA. Hayhoe and Quaglino (1965) also observed that the uptake of [5-\(^3\)H]uridine occurred at a low level in small lymphocytes whereas it was very high in blast lymphoid cells.

Most of the RNA synthesized by lymphocytes throughout a stimulatory response is of the informational type (Rubin and Cooper, 1965; Cooper, 1969; Jagus-Smith and Kay, 1976) and includes messenger RNA (mRNA) and high molecular weight heterogeneous nuclear RNA (HnRNA). Cooper (1969) estimated that over 90% of the RNA synthesized in resting lymphocytes and about 80% in proliferating lymphocytes was HnRNA. This molecular species comprises about 1% of the total RNA of the cell at any time. It is extremely labile and is degraded in the nucleus within minutes of being synthesized. Structural RNA species (ribosomal RNA (rRNA) and transfer RNA (tRNA)) are much more stable and comprise more than 90% of the RNA in most animal cells. This stable RNA is made up of 80% of rRNA and 10% tRNA (Darnell, 1968; Attardi and Amaldi, 1970) but the rates of synthesis of these RNA species in resting small lymphocytes is quite low - less than 5% of the rate of synthesis of the total RNA. The rate of synthesis of the structural RNA species in lymphocytes increases within 20 hr of PHA stimulation (Cooper, 1969). The addition of PHA to lymphocyte cultures in vitro increases both the rate of synthesis and the rate of maturation of precursors of rRNA and tRNA. (Maturation is the complex process in which RNA's synthesized by DNA-directed RNA polymerases are enzymatically processed or modified after transcription and before the functional products, mRNA's, rRNA's, and tRNA's are released).
The rates of synthesis of mRNA and HnRNA also increase during the stimulation of lymphocytes with PHA and other plant lectins. PHA also increases the activity of the major classes of RNA polymerases - Polymerase I and II. A specific inhibitor of the enzyme alkaline ribonuclease is increased in amount in lymphocytes cultivated with PHA and reaches levels that are sufficient to inhibit the activity of all the alkaline ribonuclease present in the cells. The increase in the amount of specific inhibitor in the cells correlates well with the increase in total RNA levels (Cooper, 1973; Ling and Kay, 1975).

(ii) Protein Synthesis

Lymphocyte stimulation results in an increase in the content of protein in the cells and consequently in the cellular dry mass. Some of the additional protein is present in the nucleus but most accumulates in the cytoplasm resulting in its enlargement (Pachman, 1967; Ling and Kay, 1975). Wang et al. (1976) showed by micrometric analysis that the cell volume of small lymphocytes was approximately doubled by the time the onset of DNA synthesis had occurred following mitogen stimulation.

Protein synthesis in cells is usually assessed by measuring the incorporation of labelled amino acids into various cellular protein fractions. The method is reasonably accurate, at least when representative amino acids such as leucine and phenylalanine are used. This is because the pool of amino acids from which the precursors for protein synthesis are drawn seems to equilibrate rapidly with the exogenous precursor pool (Kay, Ahern and Atkins, 1971).
The rate of incorporation of a wide variety of amino acids into proteins has been found to increase following the stimulation of lymphocytes by mitogens. The amino acids studied have included alanine, glucosamine, leucine, phenylalanine, serine and valine (Mitchell, 1964; Pogo et al., 1966; Hedeshkov, 1968; Polgar et al., 1968; Kay et al., 1971; Cooper, 1973; Ling and Kay, 1975; Parkes and Howells, 1975; Jagus-Smith and Kay, 1976; Wang et al., 1976). Amino acid transport was also found to increase during the transformation of lymphocytes stimulated with PHA (Wise, 1978). The rate of conversion of monoribosomes into polyribosomes is also increased (Kay et al., 1971) and this structural change is considered to be related to increased protein synthesis. The observed increase in protein synthesis is thought to be due to an increase in the rate of initiation of new polypeptide chains, a process in which mRNA and the first or initiating aminoacyl-tRNA are bound to the small subunit of a ribosome (Kay et al., 1971; Ahern and Kay, 1975; Kay, Ahern, Lindsay and Sampson, 1975).

Most of the proteins synthesized by stimulated lymphocytes remain associated with the cell. Only a small proportion is secreted into the culture medium (Smith, Lawton and Forbes, 1967). Mitchell (1964) also found that blast cells stored a large proportion of their newly synthesized proteins. This is consistent with the rudimentary development of the endoplasmic reticulum and the Golgi apparatus in these transforming cells. Neiman and MacDonnell (1971) suggested that a large proportion of the increased protein synthesis after stimulation resulted from a more rapid production of protein molecules which were already being synthesized. New species of protein molecules
PHA is accepted as a T cell mitogen (Roos and Loos, 1973; Tsan, Chen, Newman, Wagner and McIntyre, 1976) and these cells are not considered to participate in immunoglobulin synthesis. Mitogens which activate B lymphocytes such as pokeweed mitogens, LPS and Sepharose-bound concanavalin A (Ling and Kay, 1975) do stimulate immunoglobulin synthesis. Most of the immunoglobulin molecules produced in these circumstances are IgM. English, Adams and Morris (1976) measured the production of immunoglobulins by cells in the popliteal lymph following stimulation of the lymph node with Salmonella LPS. They found that most of the specific antibody produced by free-floating cells in the lymph and by cells within the node during the response was IgM and this was produced mainly by the blast cells. The majority of the immunoglobulin produced however, could not be shown to have any specificity for the antigen. Takahashi, Yagi, Moore and Pressman (1969) found that cultivated human lymphoid cells synthesized immunoglobulins most rapidly in late $G_1$ and early $S$ phase.

The activities of many lymphocyte enzymes change after stimulation and these changes have been well documented (Cooper, 1973; Ling and Kay, 1975).

(iii) DNA Synthesis

The normal mitotic cell cycle comprises the mitotic event followed first by a diploid growth phase, $G_1$, in which there is no DNA synthesis, then by the $S$ phase during which DNA synthesis occurs. A tetraploid phase, $G_2$, follows during which DNA replication is completed and the cells prepare for the onset of another mitosis. Unstimulated lymphocytes are
usually found to be in the early G_1 phase (Ling and Kay, 1975). Populations of stimulated lymphocytes are characterized by the large variations in the length of the S phase amongst the cells which ranges from 6 to 30 hr (Darzynkiewicz, Evenson, Staiano-Coico, Sharpless and Melamed, 1979). Usher and Reiter (1977) reported that thymidine-catabolizing enzymes which degraded thymidine to β-aminoisobutyric acid were active in lymphocytes during the G_1, G_2 and mitotic phases. The activity of these enzymes fell to very low levels just prior to the onset of the S phase and remained low throughout the S period when DNA was being synthesized. Chen, Heiniger and Kandutsch (1975) suggested that the synthesis of cholesterol is an essential prerequisite for the successful initiation and completion of the cell cycle in lymphocytes after activation. The incorporation of labelled DNA precursors such as ^3H-and ^14C-thymidine (TdR) and deoxycytidine (CdR) into DNA during the S phase when DNA synthesis is initiated, has conventionally been used as a sensitive, low background assay for studying lymphocyte transformation (Ling and Kay, 1975). This assay has the disadvantage of requiring a 24-48 hr interval between the time the stimulus is applied and the time its effect on DNA synthesis can be measured. Little information can be obtained on the early events which occur before this period (Cooper, 1973). Akasaka, Olds-Arroyo and Miller (1979) found that human blood lymphocytes stimulated in vitro by plant lectins incorporated ^3H-deoxycytidine into DNA less well than ^3H-thymidine. They concluded that there were differences between human T cells and B cells in their ability to incorporate ^3H-deoxycytidine into DNA.
Lymphocytes cultivated with PHA begin to synthesize DNA after about 24 hr. This is later than the onset of RNA and protein synthesis. DNA synthesis usually reaches a maximum after about 72 hr cultivation and falls to prestimulation levels over the ensuing 48-72 hr (Cooper, Barkhan and Hale, 1963; Hayhoe and Quaglino, 1965; MacHaffie and Wang, 1967; Polgar et al., 1968; Ling and Kay, 1975; Parkes and Howells, 1975; Staub, Antoni and Sellyei, 1976; Chen et al., 1975; Harris and Olsen, 1976; Dauphinais and Waith, 1977). Other stimulants such as staphylococcal filtrate, conc A or pokeweed mitogen, which affect a high proportion of lymphocytes in culture, induce maximum rates of DNA synthesis after 3-5 days exposure while stimulants such as antigens and allogeneic lymphocytes require 5-7 days or even longer (Ling and Kay, 1975; Hardt and Panijel, 1976; Harris and Olsen, 1976). Barlow and Ord (1975) found that there was an increased rate of entry of $^3$H-thymidine into pig lymphocytes cultured with PHA. This occurred within 12 hr of adding PHA. The increased rates of thymidine uptake into the cells were characterized by an increase in maximum velocity ($V_{max}$) without any change in the apparent Michaelis constant ($K_m$). A similar picture is observed for the uptake of deoxycytidine (Barlow, 1976). Wang et al. (1976) found that the decline in DNA synthesis in lymphocytes in culture could not be prevented by frequent changes of medium, by replenishment of the culture medium or by the continued presence of the mitogen. These results indicated that the cells undergo a discrete proliferative response to stimulation which results in a regulated series of cell divisions; this behaviour is unlike that exhibited by established culture cell lines or tumour
cells. The onset, extent and duration of DNA synthesis in stimulated cultures of lymphocytes are affected by a variety of factors such as the nature and concentration of the stimulant used, the way it is metabolized by the cells, the source and the previous history of the cultivated cells and the culture conditions. Additionally, the response of lymphocytes to stimulants as measured by DNA synthesis is heterogeneous. Only about half of the cells in most cultures of lymphocytes respond to PHA with the initiation of DNA synthesis and the rate at which they do so varies greatly between cells (Ling and Kay, 1975).

It is generally assumed that the DNA synthesis which is observed after activation of lymphocytes by mitogens is due to the normal replication of the chromosomal DNA required for mitosis. Ribas-Mundo (1966) observed that DNA synthesis in PHA stimulated human lymphocytes from peripheral blood began diffusely throughout the nucleus. The chromatin associated with the nucleolus and some other localized regions was replicated at the end of S phase. Bader, Miller and Mukherjee (1963) found that in cultivated human leucocytes, a chromosome in group 16-18 and another in group 19-20 were the first to complete DNA synthesis.

The size of the pools of deoxyribonucleotides, dATP and TTP in lymphocytes increased 20-200 fold after stimulation with PHA and enzymes concerned with the metabolism of nucleotides including thymidine and TMP kinase, dCMP deaminase and deoxycytidine kinase were more active after stimulation (Barlow and Ord, 1975; Ling and Kay, 1975; Tsutsui, Amano and Everett, 1975). The rate of nucleotide synthesis from purine
bases (adenine, hypoxanthine and guanine) and adenosine is also substantially increased (Synder, Mendelsohn and Seegmiller, 1976; Raivio and Hovi, 1978). DNA polymerase activity is also increased and this is closely linked to the initiation of DNA synthesis. The increased enzyme activity seems to be due to an increased rate of enzyme synthesis (Ling and Kay, 1975; Staub et al., 1976). The activities of two other enzymes involved in DNA replication, deoxyribonuclease and polynucleotide ligase are also increased after PHA stimulation (Ling and Kay, 1975).

DNA synthesis can be restimulated in lymphocytes when they are exposed to a second dose of PHA or staphylococcal filtrate (SF). Under these circumstances the increase in DNA synthesis occurs much more rapidly and is much more vigorous than in cells not previously exposed to the stimulants (Ling and Holt, 1967; Hardy and Ling, 1973). Wang et al. (1976) observed however that mouse lymphocytes were unresponsive to con A after undergoing a proliferative response to this mitogen and thus they concluded that stimulation by con A produced an in vitro effect similar to the final activation and differentiation of lymphocytes which occurs in vivo in response to an antigenic challenge. Wang et al. thought that it was unlikely that lymphocytes possess a capacity for continuous division in vivo.

(iv) Carbohydrate Metabolism

Lymphocytes stimulated in vitro have been shown to have an increased membrane transport of glucose (Peters and Hausen, 1971; Yasmeen, Laird, Hume and Weidemann, 1977), increased protein and nucleic acid synthesis (see previously), and increased motility (see Chapter 6). Increases in each of these
activities would require additional metabolic energy. It stands to sense that increased metabolic requirements for energy would come from an enhanced rate of carbohydrate metabolism. There have been many studies on the changes in carbohydrate metabolism that accompany lymphocyte transformation induced by various mitogens in vitro.

Addition of PHA to lymphocyte cultures stimulates glucose consumption by these cells (Pachman, 1967; Hedeskov, 1968; Sagone, Lobuglio and Balcerzak, 1974; Parkes and Howells, 1975). Similarly it has also been shown that glucose uptake by lymphocytes is increased when con A is used as the mitogen (Weidemann and Kolbuch, 1974; Puckle, Crumpton, Kolbuch and Weidemann, 1975; Culvenor and Weidemann, 1976; Wang et al., 1976).

Lactate production has often been measured to estimate the extent of glycolysis by lymphocytes in relation to Krebs cycle activity before and after the cells were stimulated. The rate of lactate production by lymphocytes is increased in the presence of mitogens to the same extent as the uptake of glucose is stimulated (Cooper et al., 1963; Pachman, 1967; Hedeskov, 1968; Polgar et al., 1968; Roos and Loos, 1970; Weidemann and Kolbuch, 1974; Parkes and Howells, 1975; Puckle et al., 1975; Culvenor and Weidemann, 1976; Wang et al., 1976).

PHA may have its stimulatory effect on glycolysis through one or more steps involving the transport and conversion of free extracellular glucose to pyruvate. Parkes and Howells (1975) studied the levels of glycolytic enzymes in peripheral lymphocytes of pigs stimulated by PHA. They found that the cells showed significantly elevated activities of phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase and pyruvate
kinase 24 hr after the addition of PHA. These increased enzyme activities were sustained over a 48 hr period.

Parkes and Howells suggested that PHA was able to influence the synthesis of specific glycolytic enzymes and this in turn would facilitate the higher rate of energy production necessary for the transformation of small lymphocytes into blasts. In studying the early sequential changes in the concentrations of intermediate products of glycolysis that accompanied PHA stimulation of thymus lymphocytes from rats, Culvenor and Weidemann (1976) suggested that reactions involving the glucose carriers, hexokinase and PFK are potential regulatory steps that undergo simultaneous or close sequential activation following the interaction of the cells with the mitogen. It appears that glucose transport is not the rate-limiting step in glucose metabolism in lymph-node cells (Helmreich and Eisen, 1959) and this transport process has been shown to be stimulated in lymphocytes by PHA and con A (Peters and Hausen, 1971; Culvenor and Weidemann, 1976; Wang et al., 1976; Yasmeen et al., 1977). Hedekov (1968) found a two to three-fold increase in glucose-6-phosphate concentration in lymphocytes after these cells were incubated for 2 hr with PHA and suggested that the hexokinase reaction was facilitated by the presence of PHA. Hedekov also observed that lactate production from glycolytic processes in human blood lymphocytes stimulated with PHA could be abolished by puromycin. This finding indicated that the activation of glycolysis by the mitogen depended on the maintenance of protein synthesis. The effect of puromycin on glycolysis might be due to an inhibition of the de_novo synthesis of hexokinase. The
increased glycolysis was not thought to be due to an increase in the rate of the PFK reaction because that would imply increased glycogen synthesis and pentose phosphate pathway activity concomitant with a decreased concentration of glucose-6-phosphate.

Experiments which have been done with 3-O-methyl-glucose, a non-metabolizable glucose analogue have indicated that the transport of glucose is a rate-limiting step in its metabolism by rat thymus lymphocytes. The stimulation that occurs in glucose transport may be due to an activation of carrier sites in the cell membrane (Peters and Hausen, 1971; Feldman, Kraetsch, Lichtman and Peck, 1974; Yasmeen et al., 1977). Yasmeen et al. claimed that if hexokinase activation occurs in lymphocytes following stimulation with mitogen it cannot account for the enhanced glucose transport, as 3-O-methyl-glucose is not phosphorylated by mammalian hexokinase. They suggested that the inhibitory effect of puromycin on glycolysis is due to its direct effect on glucose transport and this is independent of its effect on protein synthesis.

The $O_2$ consumption of lymphocytes has been found to be stimulated in the presence of PHA by some workers (Pachman, 1967; Roos and Loos, 1973), while others (Polgar et al., 1968; Weidemann and Kolbuch, 1974; Culvenor and Weidemann, 1976) have been unable to demonstrate any change in respiration during either PHA or Con A stimulation. This failure to demonstrate any change in $O_2$ consumption may be due to the fact that the studies were done at different times after stimulation. Roos and Loos (1973) studied the effect of PHA on lymphocyte metabolism during a three day culture period; they observed
no change in the $O_2$ consumption of the cells on day 0 but a significant increase was recorded on day 1 and day 3 after the addition of PHA. Pachman (1967) also observed an increased $O_2$ consumption over a period of culture that lasted 72 hr.

Roos and Loos (1970) found that the Krebs cycle activity of lymphocytes was increased within about 2 hr of adding PHA to cell cultures and it was estimated that as much as 85% of the ATP synthesized by lymphocytes may be formed as a result of oxidative phosphorylation even though only 25-30% of the glucose was oxidized completely while 60-70% was converted to lactate (Roos and Loos, 1973). Thus aerobic glycolysis is active in lymphocytes during proliferation. Roos and Loos (1970) also observed that PHA caused an early but transient fall in the levels of ATP+ADP in lymphocytes even when glycolysis was increased immediately; this indicated that the stimulation of carbohydrate metabolism by PHA requires an initial consumption of energy. Ling and Kay (1975) suggested that the increased rate of glycolysis induced by PHA was not related solely to an increased demand for ATP but may be part of the primary response by the cells to enlarge and proliferate. Wang et al. (1976) observed a 20-fold increase in aerobic glycolysis in lymphocytes by 50-60 hr after con A stimulation and the increase occurred coincidentally with the increase in DNA synthesis but not with RNA or protein synthesis. They suggested that aerobic glycolysis in stimulated lymphocytes was related to DNA synthesis and was not due to either an increase in cell size or to the process of cellular differentiation.
The majority of the glucose taken up by normal lymphocytes is converted to pyruvate or lactate (Pachman, 1967; Hedeskov, 1968; Lengle, Gustin, Gonzalez, Menahan and Kemp, 1978). About 2% is converted to glycogen and the rate of conversion of glucose to glycogen is stimulated by PHA (Pachman, 1967; Hedeskov, 1968). Glycogen accumulates in lymphocytes during the early stages of stimulation (Quaglino, Hayhoe and Fleman, 1962) but it is absent from the large lymphoblasts which appear later in the cultures. A further 2% of the glucose taken up is converted to amino acids, mainly serine and glutamate and less than 1% is converted to fats (MacHaffie and Wang, 1967; Hedeskov, 1968). Puckle et al. (1975) observed that in thymic lymphocytes stimulated with con A, there was an increased flow of carbon from [U-14C]glucose into the protein, nucleic acid, lipid and glycogen pools. Kay (1968) reported that the incorporation of [U-14C]glucose into lipid was stimulated when human lymphocytes were incubated with PHA and Inouye, Handa and Osawa (1974) observed that stimulated lymphocytes exhibited enhanced incorporation of [6-3H]glucose into polar lipids.

About 2% of the total glucose taken up has been shown to be oxidized through the pentose phosphate pathway (Hedeskov, 1968; Suter and Weidemann, 1976). PHA significantly increased the yield of 14CO₂ from D-[1-14C]glucose (MacHaffie and Wang, 1964, 1967; Hedeskov, 1968; Sagone et al., 1974; Tsan et al., 1976); this result means that the addition of PHA enhances the operation of the pentose phosphate pathway.

Thus at least in vitro, the first effect of PHA and probably other mitogens on lymphocytes is seen in the stimulation of glycolysis, followed by an increase in Krebs cycle activity. Either of these pathways can provide the energy for the PHA-induced
transformation in short-term experiments but both pathways are required for cell proliferation in long-term cultures. When glycolysis or the Krebs cycle is suppressed, the stimulation of lymphocytes by PHA is inhibited (Roos and Loos, 1973). The increased activity of the pentose phosphate pathway would result in an increased production of intermediates such as pentoses, which would be required for biosynthetic functions during the induced mitosis. Ribose-5-phosphate, synthesized in the pentose phosphate pathway is an essential component of nucleotides and RNA. Stimulation of the pentose phosphate pathway would also lead to an increase in the amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the cells. This is a key coenzyme in biosynthetic processes involving reduction, e.g. the biosynthesis of long-chain fatty acids and DNA (MacHaffie and Wang, 1967; Harper, 1973; White, Handler and Smith, 1973; Lehninger, 1975; Conn and Stumpf, 1976).

(c) STUDIES ON LYMPHOCYTES STIMULATED IN VIVO

Almost all investigations on the metabolic effects of stimulating agents on lymphocytes have been done on cells activated in vitro in the presence of convenient mitogenic agents. The proposition that lymphocytes stimulated to dedifferentiate and proliferate by plant lectins will show alterations in their metabolism similar to those that occur following antigenic stimulation is reasonable enough, but it remains for this to be formally demonstrated. The reasons why such experiments have not been done probably relate to a preoccupation with describing the effects of antigen on lymphocytes in terms of specific antibody synthesis. Additionally, in vitro responses of lymphocytes to antigens
are highly unpredictable and the initiation of an *in vitro* immune response in pure populations of lymphocytes by most antigens is difficult if not impossible to achieve. Furthermore, it is not possible to acquire pure populations of lymphocytes stimulated *in vivo* by antigen by recovering cells from the blood or the thoracic duct lymph or from the spleen or lymph nodes. As a consequence, metabolic studies done on cells from these sources represent the results obtained from a conglomerate of cells removed from their normal physiological environment.

Lymph coming from lymph nodes contains a pure population of lymphocytes that can be collected by cannulating the efferent duct of a conveniently placed node such as the popliteal, prescapular or prefemoral nodes. This technique was first applied by Linzell (1960), and by Lascelles and Morris (1961) to study lymph coming from the mammary gland of the goat and the ewe. The technique was further developed by Hall and Morris (1962) to study lymph from single nodes in the sheep before and following antigenic challenge. The virtue of this physiological approach to the study of the immune response lies in the fact that events in the node are reflected closely in the cellular composition of the lymph. Lymph can be collected continuously throughout an immune response in unanaesthetized animals and this provides an opportunity to study the evolution of the response over periods of days. The immune response can be localized in the single cannulated node provided the antigen is placed quantitatively there and provided all the cells leaving the node in the lymph are diverted from the body.
Using this experimental approach various aspects of the immune response have been investigated (Hall and Morris, 1962, 1963; Cunningham, Smith and Mercer, 1966; Hall, Morris, Moreno and Bessis, 1967; Smith, 1967; Hay, 1970; Smith, McIntosh and Morris, 1970; Smith and Morris, 1970; Cahill, Hay, Frost and Trnka, 1974; Hay, Cahill and Trnka, 1974; Poskitt, 1974; Cahill, Frost and Trnka, 1976; English, Adams and Morris, 1976; Hay and Morris, 1976).

(d) AIM AND SCOPE OF THESIS

Although the immunological role played by lymphocytes and lymphoid tissue in the mammal has been extensively investigated and despite the fact that much work has been done on the metabolism of stimulated lymphocytes in vitro there is little detailed information available on the changes in energy metabolism that occur in these cells during in vivo primary and secondary responses to antigen. The crucial events of cell proliferation and specific antibody synthesis can only occur in conjunction with energy consuming processes. A knowledge of the way in which carbohydrate is metabolized by the lymphocytes is thus as significant biochemically for the proper understanding of the immune response as is a knowledge of the mechanism of antigen recognition and antibody synthesis. The results reported in this thesis are concerned with studies into the glycolytic, respiratory and pentose phosphate pathway activities of lymphocytes collected from the efferent lymph of the popliteal node of the sheep at various times before and after in vivo challenge with Salmonella organisms. The energy yielding catabolic processes have been correlated with other activities of the cells such as DNA, RNA and protein synthesis and their intrinsic motility, which require the consumption of
energy. These determinations have to be done \textit{in vitro} on free-floating cells. The physiological significance of the results obtained \textit{in vitro} has been related to the results obtained from studies on the oxygen tension of the environment in which the cells exist \textit{in vivo} - the lymph.
(a) EXPERIMENTAL ANIMALS

Sheep

Anesthetized Merino or Merino-cross ewes 3-6 years old were used for all the experiments. The animals were confined in metabolism cages after operation and throughout the experiments.

Chickens

Outbred hens were bled to provide red blood cells (RBC) for haemagglutinating antibody assay.

(b) CHEMICALS, BUFFERS AND SOLUTIONS

All chemicals were of the Analytical Reagent grade unless otherwise stated.

Sodium chlorella solution

An aqueous solution of 0.9% NaCl prepared in distilled water.

Phosphate-buffered saline (PBS)

The following solutions were prepared separately and mixed before use: 1.0 g NaCl, 0.2 g KCl, 1.19 g CaCl₂, 0.2 g KH₂PO₄ in 500 ml distilled water. 0.1 M CaCl₂ in 100 ml distilled water. 0.3 g MgCl₂·6H₂O in 100 ml distilled water. Alsever's solution (modified)

0.089 g NaCl, 0.05 g KCl, 0.1 g MgCl₂·6H₂O, 0.06 g KH₂PO₄, 1.0 g glucose and 6 ml of 10% citric acid (pH adjusted to 6.4 with 10% citric acid) were added to 1 litre of DW. This solution was autoclaved at 112°C for 20 min and used for the collection of chicken blood.

Halide-balanced salt solution (HBS)

8.0 g NaCl, 0.4 g KCl, 0.1 g MgCl₂·6H₂O, 0.14 g CaCl₂·2H₂O, 0.069 g Na₂HPO₄·2H₂O, 0.069 g KH₂PO₄, 1.0 g glucose, and 6 ml of 10% citric acid were dissolved in 1 litre of DW. 5 ml of a 0.45 aqueous solution of amniot...
(a) **EXPERIMENTAL ANIMALS**

**Sheep**
Randomly bred Merino or Merino-cross ewes 3-5 years old were used for all the experiments. The animals were confined in metabolism cages after operation and throughout the experiments.

**Chickens**
Outbred hens were bled to provide red blood cells (CRBC) for haemagglutinating antibody assays.

(b) **CHEMICALS, BUFFERS AND SOLUTIONS**
All chemicals were of the Analytical Reagent grade unless otherwise stated.

**Sodium chloride solution** (saline)
0.9% solution of NaCl prepared in distilled water.

**Phosphate buffered saline** (PBS)
The following solutions were autoclaved separately and mixed before use: 8.0 g NaCl, 0.2 g KCl, 1.15 g Na$_2$HPO$_4$, 0.2 g KH$_2$PO$_4$ in 800 ml deionized distilled water (DDW); 0.1 g CaCl$_2$ in 100 ml DDW; 0.1 g MgCl$_2$·6H$_2$O in 100 ml DDW.

**Alsever's solution** (modified)
8.0 g sodium citrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O), 4.2 g NaCl, 20.5 g glucose and 8 ml of 10% citric acid (pH adjusted to 6.1 with 10% citric acid) were added to 1 litre of DDW. This solution was autoclaved at 112°C for 20 min and used for the collection of chicken blood.

**Hank's balanced salt solution** (BSS)
8.0 g NaCl, 0.4 g KCl, 0.1 g MgSO$_4$·7H$_2$O, 0.14 g MgCl$_2$·6H$_2$O, 0.069 g Na$_2$HPO$_4$·0.06 g KH$_2$PO$_4$ and 1.0 g glucose were dissolved in 1 litre of DDW. 5 ml of a 0.4% aqueous solution of phenol

red was added as a pH indicator. The pH of the solution was adjusted with 5% NaHCO₃. The colour of the solution gave an estimate of pH.

Krebs & de Gasquet buffer (Krebs and de Gasquet, 1964)  
The following solutions in DDW were combined: 116 ml of 0.154M (0.9%) NaCl; 4 ml of 0.154M (1.15%) KCl; 3 ml of 0.055M (0.81%) CaCl₂·2H₂O; 1 ml of 0.100M (2.46%) MgSO₄·7H₂O; 1 ml of 0.154M (2.68%) K₂HPO₄; 6.5 ml of 0.10M Na₂HPO₄-NaH₂PO₄ buffer solution, pH 7.4 at 25°C.

The 0.10M Na₂HPO₄-NaH₂PO₄ buffer solution was prepared according to the method of Gomori (1955). It had a pH of 7.4 at 25°C: 8.1 ml of 0.10M (1.42%) Na₂HPO₄ was mixed with 1.9 ml of 0.10M (1.56%) NaH₂PO₄·2H₂O or 4.05 ml of 0.20M (2.84%) Na₂HPO₄ was mixed with 0.95 ml of 0.20M (3.12%) NaH₂PO₄·2H₂O and diluted to 10 ml with DDW.

Tris-buffered isotonic ammonium chloride solution (ACT solution)(Boyle, 1968)  
9 volumes of 0.83% aqueous NH₄Cl were mixed with 1 volume of Tris buffer (20.594 g Tris base per litre adjusted to pH 7.65 with N HCl). The pH of the mixture was adjusted finally to 7.2.

One to two quick washes would lyse contaminating red blood cells efficiently without damaging the lymphocytes. The solution was most effective at 37°C.

Eagle's minimum essential medium(F-15, MEM)(Eagle, 1959)  
With Earle's salts, L-glutamine and non-essential amino acids and without sodium bicarbonate.

Manufacturer: Grand Island Biological Co.
Preparation:

10 g of the commercial powder was dissolved in 956 ml of DDW and 44 ml of 5% sodium bicarbonate was then added and the mixture gassed with CO₂ for a few minutes until the solution was acidic. The solution was sterilized by filtration through a millipore filter (0.22 µ membrane). 2.0 ml of Penicillin-Streptomycin-Neomycin (PSN) was added and the solution dispensed into sterile bottles in 100 ml portions. Before use, 1 N NaOH was added until the colour of the solution turned purple. Then 1 ml of HEPES solution (1M) was added to each 100 ml portion. The final colour was red to orange. 10 ml of foetal calf serum (heat inactivated) was added finally. The medium was used within 2 weeks of it being made up.

Dulbecco's modified Eagle's medium (H-16) (Dulbecco and Freeman, 1959)

Manufacturer: Grand Island Biological Co.

Preparation:

10 g of the commercial powder was dissolved in 926 ml of DDW. 74 ml of 5% NaHCO₃ was then added and the mixture gassed with CO₂ for a few minutes. 2 ml of PSN was added and the solution sterilized by filtration through a millipore filter. The solution was dispensed into sterile bottles in 100 ml portions. Before use the pH was adjusted with 1 N NaOH to a purple colour. 1 ml of HEPES solution (1M) was added to 100 ml of medium to give a red to orange colour.

Foetal calf serum (FCS)

Foetal calf serum (Commonwealth Serum Labs., Melb. Australia) was inactivated at 56°C for 30 min. This serum was usually used as a supplement in synthetic media (e.g. F-15 and H-16) for cell culture, particularly in circumstances where the
quality of the growth medium may be a limiting factor.

It was also used in cell washings to improve the viability of the cells. Normally the final concentration of FCS was 10%.

**HEPES**

A 1 M solution was prepared by adding 283.3 g Hepes (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (Sigma Chemicals) to 700 ml DDW. The solution was titrated to pH 8.1 with NaOH (50-70 ml of 5 N NaOH) and the final volume adjusted to 1 litre with DDW.

The solution was sterilized by filtration through a millipore filter and stored at 4°C.

**Penicillin - Streptomycin - Neomycin solution (PSN)**

To make 500 ml of PSN solution, the following quantities of antibiotics were required:

- 25 bottles of Penicillin G (Benzylopenicillin, sodium salt, 600 mg = 1,000,000 units per bottle, CSL)
- 25 bottles of Streptomycin sulphate (1 g per bottle, equivalent of 1 g Streptomycin base, Glaxo)
- 1 bottle of Neomycin sulphate (25 g per bottle, contains 2.5 moles of H₂O per molecule, anhydrous M.W. 908.7, Sigma)

The chemicals were dissolved in 500 ml of DDW, the solution sterilized by Seitz filtration and then dispensed in 5 ml portions.

Each ml of PSN contained 30 mg Penicillin G (50,000 units); 50 mg Streptomycin sulphate (50,000 units) and 50 mg Neomycin sulphate (80,000 units).

**Heparin and antibiotic mixture for lymph collection**

Two heparin antibiotic mixtures were used for the lymph collections.

A. The following chemicals were mixed:

- 5.0 ml heparin solution (1,000 units/ml); 45.0 ml PBS;
- 250 mg Pyopen; 250 mg crystalline Penicillin G.
The mixture was frozen in 2 ml portions and 1 ml was used for each 100 ml of lymph collected.

The concentration chosen was such that the lymph contained about 1 unit of heparin and 100 µg of antibiotics per ml at the end of each collection.

B. The following chemicals (powder) were mixed together:

- 1 bottle of heparin (freeze-dried, pyrogen free, 100,000 I.U., 142 units/mg, Evans Medical Ltd.);
- 1 bottle of crystalline penicillin G (sodium salt, 600 mg per bottle, 1,000,000 units, CSL);
- ½ bottle of streptomycin sulphate (1 g/bottle equivalent to 1 g streptomycin base, 1 mega unit, Glaxo);
- a small spatula covered with the mixture was enough for about 100 ml of lymph.

Azure A stain

A 0.1% Azure A (Hopkins & Williams Ltd) solution was prepared in acetate buffer, pH 5.2.

The solution was used as a counterstain in autoradiography.

Trypan blue solution

A 0.4% solution of trypan blue (Searle) was made in saline (or PBS). This stock solution was diluted to 0.1% and used to assess the viability of cells.

Leishman's stain

0.15 g of Leishman's stain (Merck) was dissolved in 100 ml of methanol. The solution was used to stain lymphocyte smears.

Manometric fluid

A. Krebs' manometric fluid.

The solution had the composition:

44 g anhydrous NaBr; 0.3 g Triton X-100 or 1.0 g of the syrupy commercially available solution of Lissapol N or Stergene. A
comparable non-ionic detergent may be substituted; 0.3 g Evans blue (or Acid Fuchsin - for red colour); 1000 ml water.

The density of the solution was 1.033 at 20°C, and P₀ (standard pressure) was 10,000 mm.

B. Brodie's manometric fluid (B. Braun, Melsungen).

The composition of the fluid was:
23 g NaCl and 5 g sodium choleate (Merck) in 500 ml water.

The solution had a density of 1.033 at 20°C and P₀ was 10,000 mm. Brodie's solution has been the standard manometric fluid, but it has generally been replaced by the Kreb's solution.

White cell dilution fluid (WDF)

A 2% acetic acid solution (or 1% HCl) was prepared and 1 ml of a 1% aqueous solution of gentian violet was added to 100 ml of the acetic acid solution.

Mercapto-ethanol solution

A 0.2M solution of 2-mercapto-ethanol (Eastman Organic Chemicals) in saline was prepared fresh before use.

D-[U-¹⁴C]glucose

The stock solution (the Radiochemical Centre, Amersham) had a specific activity of 327 mCi/mmol and was supplied in 50 µCi/250 µl amounts (i.e. 5 µl of stock solution was equivalent to 1 µCi).

D-[1-¹⁴C]glucose

The stock solution (the Radiochemical Centre, Amersham) had a specific activity of 59.9 or 61.6 mCi/mmol and was supplied in 50 µCi/250 µl amounts.
D-[6-14C] glucose

The stock solution (the Radiochemical Centre, Amersham) had a specific activity of 53.2 or 59.6 mCi/mmol and was supplied in 50-51 μCi/250 μl amounts.

All these glucose radioisotopes were dispensed together with 50 μl saline, under aseptic conditions in 1 μCi quantities. They were put into small capped plastic tubes and stored frozen. The complete contents of a plastic tube were used immediately after thawing. In no case was any unused material refrozen for subsequent use. The weight of substrate per tube could be calculated from the specific radioactivity quoted.

[Methyl-3H]thymidine

The stock (the Radiochemical Centre, Amersham) supplied was 1.0 mCi/ml and had a specific radioactivity of 5.0 Ci/mmol.

[5-3H]uridine

The stock supplied was 1.0 mCi/ml and had a specific radioactivity of 5.0 Ci/mmol.

L-[4,5-3H]leucine

The stock supplied was 1.0 mCi/ml and had a specific radioactivity of 1.0 Ci/mmol.

These three radioisotopes were diluted to a concentration of 1.0 μCi/20 μl (i.e. 0.05 mCi/ml) with Hanks and stored frozen in 2 ml portions. Each portion was thawed before use and the required amount of isotope taken. The remaining material was stored in the cold room (4°C) until used.

Carbo-Sorb II

This was an organic amine which readily trapped carbon dioxide. Carbo-Sorb II (Packard) and the trapped carbon dioxide was easily soluble in toluene scintillator solutions (e.g. Permafluor V, Packard) without the addition of alcohols.
Permaflour V
This was a liquid scintillator solvent system for counting \(^{14}\text{C}\)-labelled carbamate.

A combination of 9 ml of Carbo-Sorb II and 12 ml of Permaflour V was used for the radiorespirometry experiments.

Scintillation medium for radioactive samples in glass fibre discs
A 0.5% PPO (2,5-phenyloxazole) in toluene. 7.0 ml of the mixture was used in each scintillation vial.

Chromerge
This was a concentrated Labware cleaner used for preparing all glassware. The contents of a 25 ml bottle of Chromerge (Manostat, N.Y.) was added to a standard 9lb container of concentrated sulphuric acid. Approximately 5 ml were added at a time with shaking. A precipitate normally formed after mixing.

Perchloric acid (MW.100.46)
This was a 70% solution of the commercial product.

Siliclad
This water soluble silicone concentrate was supplied by Clay Adams and used to give a coating to glass, porcelain or enamel ware, that was physiologically inert and under normal conditions unaffected by heat, moisture and most common chemicals and compounds. A 1% aqueous solution of Siliclad was used to siliconize glassware. The object was immersed in the Siliclad solution for 5 sec and then removed immediately and rinsed thoroughly to remove any excess Siliclad from the surface. It was then air-dried or heated to a temperature of 100°C for 10 min.
Chrome alum/Gelatine solution

0.05 g chrome alum (chromium potassium sulphate, BDH) was dissolved in about 80 ml of warm water using a magnetic stirrer. 0.5 g of gelatine (Davis) was added and stirred until it dissolved. The solution was then filtered and made up to a volume of 100 ml. The fresh solution was used for coating slides for autoradiography.

Carbonic anhydrase (carbonic hydro-lyase, carbonate dehydratase; E.C. No. 4.2.1.1.)

The enzyme, supplied by Sigma, was extracted from bovine erythrocytes. It was used to decompose $^{14}$C-bicarbonate in the incubation medium to $^{14}$CO$_2$.

Cellulose diacetate (CDA)

A 7.5% solution of CDA (acetylation, 39.8%; ASTM viscosity, 25; Eastman Co., Ltd) in acetone/ethanol (1:1) mixture was used to coat the platinum cathode of the insertable oxygen electrode.

(c) ANTIGEN PREPARATION AND ADMINISTRATION

(i) Salmonella muenchen (English, 1974)

Agar slopes of the bacteria were washed with 5 ml of a sterile solution of Hanks basal salt medium and 0.5% lactalbumin hydrolysate containing 8 g per litre of nutrient broth (Difco). These suspensions of bacteria were incubated overnight at 37°C on a mechanical shaker. 5 ml of bacterial suspension was then introduced into a litre of the same nutrient solution and kept at 37°C for 48 hr with occasional agitation. A thick suspension of the bacteria was harvested and the cells separated from the medium by centrifugation at 19,000 g, washed in saline and re-centrifuged. The bacteria so obtained were killed by suspending them in acetone followed by freeze-drying.
(ii) Preparation of Lipopolysaccharide (LPS) from Salmonella muenchen Organisms (Westphal, Lüderitz & Bister, 1952)

1 g of the freeze-dried bacteria was suspended in 18 ml of distilled water and heated to 65-68°C. One volume (18 ml) of a 90% phenol solution (w/v), preheated to 65-68°C, was added and the reaction mixture stirred vigorously for 15 min before being cooled to 10°C and spun at 2,000 g for 30 min. The mixture separated into 2 phases, a lower phenol layer and an upper aqueous layer. The upper phase which contained the LPS was drawn off and the phenol layer extracted at 65-68°C with a further 18 ml of water. The combined aqueous extracts were dialyzed against running tap water for 3 days at room temperature and the precipitate collected by centrifugation. The crude LPS was dissolved in 25 ml of 0.05 M Tris/HCl buffer, pH 7.7 and 10 mg of ribonuclease A (Type IIIA, Sigma Chemical Co.) was added (Fensom and Gray, 1969). The digest was kept at 37°C for 16 hr and centrifuged at 80,000 g for 8 hr. The sediment was resuspended in water and centrifuged at 105,000 g for 90 min. The sedimented LPS, essentially free of RNA, was freeze-dried from a concentrated solution in water.

(iii) Administration of Antigen

Acetone killed freeze-dried Salmonella muenchen organisms were used as an antigen in all experiments. The concentration of organisms was determined by comparison with Wellcome opacity tubes for the standardization of bacterial suspensions. 2 mg of the freeze-dried organisms in 0.2 ml of saline was equivalent to 1.17 x 10^10 cells and was used for injection in all cases. The suspension was injected subcutaneously into the posterolateral aspect
of the leg just above the fetlock. This area was over the
course of the recurrent tarsal vein and there were several
lymphatic ducts in this region which drained to the popliteal
lymph node (Hall and Morris, 1962). To obtain a secondary
response, another dose of antigen was given at about 6 weeks
after the primary challenge.

(d) CANNULATION OF EFFERENT POPLITEAL DUCT (Hall & Morris, 1962)

All operations were carried out in a properly equipped
operating theatre under strict sterile conditions. The sheep
to be operated on was starved for at least 24 hr prior to surgery.
Anaesthesia was induced with a 5% aqueous solution of sodium
pentothal ("Intraval" Sodium, May & Baker, Ltd) (0.1 g per
5 kg body weight) and a cuffed Magill's endotracheal tube was
used for intubation. Anaesthesia was maintained with Halothane
("Fluothane", I.C.I. Ltd) and O₂ using a closed circuit
Boyles anaesthetic machine (British Oxygen Company). The
operation site was prepared by clipping the wool as close to
the skin as possible and then scrubbing the area with a 1.0%
solution of chlorohexidine ("Hibitane", I.C.I.). The operation
involved an incision through the skin and subcutaneous tissue
made from a point 3-4 cm below the sciatic tuberosity for a
distance of 10-15 cm on the posterolateral aspect of the thigh
parallel to the posterior margin of the leg. Bleeding was
controlled by electrocautery. The semitendinosus muscles and
the biceps femoris were separated with wound retractors and the
popliteal fossa exposed. Muscular branches of the posterior
femoral artery and vein were tied with two silk ligatures and
then cut. The femoral vein was then exposed by blunt dissection
and the efferent lymphatic located adjacent to it. The position
of the duct was variable but it could usually be found without tracing it back too close to the hilum of the node. In some cases more than one efferent duct was present and it was important to identify and ligate each one. Failure to ligate all the efferent ducts results in a feeble and short-lived flow of lymph. A ligature of 3/0 silk was tied around the duct as far as possible from the node and the duct cleaned of connective tissue and fat. A second ligature was loosely applied 1-2 cm below the first occluding tie and a small incision was made in the lymphatic in a region free of any valves. A cannula (either polyvinyl or polyethylene, Dural Plastics, N.S.W.) of suitable size was inserted into the duct through the incision in the direction of the node and securely tied into place with the lower ligature. Further ligatures were used to ensure the security of the cannula which was led out through a stab wound in the skin about 5 cm above the upper limit of the original incision. The cannula was secured to the skin with a purse string suture. The wound was dusted with powdered penicillin and the deep fascia and subcuticulum were repaired with interrupted silk sutures. The skin incision was closed with Michel clips. A plastic holder was secured to the skin and the cannula inserted into a bottle tied onto the holder.

(e) ISOLATION AND PREPARATION OF CELLS
(i) Collection of Popliteal Efferent Lymph

Lymph was collected in sterile plastic or glass collection bottles containing heparin and antibiotics in powdered form or in solution. The plastic bottles were tissue culture flasks (Falcon) and were sterilized by gamma irradiation. The glass bottles were sterilized by autoclaving. The lymph was normally
collected over 12-hourly periods during an immune response. A portion of cell free lymph was saved from each collection, and stored frozen. This lymph was used subsequently in antibody determinations.

(ii) Collection of Sheep Blood

Samples of sheep blood were obtained from the jugular vein with a hypodermic syringe containing heparin. If serum was needed, no heparin was used. When blood samples were required every day from a sheep, a cannula was inserted permanently into the jugular vein and blood samples were drawn from the cannula through a 3-way tap at the end of the cannula.

(iii) Collection of Chicken Red Blood Cells (CRBC)

Blood was taken from the wing vein of chickens and collected into a syringe containing 2 ml of Alsever's solution (for the collection of 2 ml of blood). The blood was drawn and mixed with the Alsever's solution and the mixture washed 3 times with PBS. The CRBC were resuspended in PBS and could be stored for future use at 4°C for up to 2 days.

(iv) Isolation of Peripheral Blood Lymphocytes (Böyum, 1968, 1976)

120 ml (2 x 60 ml) of heparinized sheep blood (approx. 1 ml heparin - 1,000 units/ml per 50 ml of blood) was taken and centrifuged at 1,500-2,000 g (approx. 2,500 rpm) for 10 min at room temperature (20°C). The buffy coat was removed carefully with a Pasteur pipette and dispensed into about 3 portions of 10-15 ml of Hanks, and mixed to avoid clotting. The mixtures were diluted to 25-30 ml with Hanks. 8 ml of Ficoll-Isopaque (Ficoll-Ip) was added gently to each sample keeping the pipette against the tube wall 5-10 mm above the fluid meniscus so as to
avoid mixing the layers. The tubes were centrifuged at 600-800 g (approx. 1,900 rpm) for 40 min. After centrifugation the blood cells separated into two fractions, a white layer consisting of mononuclear cells and platelets at the interface region, and a bottom fraction containing the erythrocytes and granulocytes. The mononuclear cells could be removed easily with a Pasteur pipette. They were located mostly around the periphery of the tube, but to ensure complete removal, it was necessary to move the pipette over the whole cross-sectional area of the tube. The lymphocytes so harvested were washed 3 times to remove platelets (1,000-1,200 rpm, 5-10 min).

(v) Column Fractionation of Cells in Lymph (Rabinowitz, 1964)

A glass column 30 cm long and 1 cm in diameter was used. It was fitted with a rubber stopper at the inlet with a Luer needle inserted through it. The outlet was 0.35 cm in diameter and was connected to plastic tubing. The column was packed to 85% capacity with glass beads (Selbys, 0.45-0.5 mm diameter). A thin layer of glass wool was used at the bottom to retain the beads. The apparatus including glass beads and wool were siliconized with Siliclad. Lymph was collected, centrifuged and the cells resuspended in 5-10 ml of supernatant lymph plasma. Excess lymph plasma provided a convenient source of fresh compatible material for washing the column. The lymph plasma was warmed to 37°C and used undiluted to elute the lymphocytes. It was diluted to 20% with Hanks for washing the column prior to eluting the polymorphonuclear leucocytes. Disodium ethylenediamine tetraacetic acid (EDTA), 0.02% in Ca++- and Mg++- free salt solution buffered at pH 7.4 was used for washing PMN leucocytes
off the column. This reagent contained disodium EDTA 0.2 g, NaCl 8.0 g, KCl 0.2 g, Na₂HPO₄ 1.15 g, KH₂PO₄ 0.2 g, and glucose 0.2 g, dissolved in one litre of DDW. The concentrated cell suspension was added to the column by syringe or Pasteur pipette through the inlet which was then incubated at 37°C for 30 min. Lymphocytes were washed from the column with approximately 100 ml fresh heparinized plasma at 37°C. The flow rate through the column was adjusted to 15-20 drops per min (approx. 1.5 ml/min).

Residual lymphocytes, RBC and platelets were removed from the column by washing it with approximately 100 ml of 20% fresh plasma in Hanks at 37°C. Most PMN leucocytes continued to adhere to the column. When the effluent from the column no longer contained RBC, platelets or lymphocytes, the column was washed with 0.02% EDTA in Ca⁺⁺- and Mg⁺⁺- free buffered saline to elute the PMN leucocytes.

(vi) Preparation of Cells for Metabolic Studies

For short-term incubations of less than 8 hr duration, cell suspensions were not prepared under sterile conditions. Lymph was centrifuged at room temperature and the lymphocytes collected and washed by centrifugation with ammonium chloride-Tris buffer (ACT solution) to remove any contaminating erythrocytes and then twice with Krebs & de Gasquet buffer. After washing, the cells were resuspended in a suitable volume of the same buffer at pH 7.4. For long-term incubations cells were prepared under sterile conditions as mentioned in the text.
(vii) **Cell Counts**

**Coulter counter method**

A model FN Coulter counter (Coulter Electronics Ltd, Dunstable, U.K.) was used to determine the cell counts. The number of large lymphocytes determined with the Coulter counter was correlated with the number of basophilic blast cells determined from Leishman's stained cell smears.

**Haemocytometer method (Dacie & Lewis, 1975)**

For more accurate cell counting, the Coulter counter results were regularly checked by counting samples in a haemocytometer chamber (Bright-line, Spencer).

(viii) **Cell Smears and Differential Counts (Dacie and Lewis, 1975)**

5 ml of lymph was spun down and the cell pellet was resuspended in an equal volume of lymph plasma or buffer. A drop was added to one end of a slide and smeared with a spreader. The cytocentrifuge (Shandon Elliott) was also used to make cell smears (Dore and Balfour, 1965). The cell concentration was first adjusted to $10^6$ cells/ml and 0.2 ml was applied to the plastic chamber with the slide and filter paper in place. The centrifugal speed was set to 80 (1,300 rpm) and the sample spun for 5 min. The circular smear was air-dried and 1 ml of Leishman's stain was added to the slide for 3 min. The stain was then diluted with 2.5 ml of tap water and washed off with distilled water 10 min later. The slide was then examined under oil immersion and the cells were counted and classified according to their morphology and staining characteristics. Differential counts were done on random fields of the smear counting a total of 1000 cells (Hall and Morris, 1963; Morris, 1966). The following categories of cells were identified:
Normal lymphocytes

These cells included small, medium and large cells. The medium lymphocytes had a diameter of 10-12 µ, a narrow rim of faintly basophilic cytoplasm and a featureless round nucleus which sometimes displayed clumped chromatin. Large lymphocytes were cells around 20 µ in diameter with a pale blue-staining cytoplasm and nucleoli. The small lymphocytes were about 8-10 µ in diameter with nuclei which stained intensely. Any cytoplasm that was visible stained palely. There was no clear distinction between the medium and small lymphocytes of the intermediate size range.

Blast cells

These were large cells with strongly basophilic cytoplasm which appeared in the efferent lymph in large numbers about 60 hr after antigenic challenge. In Leishman's stained films, these cells had a nucleus with a fine chromatin structure and several well defined nucleoli. They range in size from 10 µ to 40 µ.

Polymorphonuclear leucocytes (PMN leucocytes)

Both neutrophils and eosinophils were sometimes present in efferent lymph. The cytoplasmic granules of the neutrophils stained palely. The eosinophils had lobulated nuclei and red-staining cytoplasmic granules. Occasionally cells resembling haemohistoblasts were seen but they tended to be fragile and rarely survived the spreading process intact. Macrophages were present regularly in peripheral lymph. These cells were characterized by numerous inclusion vacuoles and extensive cytoplasmic veils.
(ix) **Cell Viability**

A cell suspension in serum free medium was added to an equal volume of 0.1% trypan blue solution. The percentage of cells which took up the dye after a period of 3-5 min was determined under the microscope by counting 500 cells on random fields of the smear. There was generally no difficulty in detecting the stained, non-refractive cells from the highly refractive unstained cells. Under certain conditions, various degrees of transitional staining were encountered which made some determinations uncertain. This was particularly the case when the proportion of stained cells was high. There was then a tendency for all the cells to gradually take up the stain so that when the count was repeated the proportion of stained cells increased. When the proportion of stained cells was low, the time element was a less important factor. In general the variations in the estimates did not exceed 5% (Papperheimer, 1917). Non-viable cells sometimes took up the stain without showing any other morphological change but a high proportion of the stained cells were grossly swollen (Gorer and O'Gorman, 1956).

(f) **HAEMAGGLUTINATION ASSAY**

(i) **Preparation of LPS-coated Chicken Red Blood Cells**

(Poskitt, 1974)

16 mg of LPS was added to 1 ml of 0.02 M NaOH and the mixture was allowed to stand overnight. The mixture was then centrifuged and the precipitate discarded. The supernatant was neutralized with 0.1 ml of 0.2 N HCl. 0.1 ml of the solution was made up to 5 ml with PBS and added to 5 ml of a 5% suspension of chicken RBC in PBS. The reaction mixture was incubated for 1 hr at 37°C, centrifuged and the cells collected. The LPS coated
CRBC were washed twice with 5 ml of PBS and resuspended to 0.5% in PBS for use in the antibody assay.

(ii) Titration of Haemagglutinating Antibody

Cell-free lymph samples were incubated at 56°C for 30 min to inactivate the complement. IgM and IgG antibodies were distinguished by incubating lymph in an equal volume of 2-mercapto-ethanol solution for 45 min at room temperature prior to titration; IgM was sensitive to this treatment. 50 µl of lymph was plated in doubling dilutions with PBS in microtitre 'V' plates (Cooke Engineering Co.). 50 µl of a 0.5% suspension of coated CRBC in PBS was added to each well of the plates. Uncoated CRBC were used as controls and were added to another set of lymph dilutions. The plates were incubated at 37°C for ½ hr before the results were read and then left at room temperature overnight and read again. The antibody titre of a given sample was taken as the last dilution at which agglutination of the red cells occurred. The first well was designated as 0 and subsequent wells 1, 2, 3, etc. The starting dilution was 1:2 but after incubation in 2-mercapto-ethanol, the starting dilution was 1:4. In situations where a comparison was drawn between titres the same starting dilution was used. If this was not possible a correction was made.

(g) OXYGEN ELECTRODES AND OXYGEN MEASUREMENTS

(i) The Rank Oxygen Electrode

The electrode (Rank Brothers, England) was designed to measure the \( \text{O}_2 \) content in a solution. It could be used instead of the manometric assay for \( \text{O}_2 \) consumption by cells. Basically, the electrode consisted of a small area of platinum which was the cathode of a circuit. It was polarized to
approximately 0.6 V with respect to the anode. The anode was a silver-silver chloride half-cell. $O_2$ diffused through a thin (0.0005) teflon membrane and was reduced at the Pt surface immediately in contact with the membrane:

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$$

The magnitude of the cathodic current so generated was directly proportional to the concentration of $O_2$ in the medium.

The reaction vessel of the electrode was made of either perspex or glass. Its dimensions were 1.5 cm internal diameter, and 3.5 cm in depth and it was surrounded by a water jacket through which water from a thermostat was circulated. The Pt and Ag half-cells were incorporated in the base of the reaction vessel. All additions of material were made through a hole 1 mm diameter in a perspex disc. The level of liquid in the reaction vessel was adjusted to reach the top of the hole in the disc and the liquid was stirred continuously with a magnetic flea. The current flowing through the electrode system was passed through a polarizing unit (Rank Brothers) which in turn fed a recorder (Houston Instrument Co.). The steps in setting up the electrode were as follows:

1. The base of the reaction vessel was detached by unscrewing the perspex locking nut.
2. Sufficient M-KCl or saturated KCl was added to wet the Ag and Pt electrodes.
3. A 1 cm square piece of teflon was placed over the Pt electrode and locked in place. Care was taken to avoid trapping any air bubbles, or twisting the membrane. The oxygen electrode was then placed on the magnetic stirrer.
To use the electrode for $O_2$ measurements the air-saturated medium (usually Krebs & de Gasquet Buffer, pH 7.4, containing 5.56 mM glucose) was added to the reaction vessel. The perspex disc was placed in position and the magnetic stirrer was switched on. The sensitivity control was adjusted to give a suitable deflection on the recorder and when a steady state had been achieved, the cell suspension was added through the small hole in the perspex disc with a hypodermic syringe. The percentage change in the deflection over time was due to the respiratory activity of the cells. Care was taken not to introduce air bubbles and the experiment was concluded when the suspension became anaerobic, i.e. when the current fell to zero or nearly so. To start the next experiment the perspex disc was removed and the vessel washed by sucking out the contents.

Since the oxygen electrode was a "percentage response" instrument, the values obtained during the course of an experiment could be used to calculate directly the initial amount of $O_2$ in the medium, and thus the number of moles of $O_2$ utilized by the sample in unit time. If

$$R_1 = \% \text{ recorder reading with air-saturated buffer},$$

$$R_2 = \% \text{ recorder reading of buffer of zero } O_2 \text{ concentration},$$

then each division of the recorder would be equivalent to

$$\frac{\text{Reaction volume} \times \text{Solubility at } R_1}{R_1 - R_2} \text{ units of } O_2$$

The solubility of $O_2$ in Krebs & de Gasquet buffer used in the present investigation was taken as 5.1 $\mu$l/ml at 37°C, 760 mm Hg, the same as the Krebs-Ringer solution (Lessler and Brierley, 1969). It was verified in preliminary experiments that the two solutions when saturated with air were more or less identical in
terms of the current monitored by the electrode. The detailed method for the determination of the concentration of O₂ in an air-saturated medium was described by Chappell (1964). The electrode was calibrated by measuring the current output from the electrode when the apparatus was filled with buffers equilibrated with gases having known values of partial pressure of oxygen, viz., pure (99.9%) O₂, air (20.9% O₂) and pure (99.9%) N₂. The oxygen tension of the equilibrated solutions (Pₒ₂) was calculated from:

\[ Pₒ₂ = (P_{bar} - P_{H₂O}) \times F_{O₂} \]

where

\( P_{bar} \) = barometric pressure,
\( P_{H₂O} \) = saturated vapour pressure of water at the ambient temperature (available from Handbook of Chemistry and Physics, R.C. Weast, ed., 1976-77),
\( F_{O₂} \) = fraction of O₂ in the equilibrated gas mixture.

Solutions with zero O₂ content could also be prepared by the addition of sodium dithionite (a few crystals or 0.1 ml of saturated aqueous solution) which reacted with dissolved O₂ to provide anaerobic situations.

When allowance was made for the residual current of the electrode, the response of the electrode was linear with respect to oxygen partial pressure as shown in Figure 2.1.

Non-linearity of electrode response was usually due to AgCl deposition on the anode. This was easily removed by wiping the anode with 8-10 N NH₄OH solution or by polishing the anode surface with "jeweller's rouge". The residual current could be tested by adding a few crystals of sodium dithionite.
FIGURE 2.1: Rank oxygen electrode calibration curve.

The buffer in the electrode reaction vessel was equilibrated with $N_2$ (○), air (△) and high purity $O_2$ (□). The electrolysis current was recorded in terms of per cent full-scale-deflection (% F.S.D.).
Electrolysis current (% F.S.D.) vs. \( P_{O_2} \) (\% \( O_2 \))

\[ \text{This was a separated oxygen electrode system in which the cathode consisted of a platinum wire (0.2 mm diameter), the tip of which was sealed in an oxygen-natural gas flame to form a soft glass capillary. The Pt wire was inserted into a teflon tube to a depth of 5-10 mm into the glass capillary. The space between the Pt wire and the teflon tube was filled with epoxy resin. The glass-sealed tip was ground into a hemisphere until about half of the Pt ball inside the glass was exposed. This exposed part of the cathode was then coated with a 1% solution of cellulose diacetate in acetone/ethanol (1:1) mixture (Hayakawa, Ishibashi, Sasaki and Kodigawa, 1967; Sasaki, 1967).} \]
(hydrosulphite) to a solution in the electrode. The electrode current should fall within 5 sec to zero or nearly so following addition of the sodium dithionite. If this did not happen, the membrane was changed and the electrical connection checked. The electrode should be removed from contact with the dithionite as soon as possible as prolonged exposure affected its performance. A "leaky" membrane often led to noise and had to be changed. For best results, the locking nuts of the apparatus should not be overtightened and the electrode should not be switched off between experiments.

Further information on the characteristics and the details of operation for this type of electrode is available in the literature (Strohm and Dale, 1961; Chapell, 1964; Fatt 1964; Estabrook, 1967; Lessler and Brierley, 1969; Brown, 1970; Beechey and Ribbons, 1972; Veefkind, Van den Camp and Maas, 1975).

(ii) Insertable Oxygen Electrode

This was a separated oxygen electrode system in which the cathode consisted of a platinum wire (0.2 mm diameter), the tip of which was melted in an oxygen-natural gas flame to form a small ball (0.3-0.5 mm diameter). This tip was sealed into a soft glass capillary. The Pt wire was inserted into a teflon tube to a depth of about half of the part sealed into the glass capillary. The space between the Pt wire and the teflon tube was filled with epoxy resin. The glass-sealed tip was ground into a hemisphere until about half of the Pt ball inside the glass was exposed. This exposed part of the cathode was then coated with a 7.5% solution of cellulose diacetate in acetone/ethanol (1:1) mixture (Hagihara, Ishibashi, Sasaki and Kamigawara,
1978). The coating procedure involved dipping the end of the cathode to a depth of 3 to 5 mm into the cellulose diacetate solution and then removing it. The coating was allowed to dry in air with the dipped end uppermost. The procedure was repeated 6 to 10 times and the tip of the cathode then exposed to acetone vapour. The cellulose diacetate which was precipitated in the membrane was dissolved by the vapour and became transparent within a few minutes. The membrane was then dried in air and heated in an electric oven at 120°C for 20 min. This resulted in a stable, relatively flow independent cathode for oxygen tension measurements which had a sufficiently rapid response (Figure 2.2).

The anode was made up of a bright silver wire (0.3 mm diameter) built into a plastic chamber, one end of which was constructed so as to accommodate an injection site (Tuta Lab., N.S.W. No. 50-251). The other end of the chamber held a small piece of porous pot. The chamber was filled with normal saline to convert the system to a Ag/AgCl electrode a few minutes before use.

Both the anode and the cathode were supplied by Professor Hagihara of the University of Osaka, Japan.

To measure the $P_{O_2}$ of popliteal efferent lymph, the anode and cathode were connected as shown in Figure 2.3. The cathode was fitted onto an injection site which was inserted into a 3-way stopcock (Pharma Plast, N.S.W. No. 451), one opening of which was connected in turn to another stopcock. The anode was connected to the second stopcock as shown in the diagram. The electrode system was filled with 2% heparin in saline and the cathode was ready for insertion into the lymphatic cannula.
FIGURE 2.2: Structure of the cathode used in lymph oxygen measurements.

C: cellulose diacetate coat
D: "Dotite"-electroconductive epoxy resin
E: epoxy resin
G: glass
P: platinum
S: stainless steel tube
T: teflon tube

Dimensions in mm.
FIGURE 2:3: The cathode, anode and connecting system for monitoring lymph PO₂.

- A: anode
- C: cathode
- I: injection site
- L: lymph duct
- M: measuring cylinder
- P: platinum cathode
- S: three-way stopcock
The cathode was inserted into the capsule so that its tip was situated at the point where the capsule started to taper close to the uterine cavity of the oviduct. The external end of the cathode was kept immersed in the Locke's solution in the catheter, and the cathode tip was first inserted into the cannula, and then the cathode was slowly advanced through the two capillaries (the second containing the cannula) and was finally collected in a measuring cylinder. A sufficient and constant conductivity between the capsule and the cathode was thus provided when a \( \text{PO}_2 \) measurement was made. The terminals of the cathode and the anode were connected to a polarizing unit (Rank Brothers, Ltd.). The capsule was connected to a pen-recorder (Houston Instruments, U.S.A., \( \text{F.} \)) when \( \text{PO}_2 \) measurements were not made. A length of tubing of smaller diameter (usually 1.5, 1.0 mm, or 1.0 mm) was disconnected to the end of the cannula by the nicotine, and the lymph collected in a bottle tied to a suture attached to the skin. This tubing was disconnected each time that \( \text{PO}_2 \) measurements were made. The cannula was cleared of any fluid or air bubbles before the cathode was inserted.
The cannula was a clear vinyl tubing (Dural Plastics, N.S.W., No. SV110; I.D. 1.5 mm, O.D. 2.5 mm). One end of the tube was pulled out so that it could be inserted into the lumen of the lymph duct. This was done by heating the tube gently over a wire gauze on top of a Bunsen burner (Heatley and Weeks, 1964).

The cathode was inserted into the cannula so that its tip was situated at the point where the cannula started to taper close to the lumen of the duct. The external end of the cannula was inserted into the injection site mounted on the cathode to provide an air-tight system. The total length of the cathode that was inserted into the cannula within the sheep was about 16-17.5 cm which was the length of the cannula plus the short tapered segment inserted into the lymph duct. Lymph flowed into the cannula, past the cathode tip first, then through the two stopcocks (the second containing the anode) and was finally collected in a measuring cylinder. A sufficient and constant conductivity between the anode and the cathode was thus provided when a $P_{O_2}$ measurement was being made.

The terminals of the anode and the cathode were connected to a polarizing unit (Rank Brothers, England) which was connected to a pen-recorder (Houston Instruments, U.S.A.). When $P_{O_2}$ measurements were not being made a length of tubing of smaller diameter (usually I.D. 1.0 mm, O.D. 1.5 mm) was connected to the end of the cannula on the outside, and the lymph collected in a bottle tied to a holder attached to the skin. This tubing was disconnected each time that $P_{O_2}$ measurements were made. The cannula was cleared of any clots or air bubbles before the cathode was inserted.
Electrode characteristics including drift, noise, residual current, linearity, response, flow dependence and temperature dependence were determined before an electrode was actually used to measure lymph \( P_{O_2} \) (Chapter 3).

(h) **MANOMETRIC TECHNIQUES**

The apparatus used was a Warburg constant volume respirometer (B. Braun, W. Germany) consisting of a detachable flask equipped with one sidearm and a centre cup, attached to a manometer. The manometer had an open and a closed end. A reference point on the closed side was chosen (150 mm), and the liquid in the closed side was always adjusted to this point before recording pressure changes. Thus the volume of gas in the flask was held constant. The flask was immersed in a water bath at a constant temperature (37°C), and between readings the system was shaken at a rate of 107 oscillations per min to promote a rapid exchange of gas between the fluid and the gas phase. It was assumed that the temperature of the manometer, which was not immersed, did not differ greatly from that of the flask. The procedure was conducted according to the method of Umbreit, Burris and Stauffer (1964).

(i) **The Direct Method of Warburg Manometry**

The method employed 2 flasks in which the respiration was occurring in exactly the same way, except that in one the \( CO_2 \) was absorbed with alkali whereas in the other it was not. Three chrome-merge cleaned Warburg flasks were prepared as in Table 2.1:
T.B.: Thermobarometer which consisted of a Warburg manometer with a flask containing water attached; the volume of the flask and the volume of water it contained was not critical. It was employed for making corrections to external changes of temperature and pressure.

A : Flask with KOH which absorbed any CO₂ present in the flask.
B : Flask without KOH.

Buffer: Krebs & de Gasquet buffer with 5.56 mM glucose (0.1%).

Cell suspension: Lymphocytes suspended in the above buffer solution at a concentration of 2.0 x 10^8 cells/ml.

The plug for the sidearm was greased and inserted. The joint on the manometer was also greased and the flask attached to it. The three systems were placed in the constant temperature bath (it was recommended by the manufacturer that 30 ml of 1.5% NaCl be added to about 10 litres of distilled water in the bath to improve the sensitivity of the thermostat) and allowed to equilibrate, with shaking, for 10-15 min. After equilibration, the manometer fluid was adjusted to the reference point (150 mm) on the closed side of the manometer with the stopcock open. The stopcock was then closed and readings begun at 20 min intervals for 1 hr and at 30 min intervals for a second hour. The amount of gas exchanged was calculated by the formula:

<table>
<thead>
<tr>
<th>Flask</th>
<th>Main vessel</th>
<th>Centre well</th>
<th>Sidearm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂O</td>
<td>Cell susp.</td>
<td>Buffer</td>
</tr>
<tr>
<td>T.B.</td>
<td>2.0 ml</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>A</td>
<td>---</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>B</td>
<td>---</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>
(1) \[ x_{O_2} = h.k_{O_2} \]

(2) \[ x_{CO_2} = (H - \frac{x_{O_2}}{K_{CO_2}})K_{CO_2} \]

(3) \[ k(K) = \frac{V_g \frac{273}{T} + V_f \alpha}{P_0} \]

where

- \( x_{O_2} \) = \( \mu l \) of \( O_2 \) exchanged (\( 0^\circ C. \), 760 mm Hg pressure),
- \( x_{CO_2} \) = \( \mu l \) of \( CO_2 \) exchanged,
- \( h \) = mm observed change corrected for thermobarometer in system with KOH in flask (Flask A),
- \( H \) = mm observed change corrected for thermobarometer in system without KOH in flask (Flask B),
- \( k_{O_2} \) = flask constant for \( O_2 \) exchange for flask with KOH,
- \( k_{CO_2} \) = flask constant for \( CO_2 \) exchange for flask without KOH,
- \( V_g \) = volume of gas phase in flask including the connecting tubes down to the reference point,
- \( V_f \) = volume of fluid in flask,
- \( P_0 \) = standard pressure (760 mm Hg or 10,000 mm Krebs' or Brodie's fluid),
- \( T \) = temperature of bath in degrees absolute,
- \( \alpha \) = the Bunsen coefficient. This is the solubility of the gas involved in the reaction fluid. For \( O_2 \) in Krebs & de Gasquet buffer, it was taken as 0.0239 ml/ml, and for \( CO_2 \) it was 0.5672, the same as for Ringer's solution (Umbreit et al., 1964) assuming that the composition of the two buffer solutions was not too different.
In expressing the rate of gas exchange, a quotient \((Q)\) was employed, \(Q = \mu l\) of gas exchanged per number of cells per hr. The respiratory quotient \((R.Q.)\) could also be calculated. This was defined as the amount of \(CO_2\) produced divided by the amount of \(O_2\) consumed.

(ii) The Indirect Method of Warburg Manometry

The method was based upon the principle that changes in the volume of 2 gases of different solubility (e.g. \(O_2\) and \(CO_2\)) could be measured simultaneously by following the manometric changes occurring over identical reaction mixtures in two flasks of different volumes or fluid volumes. Three flasks were prepared as in Table 2.2:

<table>
<thead>
<tr>
<th>Flask</th>
<th>Main vessel</th>
<th>Centre well</th>
<th>Sidearm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(H_2O)</td>
<td>Cell susp.</td>
<td>Buffer</td>
</tr>
<tr>
<td>T.B.</td>
<td>2.0 ml</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>A</td>
<td>---</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>B</td>
<td>---</td>
<td>1.0 ml</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

The two flasks, A and B, were of approximately the same volume, containing the same number of cells \((2.0 \times 10^8\) cells) but different volumes of fluid \((A = 2.0\) ml, \(B = 6.0\) ml). The gas exchange was calculated as follows:

\[
(1) \quad x_{O_2} = \frac{H.K_{CO_2} - h.k_{CO_2}}{C(O_2)}
\]

\[
(2) \quad x_{CO_2} = \frac{H.K_{O_2} - h.k_{O_2}}{C(CO_2)}
\]
Small letter symbols were used for the flask containing less fluid (Flask A) and capital letter symbols for Flask B. This method allowed the O$_2$ uptake and CO$_2$ production to be determined in the presence of adequate supplies of CO$_2$, and thus allowed a comparison to be made with the results of the "Direct Method" to determine whether or not CO$_2$ did influence the rate or the course of the process involved.

(i) DETERMINATION OF GLUCOSE UPTAKE AND LACTATE PRODUCTION

(i) Glucose Uptake

The method used for determining glucose uptake was described by Huggett and Nixon (1957) and Jakobsen (1960). The procedure was that of the Sigma Technical Bulletin No. 510 described by Bergmeyer and Bernt (1974). Sigma reagents were used. The method was based upon the following coupled enzymatic reactions:

1. Glucose + 2H$_2$O + O$_2$ \( \xrightarrow{\text{Glucose oxidase}} \) Gluconic acid + 2H$_2$O$_2$

2. H$_2$O$_2$ + O-Dianisidine \( \xrightarrow{\text{Peroxidase}} \) oxidized O-Dianisidine (colourless) (brown)

The intensity of the brown colour measured at 425-475 nm was proportional to the original glucose concentration.

The reagents required were:

PGO enzymes (Stock No. 510-6)

These were supplied in pre-weighed capsules. Each capsule contained 500 International units of glucose oxidase.
(Aspergillus niger), 100 Purpurogallin units of peroxidase (horseradish) and buffer salts. The capsules were stored in the refrigerator at 0-5°C. To prepare the Combined Enzyme-Colour Reagent Solution, the contents of one capsule were added to 100 ml distilled water in an amber bottle which was then inverted several times with gentle shaking to dissolve the contents. The Colour Reagent Solution (1.6 ml) was then added and mixed. The resulting Combined Enzyme-Colour Reagent Solution was stable for up to 1 month at 0-5°C unless turbidity or colour formed. If this happened the mixture was discarded.

O-dianisidine dihydrochloride (Stock No. 510-50)

To prepare the Colour Reagent 50 mg of O-dianisidine dihydrochloride was dissolved in 20 ml water. The reagent was stable for 3 months in the refrigerator at 0-5°C.

Glucose standard solution (100 mg/100ml)

100 mg of glucose was dissolved in distilled water and 2.5 ml perchloric acid was added and the solution diluted to 100 ml with distilled water. The solution was stored at room temperature and discarded if any turbidity developed.

(Commercially available glucose contained ca. 9% moisture, thus the true concentration of the Standard was 91 mg/100 ml).

Standards of other concentrations (e.g. 50, 150, 200 mg/100 ml etc.) were prepared in a similar way.

Sigma also provided Glucose Standard Solutions (Stock No. 635-100) which consisted of solutions of β-D-glucose, 100 mg/100 ml, in benzoic acid, 0.1%.
Perchloric acid (0.33 M)

2.85 ml of 70% perchloric acid (HClO₄) was diluted to 100 ml with distilled water. This was used for removing proteins from solutions. Excess of HClO₄ was not removed from the solutions. Allowance was made in the buffer used in the Enzyme Reagent for neutralizing the HClO₄ (Krebs, Bernett, de Gasquet, Gascoyne and Yoshida, 1963).

Cells collected from lymph were washed once with ACT solution (to lyse any red cells present) and once with Krebs & de Gasquet buffer. The washed cells were resuspended in an incubation medium (Krebs & de Gasquet buffer, pH 7.4, containing 5.56 mM glucose) to a concentration of 2.0 x 10⁸ cells/ml. 2.0 ml of the cell suspension, together with 4.0 ml of incubation medium were put into a Warburg flask attached to a manometer. The system was placed in a water bath at 37°C and incubated with gentle shaking for 20 min before sampling started. During the 2 hr incubation 0.2 ml of the cell suspension was removed each 30 min and deproteinized with 2 ml of 0.33 M HClO₄. Before removing the cells, the cell suspension was mixed gently by aspiration through a Pasteur pipette. The deproteinized mixture was centrifuged and 0.2 ml of clear supernatant was transferred to test tubes for assay. Assays were run in duplicate series giving four determinations for each time of sampling. 2.0 ml of Combined Enzyme-Colour Reagent Solution was added to each test tube containing the sample. After mixing, the tubes were incubated at 37°C for 30 min or at room temperature for 45 min. Absorbance was read at 450 nm together with the Standard and the Blank (containing HClO₄ instead of samples). Glucose was also measured in samples of lymph and serum. For this, 0.2 ml samples were
deproteinized with 2 ml of HClO₄ and centrifuged. 0.2 ml of the supernatant was assayed for glucose concentration as mentioned above. Readings were completed within 30 min. The glucose level in each sample was calculated as follows:

\[
\text{Glucose (mg/100 ml)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100, \text{ or}
\]

\[
\text{Glucose (mM)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 5.56
\]

and glucose uptake was calculated as

\[
(G₀ - Gₓ) \times 6.0 \mu\text{moles, where}
\]

\[
G₀ = \text{glucose level (mM) at time zero and}
\]

\[
Gₓ = \text{glucose level at time } x.
\]

The rate of glucose uptake was determined by plotting uptake of glucose (mean of 4 determinations for each sampling) versus time (Figure 2.4). The linear relationship between glucose concentration and absorbance was verified with a series of primary standard solutions of glucose equivalent to 50, 100, 150, 200 and 300 mg/ml. A calibration curve was prepared by plotting the concentrations of the primary standards versus their respective absorbances (Figure 2.5).

For the assay a new blank was prepared for each series of tests. Serious error could result if an "old" blank was reused, because the colour of the blank increased with time. Glassware that became stained by the reagents was easily cleaned by rinsing with acetone or washing with a detergent. It was important to avoid contact between the reagents or the water used in preparing the reagents and rubber, as certain substances in rubber inhibited the reaction. Erroneously high values in assay might result if deproteinized samples were stored and not assayed shortly after
FIGURE 2.4: Determination of rates of glucose uptake and lactate production.

The incubation medium was sampled at 30 min intervals and the amount of glucose consumed (●) and lactate produced (▲) was determined. The rate of metabolism was calculated from the slope of the straight line through corresponding points on the graph, each point representing the mean of 4 determinations.
FIGURE 2.5: Glucose calibration curve.
preparation because prolonged storage in acid solution leads to hydrolysis of the polysaccharides contained in the samples (Bergmeyer and Bernt, 1974).

(ii) Lactic Acid Determination

This method was described by Peleiderer and Dose (1955) and Hohorst (1957). The present procedure was conducted according to Sigma technical bulletin No. 826-uv and the procedure described by Gutmann and Wahlefeld (1974). The method was based upon the following reversible reaction:

\[
\text{Lactic acid} + \text{NAD}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{Pyruvic acid} + \text{NADH} \quad \text{(low} \quad \text{A}_{340} \quad \text{)} \quad \text{(high} \quad \text{A}_{340} \quad \text{)}
\]

In the presence of excess \text{NAD}^+, substantially all the lactic acid was converted to pyruvic acid which was trapped with hydrazine. The increased absorbance at 340 nm due to NADH formation was a measure of the lactic acid originally present in the sample. The following reagents were used:

**Lactic dehydrogenase** (Stock No. 826-6)

This was supplied in 2.5 ml bottles which contained an LDH suspension isolated from beef heart in ammonium sulphate. It was approximately 1000 units per ml when prepared. The suspension was stored at 0-5\(^\circ\)C and had to be mixed to make the suspension uniform before use.

**Glycine buffer** (Stock No. 826-3)

This was supplied in 100 ml bottles containing glycine and hydrazine, pH 9.2. Chloroform was added as preservative. It was stored at 0-5\(^\circ\)C.

**NAD** (\(\beta\)-nicotinamide adenine dinucleotide, Stock No. N-7004)

This was prepared from yeast, Grade III (approx. 98% pure) and supplied in 600 mg bulk packages. It was stored desiccated below 0\(^\circ\)C.
Lactic acid standard solution (Stock No. 826-10)

This contained L(+)-lactic acid, 0.40 mg/ml with preservative added. It was stored at 0-5°C and diluted 1:5 times (1 ml Standard Solution to 5.0 ml with water) before use. The diluted solution was discarded after 1 day.

Perchloric acid 8% (w/v)

This was prepared by diluting 7 ml of 70% (w/v) perchloric acid to 100 ml with water.

Cells were prepared and incubated as for the glucose uptake assays. 0.4 ml of ice-cold 8% HClO₄ and 0.1 ml of clear supernatant was used for the assay. Two blank solutions were also prepared which contained HClO₄ instead of sample. (Lactic acid in the HClO₄ supernatant was stable for at least 1 week at 0-5°C). To each test tube containing the supernatant or blank, 1.4 ml of Lactic Acid Reagent mixture was added. The reagent was prepared by mixing the following:

100 mg β-NAD; 20 ml glycine buffer; 40 ml distilled water and 1.0 ml LDH suspension.

These quantities were enough to assay 20 samples. After mixing with a vortex mixer the tubes were incubated either at 37°C for 30 min or at room temperature for 45 min. Absorbance was read at 340 nm with the blank as a reference. If the reaction was complete, the absorbance did not increase with further incubation. If the absorbance continued to increase by more than 0.002 per min, another incubation period of approximately 15 min was allowed.

Since the spectrophotometer (Unicam, SP 1800) used had a narrow band width with a 1 cm cuvet, the concentration of lactic acid was calculated directly from the absorbance readings as follows:
Lactic acid (mM) = $A_{340} \times 7.23$, or

Lactic acid (mg/100 ml) = $A_{340} \times 65.1$

If a wide-bandwidth spectrophotometer was employed, values for the concentration of lactic acid had to be determined from a calibration curve which was prepared for the purpose (Figure 2.6). The factor 7.23 in the above formula was obtained as follows:

$$7.23 = \frac{1.5}{6.22 \times 0.03333 \times 1}$$

where

$1.5 = \text{reaction volume (ml),}$

$6.22 = \text{millimolar extinction coefficient of NADH at 340 nm,}$

$0.03333 = \text{volume (ml) of original sample in cuvet,}$

$1 = \text{light path (cm)}$

The rate of lactic acid production was calculated in a similar way to the rate of glucose uptake (Figure 2.4).

(j) **RADIORESPIROMETRY**

Short-term radiorespirometry was done according to Duncombe (Duncombe, 1974). The method enabled the metabolism of labelled $^{14}$C-glucose to be followed closely by recording the production of $^{14}$CO$_2$. High cell concentrations were used, with labelled substrates of the highest specific radioactivities available.

A small amount of carbonic anhydrase was added to the incubation medium to decompose the $^{14}$C-bicarbonate formed during oxidation. The resulting $^{14}$CO$_2$ was swept from the medium by a stream of air which was bubbled through a CO$_2$-absorbing solution held in containers which were easily interchangeable. The apparatus (Figure 2.7) consisted of an incubation flask (A) made from a Quickfit screw-thread joint (Catalogue No. SQ 18) on which a small bulb with a short sidearm (B) was blown. A serum cap (C) was
FIGURE 2.6: Lactic acid calibration curve.
LACTIC ACID CONCENTRATION (mg/100 ml)

ABSORBANCE (340 nm)
FIGURE 2.7: Apparatus for radiorespirometry.

A: incubation flask
B: sidearm of incubation flask
C: serum cap
D: sidearm with cone
E: two-way capillary bore stopcock
F: glass tubing
G: scintillation-counting vial
H: centre tube
Flexible over the shoulder. A shroud with a 3/16/3/16 Quickfit cone (G) was attached near the top of the flask, onto which a 10/19 socket (Quickfit) and a two-way capillary bore stopcock (E). The two outlets from this were connected by lengths of glass tubing (F) which dipped into the CaO- absorbing medium (D). One ml of Carbo-Sorb (D) contained 10 standard liquid scintillation counting vials (F) which rested on vertically movable platforms. The two-way stopcock and the movable platform allowed a rapid changeover and replacement of the vials. The lower end of the incubation flask was immersed in a thermostatically controlled water bath (37°C, Medical air circulating water bath, Ellis, No. 480) was bubbled into the system at the rate of 100 ml per min through a centre hole (H) in the shroud. The air was filtered through a glass tube, filled with distilled water. The rate of air was regulated by a flowmeter (Fisher Scientific, Ltd., Australia). The entire vessel was prepared for the Krebs & de Gasquet buffer at a concentration solution of 10 ml as for the glucose and TCA solutions. The cells set up with the 10/19 Shunt greased. The incubation flask was set up at 37°C and the vials charged with Carbo-Sorb. CaO anhydrate was weighed and dissolved in 0.5 ml of Krebs & de Gasquet buffer. The enzyme solution was transferred to the incubation flask with a Pasteur pipette and washed inulin (F) further 0.5 ml of buffer. The required amount (0.1 ml) of labeled substrate was added to the flask with a microsyringe.
fitted over the sidearm. A sidearm with a B10/19 (Quickfit) cone (D) was attached near the top of the flask, onto which fitted a B10/19 socket (Quickfit) and a two-way capillary bore stopcock (E). The 2 outlets from this were connected by lengths of glass tubing (F) which dipped into the CO\textsubscript{2}-absorbing medium (9 ml of Carbo-Sorb II) contained in standard liquid scintillation-counting vials (G) which rested on vertically movable platforms. The two-way stopcock and the movable platforms allowed a rapid changeover and replacement of the vials. The lower end of the incubation flask was immersed in a thermostatically controlled water bath (37°C). Medical air (CIG, Australia, No. 460) was bubbled into the system at the rate of 200 ml per min through a centre tube (H) which was fixed to the flask with a screw cap (Quickfit QC 18/11) fitted with rubber ring (Quickfit QR 18/7) and washer (Quickfit QW 18/7). The air was freed of CO\textsubscript{2} by passing it through Vivalyme (Indicating soda lime VSP, Medishield) in a gas absorption tower and saturated with water vapour by passing it through a wash bottle filled with distilled water. The flow rate of air was regulated by a flowrator (Fischer & Porter Pty. Ltd, Australia). The cells were prepared in a glucose-free Krebs & de Gasquet buffer at a concentration of 1.0 x 10\textsuperscript{8} cells/ml as for the glucose and lactic acid assays. The apparatus was set up with the B10/19 joint greased, the water bath running at 37°C and the vials charged with Carbo-Sorb II. 2 mg of carbonic anhydrase was weighed and dissolved in 0.5 ml of Krebs & de Gasquet buffer. The enzyme solution was transferred to the incubation flask with a Pasteur pipette and washed in with a further 0.3 ml of buffer. The required amount (1 µCi) of labelled substrate was added to the flask with a microsyringe. 150 µl of
buffer was used to wash in any substrate that was left in the plastic tubes containing the substrates. The air flow was turned on and the time recorded. A Blank collection was done to check that there was no significant non-enzymic release of $^{14}$CO$_2$ from the substrate. 1.0 ml of cell suspension in buffer was injected through the serum cap and sampling continued at the regulated time intervals. At the end of each interval the two-way stopcock was turned and the used vial removed and replaced by another vial already charged with Carbo-Sorb solution. 12 ml of Permafluor V from a dispenser was added to the used vials immediately. 1 ml of 8% HClO$_4$ was added at the end of the experiment to kill the cells and stop any further release of $^{14}$CO$_2$. The radioactivity in the vials was counted in a Packard liquid scintillation counter. For comparative purposes the observed counting rates (c.p.m.) were sufficiently accurate to use for comparison without being converted into absolute radioactivity measurements because quenching was almost identical between samples.

(k) INCORPORATION OF TRITIATED ISOTOPES BY CULTIVATED CELLS

These experiments were done under sterile conditions. Cells were washed 3 times with Hanks and resuspended in F-15 medium supplemented with 10% FCS. The concentration of cells was adjusted to $5 \times 10^6$ cells per ml. Triplicate samples (0.2 ml) of cell suspensions were incubated in multi-dish disposo trays (Linbro Scientific Co.) with 20 µl of tritiated isotope ($^3$H-TdR, -UR or -Leu; 1.0 µCi/20 µl) at 37°C in culture boxes. The gas phase was a mixture of 10% CO$_2$, 7% O$_2$ and N$_2$. After 4 hr incubation, the cells were harvested with a MASH II cell harvester (Multiple Auto Systems Harvester, Microbiological Associates) onto glass fibre filters (1½" x 12", Grade 934-AH,
Reeve Angel, Whatman Inc.) using distilled water to wash the tray wells. The filters were dried at 100°C for 45 min. The sections of filter containing the cells were cut out, placed in 15 ml scintillation vials and counted with 7.0 ml of 0.5% PPO in toluene. 

(1) PREPARATION OF AUTORADIOGRAPHS (Rogers, 1967; Hay, 1970) 

(i) Preparation of Slides

Cells were first washed 3 times with Hanks plus 5% FCS and resuspended in Hanks with 10% FCS. The cell concentration was adjusted to 5 x 10⁶ cells per ml. 0.6 ml of the cell suspension (for the preparation of 4 slides) was incubated with 60 µl of tritiated isotope (1.0 µCi per 20 µl of ³H-TdR, ³H-UR or ³H-Leu) for 1 hr at 37°C. The cells were washed 3 times with 5 ml of Hanks+5% FCS at 4°C and then resuspended in FCS, the cell concentration being adjusted to 1.0 x 10⁶ cells per ml. Quadruplicates of 0.1 ml of the samples were transferred to chambers of the cytocentrifuge and a further 0.1 ml FCS was added to each chamber. The slides thus prepared were air-dried and fixed in methanol for 10-15 min. A pencil dot was placed at the right bottom corner of the slides so that the correct surface of the slide could be judged in the dark room when the autoradiographs were prepared. A black circle was also drawn around the circular smear on the slide, so that the stripping film could be positioned correctly. 

(ii) Stripping Film Method

The chrome alum/gelatine solution was made up and put in a small beaker filled to the top. Any air bubbles in the solution were removed with a filter paper. The slides were dipped into the solution and left to dry in air. Kodak AR 10 stripping film
was taken from the refrigerator 1 hr before it was used. The emulsion of the stripping film was cut with a scalpel into 16 sections. After discarding 1 cm of the edges of the emulsion, six sections were stripped off with curved forceps and floated up-side-down on a water bath at 25°C for 3 min. The slide was passed underneath the film and then lifted so that the film attached smoothly onto the slide over the smear. The coated slides were dried in front of a fan for 1 hr in total darkness. They were then put in light-tight boxes containing silica gel and stored in the refrigerator. The boxes were wrapped in black paper and labelled.

(iii) Development of Autoradiographs

The slides were developed at various times after they were covered with film so as to obtain the desired degree of exposure. Kodak K19 developer and diluted Hypam Ilford fixer (50 ml fixer +200 ml water) were used. Development was done under a red safety light. Autoradiographs were counter-stained by dipping them into 0.1% Azure A in acetate buffer. The slides were then dried overnight.

(m) MISCELLANEOUS METHODS

(i) Quench Corrections

In radiorespirometric experiments, the observed radioactivity counting rates (c.p.m.) were used directly without correcting them for quenching. This was justified experimentally as mentioned in the text.

In case absolute radioactivities were required, the counting efficiency of a sample was established with the internal standardization procedure. The unknown sample was first counted, then a reference standard of known d.p.m. (Packard, Carbon Standard, $^{14}$C-hexadecane) was added to the counting sample
and the sample recounted. From the additional counts due to
the Standard, the counting efficiency of the sample and thus
the sample d.p.m. could be calculated:

\[
\text{Efficiency} = \frac{(\text{Internal standard + Sample}) \text{c.p.m.} - \text{Sample c.p.m.}}{\text{Internal standard d.p.m.}}
\]

Sample d.p.m. = Sample c.p.m. - Background c.p.m.

Efficiency

The corrected disintegration rate thus obtained was directly
comparable for all samples.

(ii) Scintillation Counting

All scintillation counting was done with a Packard Tri-Carb
Liquid Scintillation Spectrometer model 3320. Samples were
allowed to cool in the counter before counting began and counts
were accumulated for 1 or 5 min. The channel settings were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maximum beta-particle energy</th>
<th>Gain</th>
<th>Window</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H Sample</td>
<td>0.018 MeV</td>
<td>50%</td>
<td>50-1,000</td>
</tr>
<tr>
<td>$^{14}$C Sample</td>
<td>0.156 MeV</td>
<td>6%</td>
<td>50-1,000</td>
</tr>
</tbody>
</table>

(iii) Photomicroscopy (Transmitted-Light Brightfield)

Photomicrographs were taken with an Orthomat-W (Leitz)
fully automatic microscope camera attached to an Orthoplan
microscope (Leitz).

(iv) Sterilization

Glassware was sterilized by autoclaving and plastic materials
by gamma irradiation. Solutions were either autoclaved or Seitz
filtered. Smaller volumes were filtered through 0.45 µ millipore
filters.

(v) Statistical Methods (Bailey, 1965; Croxton, Cowden and
Klein, 1969)

Standard deviation ($s$)

The standard deviation was expressed for the mean ($\bar{x}$) of a
series of observations ($x$) in some of the results. It was
calculated as the square root of the variance \((s^2)\) which is given by \(\Sigma(x-\bar{x})^2/(n-1)\) where \(n\) is the number of observations.

Thus
\[
s = \sqrt{s^2} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}
\]

**F-test (variance ratio test)**

The F-test was used to determine whether the standard deviations of 2 sets of observations were legitimate estimations, within reasonable limits, of the same population standard deviation (\(\sigma\)). The ratio of the variances \(s_1^2\) and \(s_2^2\) associated with the two sets is designated as F and is compared with critical values that will be exceeded by valid estimations of \(\sigma\) with a given frequency. Thus
\[
F = \frac{s_1^2}{s_2^2},
\]
with \(n_1-1\) degrees of freedom in the numerator and \(n_2-1\) in the denominator and where the larger variance is always placed in the numerator. If an experimental F value is larger than the critical value, then there is basis for questioning the hypothesis that \(s_1\) and \(s_2\) are alike and each is a measure of the same \(\sigma\).

"Student's" t-test

The significance of the difference between means of 2 small sets of observations was calculated using the t-test which was given by
\[
t = \frac{\bar{x}_1 - \bar{x}_2}{s'} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}
\]
where
\[
s' \text{ (pooled standard deviation)} = \sqrt{\frac{\Sigma_1 (x-\bar{x}_1)^2 + \Sigma_2 (x-\bar{x}_2)^2}{n_1 + n_2 - 2}}
\]

When \(n_1 = n_2\),
\[
t = \frac{\bar{x}_1 - \bar{x}_2}{s'} \sqrt{\frac{n}{2}}
\]
The calculated t value, with \( n_1 + n_2 - 2 \) degrees of freedom was compared with critical values in a table and the probability of agreement between the 2 means was established.

It is necessary to establish by the F-test that the standard deviation of both sets are legitimate estimates, within reasonable limits, of the same \( \sigma \) before the above formula for the calculation of \( t \) can be used. If a significant difference between \( s^2_1 \) and \( s^2_2 \) is found, the following method of comparison of the 2 means was employed instead of the "t" test.

Calculate

\[
d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s^2_1}{n_1} + \frac{s^2_2}{n_2}}}
\]

and treat this value as being distributed approximately like "Student's" \( t \) with \( f \) degrees of freedom, the latter being given by

\[
f = \frac{1}{\frac{u^2}{n_1-1} + \frac{(1-u)^2}{n_2-1}}
\]

where

\[
u = \frac{s^2_1 / n_1}{\frac{s^2_1}{n_1} + \frac{s^2_2}{n_2}}
\]

**Regression analysis**

For two sets of related observations, \( y \) and \( x \), it is often possible to assume that the true \( y \)-means for each value of \( x \) all lie on a straight line, the line which exhibits the linear regression of \( y \) on \( x \) and is expressed as \( y = a + bx \). The slope of the line is the regression coefficient (\( b \)) of \( y \) on \( x \), and measures the average amount by which \( y \) increases for a unit increase in \( x \) and is given by
\[ b = \frac{\Sigma(x-x) (y-y)}{\Sigma(x-x)^2} \]

The intercept of the line on the y-axis (a) is given by

\[ a = \bar{y} - b\bar{x} \]

\[ = \frac{(\Sigma x)(\Sigma xy) - (\Sigma y)(\Sigma x^2)}{(\Sigma x)^2-n\Sigma x^2} \]

In order to test whether the observed regression coefficient \( b \) is significantly different from the hypothetical value zero, the standard error \( s_0 \) of \( b \), if the number of observations \( n \) is greater than 30, is estimated by calculating

\[ s_0 = \frac{s}{\sqrt{\Sigma(x-x)^2}} \]

where

\[ s = \sqrt{\frac{1}{n-2} \left( \Sigma(y-y)^2 - \frac{[\Sigma(x-x)(y-y)]^2}{\Sigma(x-x)^2} \right)} \]

The ordinary test for normally distributed estimates then applies:

\[ t = \frac{b}{s_0} \]

with \( n-2 \) degrees of freedom and is compared with critical values in a table in order to determine whether the regression is significant or not.

**Coefficient of correlation and coefficient of determination**

The relationship between 2 variables, \( y \) and \( x \) can be determined by calculating the value \( r \), the coefficient of correlation which is equal to:
\[ r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{n\Sigma x^2 - (\Sigma x)^2}[n\Sigma y^2 - (\Sigma y)^2]} \]

where \( n \) = number of observations,

\( x \) = independent variable,

\( y \) = dependent variable (the variable to be estimated).

The calculated value of \( r \) tells the closeness of the correlation. The statistical significance of the \( r \) value can be determined with reference to a Table of Correlation Coefficients.

The coefficient of determination \( (r^2) \) is the square of the coefficient of correlation. This value gives an estimate of the percentage variation in the dependent variable "y" that has been accounted for by the regression equation (i.e. \( y = a + bx \)).
CHAPTER 3

IN VIVO MEASUREMENT OF THE PARTIAL PRESSURE OF OXYGEN IN THE EFFERENT LYMPH FROM THE POPLITEAL NODE
INTRODUCTION

All metabolic activities of lymphocytes are, to a varying degree, affected by the oxygen tension (PO₂) of the environment in which the cells exist. Thus it was an essential prerequisite to measure the PO₂ of pleural lymph in vivo so that the experimental results obtained from subsequent metabolic studies could be analyzed and interpreted in a physiological sense.

Attempts to measure the PO₂ that exists in animal tissues can be traced back as far as the early eighteen hundreds when Davy (1823) tried to estimate the extracellular PO₂ in vivo by taking periodic samples from an air bubble introduced into the pleural space. Davis and Brink (1942), used a separated oxygen electrode system consisting of a Pt cathode and a calomel anode placed in different parts of the body, to measure the tissue PO₂. The first successful duplication of a separated oxygen electrode system to the measurement of the PO₂ of whole blood in vitro and in vivo was attempted in 1953 by Clark, Wolf, Birnger and Taylor (1953).

From this time on the measurements of the PO₂ of blood have been made with intravascular separated or compartment electrodes covered with various materials such as cellophane, teflon, polyvinyl chloride, polyethylene, polyurethane or coated with collection cellophane, hydroxy polyvinyl chloride or rhoplex (Clark et al., 1953; Clark, 1954; Birnger, Norris and Nessler, 1960; Severinghaus, 1962; Heller, J adalah, Warde and Watson, 1972; Mc Hê, Neuman and Hoith, 1962; Remy, Forte, Schueller, Knöpf, Kirchner and Levivitte, 1977; Brown, Liu, McWilliam, Newman and Smith, 1973; König and Vogt, 1973; Woodward, Keith, Perousitch, Rubinstein, Noyke and Scopes, 1974; Fuxaule and Fatt, 1974; Smout, Cogman
All metabolic activities of lymphocytes are, to a varying degree, affected by the oxygen tension \( P_{O_2} \) of the environment in which the cells exist. Thus it was an essential prerequisite to measure the \( P_{O_2} \) of popliteal lymph in vivo so that the experimental results obtained from subsequent metabolic studies could be analyzed and interpreted in a physiological sense.

Attempts to measure the \( P_{O_2} \) that exists in animal tissues can be traced back as far as the early eighteen hundreds when Davy (1823) tried to estimate the extracellular \( P_{O_2} \), in vivo by taking periodic samples from an air bubble introduced into the pleural space. Davis and Brink (1942), used a separated oxygen electrode system consisting of a Pt cathode and a calomel anode placed in different parts of the body, to measure the tissue \( P_{O_2} \). The first successful application of a separated oxygen electrode system to the measurement of the \( P_{O_2} \) of whole blood in vitro and in vivo was attempted in 1953 by Clark, Wolf, Granger and Taylor (1953).

From this time on the measurements of the \( P_{O_2} \) of blood have been made with intravascular separated or combined electrodes covered with various materials such as cellophane, teflon, polyvinyl chloride, polyethylene, polystyrene or coated with collodion, cellophane, hydron, polyvinyl chloride, or rhoplex (Clark et al., 1953; Clark, 1956; Kreuzer, Harris and Nessler, 1960; Severinghaus, 1968; Heller, Imredy, Shafer and Watson, 1972; Huch, Huch, Neumayer and Rooth, 1972; Requena, Forte, Scheller, Knopf, Kirchner and Levowitz, 1972; Brown, Liu, McDonnell, Neuman and Sweet, 1973; Harris and Nugent, 1973; Goddard, Keith, Marcovitch, Robertson, Rolfe and Scopes, 1974; Huxtable and Fatt, 1974; Soutter, Conway

The determination of the gaseous tension of blood has now reached the stage where external monitoring systems can be used. The use of transcutaneous electrodes has been reported and some systems of this type are commercially available. The development of such electrode systems has been reviewed by Indyk (1975). An extensive review by 12 authors on the use of transcutaneous oxygen electrodes has been published recently (Acta Anaesthesiol. Scand. (Suppl.) 68, 1978).

The first attempts to measure the $P_{O_2}$ of lymph were made by Bergofsky, Jacobson and Fishman (1962). They estimated the concentrations of the respiratory gases in the interstitial fluid and the tissues by measuring the tensions of $O_2$ and $CO_2$ of lymph collected from different lymph ducts. A variety of lymph ducts, including the thoracic duct, the femoral duct in the groin, the cysterna chyli and the cervical duct high in the neck were cannulated in anaesthetized dogs with polyethylene tubing and the $P_{O_2}$ of the lymph collected was measured by a modified Clark needle electrode which was housed in a glass cuvette in such a way that samples of lymph and of calibrating solutions could be drawn over the tip of the electrode. Since then various workers have tried to measure lymph $P_{O_2}$ by means of different electrode systems. The methods that have been used can be classified into two categories:
(1) Cannulation of the lymph duct with plastic tubing and the measurement of the $P_{O_2}$ of the lymph after collection (Bergofsky et al., 1962; Said, Davis and Banerjee, 1965; Witte, Clauss and Dumont, 1967; Witte, Cole, Clauss and Dumont, 1968; Staub and Schultz, 1974).

(2) In situ measurement by means of an oxygen electrode inserted into the lumen of the lymph duct (Witte et al., 1976).

The problems inherent in these two methods when applied to the measurement of $P_{O_2}$ of lymph from single lymph nodes are discussed in this chapter and a totally different method of in vivo measurement of lymph $P_{O_2}$ in conscious sheep is described.
SECTION B

PROBLEMS INHERENT IN THE MEASUREMENT OF LYMPH

OXYGEN TENSION

The measurement of the $P_{O_2}$ of lymph collected from cannulated lymph ducts would seem to be simple enough in practice. There are however several problems associated with this approach. Many $P_{O_2}$ measurements have been made on lymph collected with polyethylene cannulae [Borgofsky et al., 1962; Said et al., 1965; Witte et al., 1967; Witte et al., 1968] without due acknowledgement of the fact that the materials from which these tubes are fabricated are permeable to $O_2$ to a variable extent. This intrinsic permeability of the tubing makes it difficult to maintain the gaseous tensions of lymph stable.

Steub and Schultz [1964] experiments to measure the penetration of $O_2$ through the walls of polyethylene tubing and found that there was a hyperbolic (inverse) relationship between the flow rate of lymph and the exchange of $O_2$ between the fluid inside the tubing and the atmosphere. At high flow rates (60 ml/hr) the exchange of $O_2$ was very small. At slow flow rates (10 ml/hr) the exchange was very significant. Because of this the gaseous tension of the lymph in any length of tubing within the body would tend to reach equilibrium with the surrounding tissue fluid.

Similar experiments to those of Steub and Schultz were done to test the gaseous exchange across different types of plastic tubing of different lengths. A reservoir bottle of saline was saturated with high purity $N_2$ (C.I.S., Australia, No. 6244). Fluid was introduced at predetermined flow rates (controlled by a peristaltic pump, LKB, 12000 Varioperist) from the sidearm of the bottle through different lengths of polyethylene...
PERMEABILITY OF POLYETHYLENE AND CLEAR VINYL TUBINGS TO GASES

The measurement of the $P_{O_2}$ of lymph collected from cannulated lymph ducts would seem to be simple enough in practice. There are however several problems associated with this approach. Many $P_{O_2}$ measurements have been made on lymph collected with polyethylene cannulae (Bergofsky et al., 1962; Said et al., 1965; Witte et al., 1967; Witte et al., 1968) without due acknowledgement of the fact that the materials from which these tubes are fabricated are permeable to $O_2$ to a variable extent. This intrinsic permeability of the tubing makes it difficult to maintain the gaseous tensions of lymph stable during the processes of collection and analysis. Staub and Schultz (1974) set up model experiments to measure the penetration of $O_2$ through the walls of polyethylene tubing and found that there was a hyperbolic (inverse) relationship between the flow rate of lymph and the exchange of $O_2$ between the fluid inside the tubing and the atmosphere. At high flow rates (60 ml/hr) the exchange of $O_2$ was very small. At slow flow rates (10 ml/hr) the exchange was very significant. Because of this the gaseous tension of the lymph in any length of the tubing within the body would tend to reach equilibrium with the surrounding tissue fluid.

Similar experiments to those of Staub and Schultz were done to test the gaseous exchange across different types of plastic tubing of different lengths. A reservoir bottle of saline was saturated with high purity $N_2$ (C.I.G., Australia, No. 034). Fluid was introduced at predetermined flow rates (controlled by a peristaltic pump, LKB, 12000 Varioperpex) from the sidearm of the bottle through different lengths of polyethylene.
(I.D. 1.0 mm, O.D. 1.5 mm; Dural Plastics, Cat. No. SP70) and clear vinyl (I.D. 1.0 mm, O.D. 1.5 mm; Dural Plastics, Cat. No. SV70) tubing into a home-made oxygen electrode (Ag anode, Pt cathode coated with cellulose diacetate). Figure 3.1 summarizes the results of this experiment. Both polyethylene and clear vinyl tubings were quite permeable to $O_2$ at slow rates of flow (4.5 ml/hr). Polyethylene was less permeable than clear vinyl. The permeability was more or less linear according to the length of the tubing used. At high rates of flow (45 ml/hr), the exchange of $O_2$ from atmosphere to the saline was relatively small.

Since popliteal lymph flows at rates below 10 ml/hr, an incorrect estimate of the in vivo $P_{O_2}$ of the lymph would be obtained by collecting the lymph after it had flowed through a long length of polyethylene or clear vinyl cannula. Staub and Schultz (1974) devised an oxygen-impermeable, external catheter system to measure the steady state $P_{O_2}$ of lymph in the efferent duct of the caudal mediastinal lymph node in sheep. The system consisted of a silastic cannula with a glass sheath in which the space between the glass sheath and the silastic catheter was filled with agar to eliminate the exchange of $O_2$ with the atmosphere in the length of tubing outside the body. The deficiencies of this method will be discussed later.

Another approach would be to use special synthetic tubings that were impermeable to $O_2$ or to use ordinary polyethylene or clear vinyl tubings which were coated in such a way as to make them no longer permeable to $O_2$. Synthetic tubing that is small in diameter, reasonably flexible and non-permeable to $O_2$ is not commercially available. Attempts were made to coat
FIGURE 3.1: Change in oxygen tension of saline saturated with nitrogen passing through polyethylene and clear vinyl tubings as a function of tube length.

The saline solution in the tubing was at 16°C and flowed at two different rates. The change in PO₂ of the saline due to the permeability of the tubing was monitored by an oxygen electrode.

Vinyl:
- 4.5 ml/hr (●)
- 45 ml/hr (○)

Polyethylene:
- 4.5 ml/hr (▲)
- 45 ml/hr (△)
OXYGEN TENSION CHANGE (mm Hg)

LENGTH (cm)

polystyrene and clear vinyl tubing with gold using a sputtering device (Balzers Union) such as is used for coating specimens for electron microscopy. The gold layer (1000 Å) was coated with plastic spray (Allen and Monbursys, Sweden) to facilitate the coating. The coating was, unexpectedly, still quite permeable to O₂ and in fact little better than uncoated tubing.

A second method by which the P0₂ of lymph has been measured involved the introduction of an electrode into the thoracic duct. Atkinson et al. (1978) developed a technique to measure the P0₂ of lymph in percutaneous plastic lymphangiography, and in the lymph trunk running collateral to the superior epigastric vein. The electrode was inserted into the lymphatic through a laparotomy in the latter point. Lymph was allowed to freely pass the electrode and escape from the hole in the lymphatic wall. In present experimental situations the popliteal efferent lymph ducts are small, fragile and, especially when a lymphangiographic needle was inserted, the duct would be impossible to introduce without damaging it, especially when the animal is anaesthetized and measurements are required at intervals or over periods of several days. To avoid the problem inherent in the use of plastic tubing and intraluminal electrodes, an attempt was made to fabricate an implantable oxygen electrode out of percutaneous plastic tubing and then implant it inside the duct. Such a device passed through a hole in the popliteal duct, to be mounted in a suitable positioning device, and when the animal was anaesthetized and prepared for implantation, the whole electrode chamber was designed to be implanted inside the animal as close to the duct as possible so that the duct could be connected directly to the chamber.
polyethylene and clear vinyl tubings with gold using a sputtering device (Balzers Unions) such as is used for coating specimens for electron microscopy. The gold layer (1,000 Å gold atoms) was coated with plastic spray (Allen and Hanburys, Sweden) to stabilize the coating. The tubing was, unexpectedly, still quite permeable to O₂, and in fact little better than uncoated tubing.

(b) INTRALYMPHATIC AND IMPLANTABLE ELECTRODES

A second method by which the PₐO₂ of lymph has been measured involved the introduction of an electrode into the lumen of a lymphatic duct. Witte et al. (1976) used a teflon-coated oxygen electrode to measure the PₐO₂ of lymph in periportal hepatic lymphatics and in the lymph trunk running parallel to the superior mesenteric vein. The electrode was inserted into the lymphatic through an incision in the duct and lymph was allowed to flow freely past the electrode and escape from the hole in the lymphatic.

In the present experimental situation the popliteal efferent lymphatic ducts are small in diameter and fragile; passing an electrode probe intraluminally into the duct would be impossible without blocking the duct and damaging it, especially when the animal is unanaesthetized and measurements are required at intervals over periods of several days. To avoid the problems inherent in the use of plastic tubing and intraluminal electrodes, an attempt was made to fabricate an implantable oxygen electrode out of perspex. The Pt anode and the Ag cathode were sited inside a small chamber of perspex through which lymph passed from the duct. The whole electrode chamber was designed to be implanted inside the animal, as close to the lymph node as possible so that the duct could be connected directly to an inlet of the chamber.
After preliminary trials, this electrode system was abandoned because clotting problems occurred within the chamber and these could not be diagnosed and removed from the implanted chamber. The clots also made it impossible to calibrate the electrode correctly for each successive measurement.

The solution to the problem was found in a thin, flexible cathode designed by Professor Hagihara, which was modified for insertion into a tapered plastic cannula inserted into the popliteal efferent lymphatic.
SECTION C

DIRECT ON-LINE MONITORING OF THE OXYGEN TENSION IN
POPLITEAL EFFERENT LYMPH

EXPERIMENTAL

The cathodes used for monitoring the \( P_{O_2} \) in popliteal efferent lymph directly were supplied by Professor Negmara who has designed and applied these polarographic electrodes for intravascular \( P_{CO_2} \) measurements (Negmara, 1960). These types of electrodes had not been used previously for the measurement of lymph \( P_{O_2} \). The original electrode system consisted of a Pt cathode (the Type A cathode used are described in Materials and Methods) and a Ag anode with 0.95% NaCl. The anode was converted to a Ag/AgCl electrode for use. In order to obtain a sufficient and constant conductivity of the salt bridge between the anode and the cathode during \( P_{O_2} \) measurements, 0.9% saline containing heparin (2 IU per ml) was passed slowly along an instillation system through the cathode catheter and into the blood. The catheter tip was placed in an artery or a vein with a needle at the elbow and a Mandrin tube. The cathode was usually set up in a position so that its tip was just exposed at the end of the catheter or protruded into the bloodstream through the catheter.

The electrode system used for lymph \( P_{O_2} \) measurements differed in that the direction of flow of fluid inside the tubing was reversed. Lymph flowed from the lymphatic into the catheter. First, passing the cathode tip, and along the tubing into the 3-way stopcock system connected to the anode. Finally, the lymph was collected in a measuring cylinder. Since the cathode tip was not constantly washed with Amperia-saline as when it was located in a blood vessel, the lymph tended to clot on the cathode if it was left in the lymph stream for longer than 20 min at a time. Fortunately, it usually took less than 5 min to obtain a steady and reproducible \( P_{O_2} \) reading once
(a) **EXPERIMENTAL**

The cathodes used for monitoring the $\text{PO}_2$ in popliteal efferent lymph directly were supplied by Professor Hagihara who has designed and applied these polarographic electrodes for intravascular $\text{PO}_2$ measurements (Hagihara, 1980). These types of electrode had not been used previously for the measurement of lymph $\text{PO}_2$. The original electrode system consisted of a Pt cathode (the Type A cathodes used are described in Materials and Methods) and a Ag anode with 0.85% NaCl. The anode was converted to a Ag/AgCl electrode for use. In order to obtain a sufficient and constant conductivity of the salt bridge between the anode and the cathode during blood $\text{PO}_2$ measurements normal saline containing heparin (2 I.U. per ml) was passed slowly along an instillation system, through the cathodic catheter and into the blood. The catheter (teflon or polyvinyl chloride tube) was inserted into either an artery or a vein with the aid of a syringe needle and a Mandrin tube. The cathode was usually set up in a position so that its tip was just exposed at the end of the catheter or protruded into the bloodstream through the catheter.

The electrode system used for lymph $\text{PO}_2$ measurements differed in that the direction of flow of fluid inside the tubing was reversed. Lymph flowed from the lymphatic into the catheter, first passing the cathode tip, and along the tubing into the 3-way stopcock system connected to the anode. Finally the lymph was collected in a measuring cylinder. Since the cathode tip was not constantly washed with heparin-saline as when it was located in a blood vessel, the lymph tended to clot on the cathode if it was left in the lymph stream for longer than 20 min at a time. Fortunately it usually took less than 5 min to obtain a steady and reproducible $\text{PO}_2$ reading once
the cathode was inserted into the cannula. Clots on the surface of the cathode could easily be removed after the cathode was withdrawn from the cannula. The tip of the cathode was always kept in saline when not in use. The details of the set-up and operation of the electrode system are mentioned in Chapter 2. The electrode characteristics mentioned below were tested before an electrode was used for the measurement of lymph P0₂ for the first time.

(b) CHARACTERISTICS OF THE ELECTRODE

The characteristics of the electrode were determined from the recording of the electrolysis current with air-saturated saline (or saturated with other gas mixtures) in a reservoir bottle at 39.5°c or at other specific temperatures. The saline was usually pumped through the electrode system at 10 ml/hr in the same direction of flow as the lymph. The cathode was inserted into a length of tubing of the same diameter as that used for the lymphatic cannulations. The end of this tubing was immersed in the fluid in the reservoir bottle.

(i) Stability

The sensitivity of the electrode changed considerably during the initial 30-120 min of use, and then stabilized. Subsequent drift in its sensitivity was always very small, at least within 6 hr periods.

(ii) Noise

When this was expressed as the ratio of the percentage of average noise width to the full electrode response, it was usually around 1% (Figure 3.2a).
FIGURE 3.2: Calibration of the electrode equilibrated with saline at different oxygen tensions.

Saline solution at 39.5°C and saturated with different gas mixtures was passed through the electrode system at 10 ml/hr. The respective electrolysis current was recorded as per cent full-scale-deflection (% F.S.D.).

(a) original trace recorded.

(b) plot of electrolysis current (% F.S.D.) $P_{O_2}$ versus $P_{O_2}$ of gas mixtures, $N_2$ (□), 7.72% $O_2$ (○) and air (△) in terms of % $O_2$. 
(iv) Linearity

Figure 3.4: Graphs demonstrating the linearity of the electrode to changes in \( P_{O_2} \). In this experiment the electrode was calibrated through the electrode system. The electrolysis current was plotted against the \( P_{O_2} \) of gas solutions found to be linear.

(v) Flow Dependence

The relationship between the flow rate of liquid through the cannula and the current generated is shown in Figure 3.3. The effect of different flow rates between 2.5-25 ml/hr [the usual range of flow rates for popliteal arterial flow] was examined. The electrode was calibrated, each time a measurement was made with different \( P_{O_2} \) solutions at the same rate of flow as that which was obtained from the popliteal artery during the lymph \( P_{O_2} \) measurements.
(iii) **Residual Current**

The residual current was measured in saline saturated with high purity N\textsubscript{2}. It was expressed as the percentage of the current measured under anaerobic conditions to the current measured in an air-equilibrated solution at the same temperature. It was \(< 2.5\%\) for all the electrodes used (Figure 3.2a).

(iv) **Linearity**

Figure 3.2 shows a typical result demonstrating the linear response of the electrode to changes in \(\text{P}_{\text{O}_2}\). In this experiment saline saturated with different gas mixtures (air, 7.72\% O\textsubscript{2} and N\textsubscript{2}) was passed alternately through the electrode system. The electrolysis current was plotted against the \(\text{P}_{\text{O}_2}\) of the solution (see Figure 3.2b) and was found always to be linear.

(v) **Response**

Since suitable equipment was not available, the actual response time of the electrode to changes in \(\text{P}_{\text{O}_2}\) in the solution was not measured. It was found during the process of calibrating the electrode, that the electrolysis current responded within 5-10 sec of a different gas mixture being bubbled through the medium in the reservoir.

(vi) **Flow Dependence**

The relationship between the flow rate of liquid through the cannula and the current generated is shown in Figure 3.3. The effect of different flow rates between 2.5-15 ml/hr (the usual range of flow rates for popliteal efferent lymph) was small. The electrode was calibrated, each time a measurement was made with different \(\text{P}_{\text{O}_2}\) solutions at the same rate of flow as that which obtained from the popliteal duct during the lymph \(\text{P}_{\text{O}_2}\) measurements.
FIGURE 3.3: Flow dependence of electrolysis current.

Air-saturated saline at 39.5°C was passed through the electrode system at 2.5, 5, 10 or 15 ml/hr. The respective electrolysis currents were recorded.

(a) original trace recorded.

(b) plot of electrolysis current versus flow rate of saline solution through the electrode system.
(11) Temperature Dependence

The effect of temperature on the electrolysis current is shown in Figure 3.4a. The current developed was affected by the temperature of the fluid in contact with the electrode (Figure 3.4a), when the two parameters were plotted (Figure 3.4b), a linear relation was obtained. Within the temperature range of 31-41°C, the oxygen electrode demonstrated a temperature coefficient of plus 2.65% per °C. In all subsequent $P_{O_2}$ measurements, the electrode was always calibrated at the body temperature (37.5°C) temperature of the experimental sheep. The stability of the $P_{O_2}$ measurements monitored by the electrode was tested directly by comparing the $P_{O_2}$ readings from arterial and venous blood recorded with the electrode with the $P_{O_2}$ measurements on blood samples taken simultaneously and analyzed in an independent laboratory (Peters Laboratory, Calver's Hospital). A simple technique was used to obtain a different arterial blood sample for this measurement. In order to obtain a sample, a needle was inserted into the femoral vein of a sheep such that it could be used to measure $P_{O_2}$ and at the same time the blood samples could be collected simultaneously after the arterial blood sample was obtained. It was found that the values obtained with the two methods always agreed closely.

(12) Loss of Electrode Stability

The characteristics of the electrodes described above were stable at least for the first 20-30 days; the electrodes were used for $P_{O_2}$ measurements. With use the stability of the electrodes deteriorated, the response time became slow, the current tended to drift significantly within short periods of time and linearity was no longer maintained in calibration.
(vii) Temperature Dependence

The effect of temperature on the electrolysis current is shown in Figure 3.4. The current developed was affected by the temperature of the fluid in contact with the electrode (Figure 3.4a). When the two parameters were plotted (Figure 3.4b), a linear relation was obtained. Within the temperature range of 31-41°C, the oxygen electrode demonstrated a temperature coefficient of plus 2.6% per °C. In all subsequent \( P_{O_2} \) measurements, the electrode was always calibrated at the body temperature (deep anal temperature) of the experimental sheep.

(viii) Reliability

The reliability of the \( P_{O_2} \) measurements monitored by the electrodes was tested indirectly by comparing the \( P_{O_2} \) readings of femoral venous blood recorded by the electrode with \( P_{O_2} \) measurements on blood samples taken simultaneously and analysed by an independent laboratory (Pathology Laboratory, Canberra Hospital) using a different method of measurement. In order to do this a cannula was inserted into the femoral vein of a sheep so that an electrode could be placed into the vein to measure \( P_{O_2} \) and at the same time blood samples could be collected anaerobically after the cathode was withdrawn. It was found that the values obtained with the two methods always agreed closely.

(ix) Loss of Electrode Stability

The characteristics of the electrodes described above were stable at least for the first 20-30 times the electrodes were used for \( P_{O_2} \) measurements. With use the stability of the electrodes deteriorated, the response time became slow, the current tended to drift significantly within short periods of time and linearity was no longer maintained in calibration.
FIGURE 3.4: Temperature dependence of the electrolysis current.

Air-saturated saline solution at 31, 33, 35, 37, 39 or 41°C was passed through the electrode system at 10 ml/hr. The respective electrolysis currents were recorded.

(a) original trace recorded.

(b) plot of electrolysis current versus temperature of saline solution.
Once this happened, the cathode was discarded and replaced with a new one.

3. OXYGEN TENSION OF POPILITAL EFFERENT LYMPH

Figure 3.5 shows an example of the measurement of the \( P_{O_2} \) of popliteal efferent lymph. It took a very short time (Figure 3.6a) for the current to stabilize after the cathode was inserted and the readings that resulted were very reproducible. The "pulsing" observed on the trace were not electrical noises but were caused by the pulsatile flow of the blood. The electrode current is somewhat dependent on how the current output appeared in the tube. Actually, the "pulsing" on the trace could be related to the number of drop back of the blood coming from the end of the skin. The values in terms of \( \% P_{O_2} \) could be obtained readily by comparing the cathode in vitro experiments with different \( P_{O_2} \) levels at the same temperature as the body temperature of the sheep. The rate of lymph flow at the time the readings were made (Figure 3.5b, c). Concentration of \( \% P_{O_2} \) into a pneumotachygraph using the methods described in Chapter 2.

The values in terms of \( P_{O_2} \) of popliteal efferent lymph antigens challenge were obtained from 3 conscious sheep after they recovered from operation and while they were kept in metabolism cages. They were standing and feeding while the \( P_{O_2} \) measurements were made. The results obtained were found to lie in the range of 41-54 mm Hg with a mean and standard deviation of 46.8 ± 3.3 mm Hg. This is equivalent to 6.2 ± 0.4 and 7.1 ± 0.65 \( \% P_{O_2} \).
Once this happened, the cathode was discarded and replaced with a new one.

(c) **OXYGEN TENSION OF POPLITEAL EFFERENT LYMPH**

Figure 3.5 shows an example of the measurement of the $P_{O_2}$ of popliteal efferent lymph. It took a very short time (Figure 3.5a) for the current to stabilize after the cathode was inserted and the readings that resulted were very reproducible. The "pulses" observed on the trace were not electrical noises but were caused by the pulsatile flow of the lymph. The electrode current was somewhat dependent on flow and the current output appeared in wave form. Actually the number of crests on the trace could be related to the number of drops of lymph coming from the end of the cannula.

$P_{O_2}$ values in terms of $\% O_2$ could be obtained readily by calibrating the cathode in vitro afterwards with different $P_{O_2}$ solutions at the same temperature as the body temperature of the sheep and at the rate of lymph flow at the time the $P_{O_2}$ was measured (Figure 3.5b, c). Conversion of $\% O_2$ into $P_{O_2}$(mm Hg) could be done using the formula mentioned in Chapter 2.

The values for the $P_{O_2}$ of popliteal efferent lymph before antigenic challenge were obtained from 3 conscious sheep after they recovered from operation and while they were kept in metabolism cages. They were standing and feeding while the $P_{O_2}$ measurements were made. The results obtained were found to lie in the range of 41-54 mm Hg with a mean and standard deviation of $46.6 \pm 5.3$ mm Hg; this is equivalent to $6.3 - 8.2$ and $7.1 \pm 0.8\% O_2$. 
FIGURE 3.5: Measurement of the oxygen tension in the popliteal efferent lymph of sheep.

(a) original trace recorded. Arrow indicates time of insertion of cathode into the cannula. (1) and (2) represent two successive $P_{O_2}$ determinations.

(b) in vitro calibration of the electrode after lymph $P_{O_2}$ determination with a saline solution at the body temperature of the animal. The saline was saturated with different gas mixtures and passed through the electrode system at the same rate as the lymph flow.

(c) plot of the electrolysis current versus $P_{O_2}$ of calibration gas mixtures, $N_2$ (□), 7.72% $O_2$ (○) and air (△). The $P_{O_2}$ of lymph (1) and (2) could be read off from the calibration curve.
The changes that occurred in the $P_{O_2}$ of popliteal efferent lymph after antigenic challenge will be described in Chapter 4.

(d) THE EFFECT OF ALTERING THE OXYGEN CONTENT OF THE INSPIRED AIR ON THE OXYGEN TENSION OF POPLITEAL EFFERENT LYMPH

Two sheep were given various gas mixtures to breathe (air or 100% $O_2$) while anaesthetized. This evoked a gradual but reversible change in the $P_{O_2}$ of the popliteal efferent lymph which occurred within 10 min of changing the respiration mixtures (Figure 3.6). The $P_{O_2}$ value of the lymph measured when the animal was under anaesthesia and breathing air was 42 mm Hg. This was within the range recorded in conscious animals. When 100% $O_2$ was given the $P_{O_2}$ rose to around 50 mm Hg, close to the upper limit of the normal range in conscious sheep.
FIGURE 3.6: Effect of varying the composition of the resired gas on the oxygen tension of the popliteal efferent lymph.

A: electrode equilibrated with air-saturated saline at 39.5°C.

B: P_{\text{O}_2} of lymph, animal breathing pure O_2.

C: P_{\text{O}_2} of lymph, animal breathing air.

Arrow indicates time when the gas mixture was changed.
(4) THE ELECTRODE SYSTEM USED TO MEASURE OXYGEN TENSION

The separated electrode system which was used in the present investigations aims at providing a thin, flexible, rapidly responding electrode for intravascular $P_{O_2}$ monitoring. Combined Clark-type oxygen electrodes, in which all the galvanic components, including the cathode, anode and carrier electrolyte are combined in a closed system beneath a thin hydrophobic membrane are in common use. These types of electrode have several advantages. Firstly, the active surface of the cathode is completely protected from any change in its characteristics due to the absorption of proteins, because the hydrophobic membrane is only permeable to the gases in solution. Secondly, the anode potential is stable, because the electrolyte solution reacting with the anode is kept constant. Thirdly, the conductivity of the salt bridge between the internal solution remains stable. Finally, the hydrophobic membrane resists the formation of blood clots. Despite these advantages, combined Clark-type electrodes have certain deficiencies for monitoring blood and lymph $P_{O_2}$. Due to their complicated structure, it is very difficult to make a sensor thin enough to introduce into small blood vessels and lymphatics of the size of the post-litha efferent lymph duct. Since the membrane needs frequent renewal and the carrier electrolyte must be recharged from time to time, it has proved inconvenient to make such electrodes very small. It is also almost impossible to make a stable combined electrode with a rapid response since this characteristic requires a very thin membrane. Membranes cannot be made too taut over an electrode-tip and because of this some variation occurs in the thickness of the fluid layer beneath the membrane.

SECTION D

DISCUSSION AND CONCLUSIONS
(a) **THE ELECTRODE SYSTEM USED TO MEASURE OXYGEN TENSION**

The separated electrode system which was used in the present investigations aims at providing a thin, flexible, rapidly responding electrode for intravascular $P_{O_2}$ monitoring. Combined Clark-type oxygen electrodes, in which all the polarographic components including the cathode, anode and carrier electrolyte are combined in a closed system beneath a thin hydrophobic membrane are in common use. These types of electrode have several advantages. Firstly, the active surface of the cathode is completely protected from any change in its characteristics due to the adsorption of proteins, because the hydrophobic membrane is only permeable to the gases in solution. Secondly, the anode potential is stable, because the electrolyte solution reacting with the anode is kept constant. Thirdly, the conductivity of the salt bridge between the anode and the cathode remains stable. Finally, the hydrophobic membrane resists the formation of blood clots. Despite these advantages, combined Clark-type electrodes have certain deficiencies for monitoring blood and lymph $P_{O_2}$. Due to their complicated structure, it is very difficult to make a sensor thin enough to introduce into small blood vessels and lymphatics of the size of the popliteal efferent lymph duct. Since the membrane needs frequent renewal and the carrier electrolyte must be recharged from time to time, it has proved inconvenient to make such electrodes very small. It is also almost impossible to make a stable combined electrode with a rapid response since this characteristic requires a very thin membrane. Membranes cannot be held too taut over an electrode tip and because of this some variation occurs in the thickness of the fluid layer beneath the membrane.
This affects the sensitivity of the electrode. Actually the membrane has to be sufficiently "slack" over the cathode to allow electrochemical continuity between the anode and the cathode on the inside of the hydrophobic membrane. This slackness creates electrical noise when the electrodes are moved or rotated. Although the protection of the cathode surface by the membrane is complete, there remains the possibility that the electrode may become less sensitive due to the adherence of lipids or particles onto the membrane itself.

For the separated electrode system, such as the one used in the present investigations, only the cathode is inserted into the vessel or into the catheter. It is much easier to make such an electrode very thin and flexible. No carrier electrolyte layer is required between the cathode surface and the membrane. While this design overcomes some of the inherent weaknesses in combined electrodes it has some important deficiencies. The coated cathodes cannot be protected completely from damaging substances since the coating has to be of a hydrophilic semipermeable material which allows access of the carrier electrolyte and O\textsubscript{2} to the cathode. With such coatings some interference to the function of the cathode by protein molecules is likely to occur. Since the anode and cathode are separated from each other the conductance between them through complex salt bridge structures and other components is not always adequate and stable.

According to Hagihara (Hagihara, 1980), these problems are overcome in the separated electrode system devised and
constructed in his laboratory. Cellulose diacetate (CDA) is used as the coating material and this is both mechanically strong and stable, and protective of the cathode against interference by blood constituents (Hagihara et al., 1978). However, this material is not perfect and the response time, sensitivity and linearity of current were found to deteriorate significantly after the electrode had been in use for some time. This seemed to be due to certain constituents of the lymph, which may or may not be present in blood.

The CDA coat of the cathode could be removed and the cathode recoated according to the following procedure suggested by Hagihara (personal communication). A solution is made by adding 1 ml of commercial heparin solution (1,000 I.U.) slowly with stirring to 9 ml of 7.5% CDA in 1:1 acetone ethanol mixture. The electrode tip is first coated with 7.5% CDA as described by Hagihara et al. (1978) and then twice with the above heparin-CDA solution. The electrode is dried in air for 30 min.

In the original electrode design, the tip of the cathode coated with CDA and exposed to blood is constantly washed with the electrolyte solution mentioned in Section C. This helps prevent clotting on the cathode tip. In the present investigations, since lymph flowed down the catheter from its tip towards the base, the instillation system used in monitoring blood $P_{O_2}$ was not feasible. Fortunately the lymph did not clot on the cathode surface provided the cathode was left in the lymph stream for no longer than 20 min. In blood $P_{O_2}$, $P_{O_2}$ measurements, the potential of the anode and the conductance of the salt bridge between the electrodes are maintained stable by the saline solution filling the instillation system of
the electrode. In the case of lymph \( P_{O_2} \) measurements, the saline was replaced by the lymph.

(b) THE OXYGEN TENSION OF LYMPH

Since Bergofsky and his co-workers (1962) measured the \( P_{O_2} \) of lymph in dogs, other workers have attempted to measure the \( P_{O_2} \) of lymph from various lymphatics of different animals and man. But as can be seen in Table 3.1, the values obtained by different workers for the \( P_{O_2} \) of the thoracic duct lymph of the dog, the lymph most commonly studied, are very variable.

As mentioned in Section B of this chapter, most of the measurements have been done on lymph samples collected from polyethylene cannulae. Since these tubings are fairly permeable to \( O_2 \), the validity of these \( P_{O_2} \) values and others obtained by so called "anaerobic sampling" methods is doubtful. The oxygen-impermeable, external catheter system used to measure steady state \( P_{O_2} \) in unanaesthetized sheep reported by Staub and Schultz (1974) seems to be more valid, but this method also neglected several factors which would cause the \( P_{O_2} \) measurements to be physiologically inaccurate. Firstly, the permeability of the length of tubing inside the animal was neglected. Secondly, while the lymphatic cannula outside the animal's body was made impermeable to \( O_2 \) by means of an external sheath, the \( P_{O_2} \) of lymph flowing along this length of tubing would be lowered by the respiratory activity of the cells in it, especially when the flow of lymph was slow. This effect is also complicated by variations in the number of cells present in the lymph. No previous attempts have been made to measure the \( P_{O_2} \) of lymph from peripheral lymphatics such as the popliteal efferent duct. As a consequence there are no results in the literature to which the \( P_{O_2} \) values reported in Section C
<table>
<thead>
<tr>
<th>Reference</th>
<th>Oxygen tension (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>31.1</td>
</tr>
<tr>
<td>3</td>
<td>34 ± 20</td>
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<tr>
<td>4</td>
<td>44 ± 8</td>
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<td>5</td>
<td>45</td>
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<tr>
<td>6</td>
<td>45 - 50</td>
</tr>
<tr>
<td>7</td>
<td>47</td>
</tr>
</tbody>
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**TABLE 3.1:** Results reported in the literature for the oxygen tension of thoracic duct in anaesthetized dogs breathing air.

Reference: 1. Bergofsky et al., 1962
2. Nagy et al., 1969
3. Said et al., 1965
4. Cockett, 1967
5. Bergofsky et al., 1964
6. Witte et al., 1968
7. Witte et al., 1967
of this chapter can be compared. The $P_{O_2}$ values of popliteal lymph were found to be quite close to the $P_{O_2}$ of femoral venous blood. Because of the precautions taken the $P_{O_2}$ values of popliteal efferent lymph reported here are believed to be a close estimate of the physiological $P_{O_2}$ of lymph as it leaves the popliteal node and close to the $P_{O_2}$ that exists within the popliteal lymph node.

Nearly all $P_{O_2}$ measurements made by previous workers have been done on subjects under anaesthesia. The effect of anaesthetic agents and the depth of anaesthesia on the $P_{O_2}$ of lymph has not been studied comprehensively. In part (d) of Section C of this chapter, it was noted that $P_{O_2}$ values for the popliteal efferent lymph of sheep obtained while the animal was under anaesthesia agreed well with those measured when the animal recovered consciousness. It was also shown that changing the $O_2$ content of the inspired gas from 100% $O_2$ to air produced changes in the $P_{O_2}$ of popliteal efferent lymph. This observation is consistent with the results of Witte et al. (1967) and Witte et al. (1976). It was considered that the $P_{O_2}$ of the regional lymph provided a good estimate of the $O_2$ saturation of the adjacent tissue fluid.

There are few values in the literature of the $P_{O_2}$ of lymph from different lymphatics of different animal species. More work on this topic would provide information on the differences in the gaseous tensions that exist in the interstitial fluid of the different tissues. Other characteristics of lymph such as pH and $P_{CO_2}$ (Carlsten and Söderholm, 1960; Altman and Dittmer, 1971) also need to be studied.
THE USE OF LYMPH OXYGEN TENSION AS AN ESTIMATE OF INTERSTITIAL FLUID AND TISSUE OXYGEN TENSION

Most of the lymph \( P_{O2} \) measurements done by previous workers have aimed at estimating the gaseous tensions in the interstitial fluid and tissues in which the lymph originates e.g. thoracic duct lymph \( P_{O2} \) as an index of gaseous exchange in splanchnic tissues (Bergofsky et al., 1962; Witte et al., 1967 and Witte et al., 1968), pulmonary lymph \( P_{O2} \) as an index of gaseous tensions in lung tissues (Said et al., 1965; Staub and Schultz, 1974). Gaseous tensions in the interstitial fluid and tissues are of interest for at least 3 reasons (Bergofsky et al., 1962).

1. As an index of the relationship between the oxidative metabolism of the tissue and its blood supply,
2. As a measure of the gaseous tensions under which the metabolic processes of the different tissues operate. In the present investigations this relates to the popliteal lymph node and the free-floating cells in the lymph,
3. For an understanding of the interplay between external respiration, blood, oxygen levels, and tissue respiration.

The use of lymph \( P_{O2} \) as an index of tissue \( P_{O2} \) avoids "unreliable \( P_{O2} \) measurements due to artefacts incident to the introduction of electrodes into tissues, the lack of dependable instruments for the precise measurement of the small quantities of oxygen in biological fluids, and the inability to calibrate electrodes implanted in tissues" (Bergofsky et al., 1962).

This method of estimating gaseous tensions in interstitial fluid and tissues is justified on the presupposition that interstitial fluid is in equilibrium with the tissues it bathes and that interstitial fluid and lymph are in effect one
continuous phase. The presupposition is also held that the
gaseous composition of lymph does not undergo significant
change as it passes through the lymphatic chain. The first
part of the presupposition is generally correct, but the
second is patently not so. For this reason the thoracic duct
\( P_{O_2} \) is probably an index of the \( P_{O_2} \) in the tissues (peritoneal
or pleural) adjacent to the cannulation site. The observation
that lymph \( P_{O_2} \) is affected by the composition of the inspired
air, supports the idea that regional lymph \( P_{O_2} \) measurements
are a dynamic indicator of tissue perfusion and tissue
oxygenation. The respiratory activities of the cells contained
in the lymph inside the lymph duct will also change the lymph
\( P_{O_2} \) and this effect will be greatest at slow rates of flow and
high cell concentrations. According to Bergofsky et al. (1962)
and Staub and Schultz (1974), the respiratory activities of
lymphocytes do not account for any sizeable change in the \( P_{O_2} \).

Although the transit time from a peripheral site in the leg to
the jugular venous confluence may be several minutes there is
no direct experimental evidence on the extent of exchange of
\( O_2 \) across the lymphatic wall but it can be assumed that this
would be quite rapid. It would be possible to study this
experimentally by cannulating the lymphatic as close to the
lymph node under study as possible, and at a second site further
removed from the node. In the present investigations the object
was to have a measurement of lymph \( P_{O_2} \) taken as close to the
lymph node as possible. In most experiments this was
obtained within a distance of 1-2 cm from the hilum of the node.
CHAPTER 4

SECTION A

THE RESPIRATORY ACTIVITIES OF LYMPHOCYTES IN POPLITEAL EFFERENT LYMPH DURING AN IMMUNE RESPONSE
Most studies on the respiratory activity of lymphocytes isolated from different sources have been concerned with establishing the rate of \( O_2 \) consumption of these cells in normal and in vitro stimulated states (Kennedy and Esham, 1966; Yackich, 1967; Palgat et al., 1968).

Roos and Laco, 1973; Calvecchi and Weidenbach, 1974). These studies were done either by Warburg manometry or by means of polarographic oxygen electrodes. The rate of \( O_2 \) production by lymphocytes has rarely been studied.

MANOMETRIC METHODS involving the use of the Warburg constant-volume respirometer depend on the principle that at constant temperature, any changes in the pressure of a gas present in the system can be measured by changes in its pressure. While this apparatus has been used to study both chemical and biological reactions for many years, it has most commonly been applied to the measurement of \( O_2 \) uptake.

Oxygen electrode methods for measuring respiration depend on the electrochemical reduction of dissolved \( O_2 \) at a negatively charged electrode. The electrode produces a current the magnitude of which is directly proportional to the \( O_2 \) concentration in the reaction medium. The Clark-type membrane-covered electrode, due to its excellent performance and robustness, has been widely used for \( O_2 \) measurement. Lester and Briscoe (1969) and Beechey and Rippin (1972) have published reviews of oxygen electrode measurements in biological research.

While manometric methods are simple and reliable, the relatively long time required for both equilibration and measurement of the pressure changes makes it difficult to follow rapid alterations in the gas phase. The volumetric
Most studies on the respiratory activities of lymphocytes isolated from different sources have been concerned with establishing the rate of $O_2$ consumption of these cells in normal and in *in vitro* stimulated states (Hedekov and Esmann, 1966; Pachman, 1967; Polgar et al., 1968; Roos and Loos, 1973; Culvenor and Weidemann, 1976). These studies were done either by Warburg manometry or by means of polarographic oxygen electrodes. The rate of CO$_2$ production by lymphocytes has rarely been studied.

Manometric methods involving the use of the Warburg constant volume respirometer depend on the principle that at constant temperature and constant gas volume, any changes in the amount of a gas present in the system can be measured by changes in its pressure. While this apparatus has been used to study both chemical and biological reactions for many years, it has most commonly been applied to the measurement of $O_2$ uptake.

Oxygen electrode methods for measuring respiration depend on the electrolysis of dissolved $O_2$ at a weakly negative cathode. The electrode produces a current the magnitude of which is directly proportional to the $O_2$ concentration in the reaction medium. The Clark-type membrane-covered electrode, due to its excellent performance and robustness, has been widely used for $O_2$ measurement. Lessler and Brierley (1969) and Beechey and Ribbons (1972) have published reviews on oxygen electrode measurements in biological research.

While manometric methods are simple and reliable, the relatively long time required for both equilibration and measurement of the pressure changes makes it difficult to follow rapid alterations in the gas phase. The volumetric
data obtained indicate only the average exchange of gas in a sample over the period measured. Because of this the kinetic aspects of the respiratory activity of cells cannot be analyzed by this method. The addition of several substrates and/or inhibitors is usually not practicable within a single experiment. Finally, the calculation of results and the calibration of the apparatus require a considerable amount of time. These limitations do not obtain with oxygen electrodes. The amount of biological material required is about the same for both methods.

In the present investigations, the uptake of $O_2$ by lymphocytes before and after antigenic challenge was measured by both manometric and polarographic methods, so that comparisons could be made between the two techniques. CO$_2$ production was also measured by the "Indirect Method" of Warburg, so as to enable values to be calculated for the Respiratory Quotient of lymphocytes. This provided a better insight into the energy metabolism of the cells. The Clark Oxygen electrode was also used to study several aspects of the respiratory activity of lymphocytes including the effect of $P_{O_2}$ on the rate of respiration and the affinity of these cells for $O_2$. 

SECTION B

THE IMMUNE RESPONSE TO SALMONELLA MUECHEN ORGANISMS
(a) **INTRODUCTION**

The changes which occur in the cell population of the lymph following antigenic stimulation can be studied by establishing a chronic fistula in the efferent duct of a lymph node of a sheep. The antigen under study is injected subcutaneously into the region drained by the node or directly into an afferent lymphatic. The fate of the antigen after injection depends on its physical nature, on the amount injected and on previous antigenic experiences. A particulate antigen, such as *Salmonella muenchen* organisms, will be quantitatively retained by the node (Drinker, Field and Ward, 1934; Hall and Morris, 1963; Fahy, Gerber, Morris, Trevella, Zukoski, 1980), whereas nearly all of a soluble antigen (e.g. serum albumin) will pass through the node and appear in the efferent lymph (Hall and Morris, 1963; Trevella and Morris, 1980). Following a primary antigenic challenge a characteristic cellular response occurs in the efferent lymph. When a second dose of antigen is injected a secondary response occurs which is of shorter duration but of a more vigorous intensity than the primary response. The difference between a primary and a secondary response is more obvious in the case of particulate antigens (Hall and Morris, 1963).

The sequence of events which occurs in the efferent lymph from a lymph node following antigenic stimulation is essentially similar regardless of dose, the type of antigen or whether the response is primary or secondary. This sequence has been systematically studied and documented for different antigens (Hall and Morris, 1963; Cahill, Hay, Frost and Trnka, 1974). The salient features of the immune response relate to changes in the cell population in the lymph. Initially the cell output from the node is drastically reduced. The output increases over the
ensuing 3-4 days and around 72 hr in a primary response and somewhat earlier in a secondary response, blast cells appear in the lymph. These cells are responsible for much of the antibody formed during the immune response (Cunningham, Smith and Mercer, 1966; Hay, 1970; English, 1974). In addition to the cellular changes in the lymph, a variety of biologically active factors such as macrophage inhibition factor (MIF) and mitogenic factor (MF) also appear in the lymph (Hay, Lachmann and Trnka, 1973a, b; Cahill et al., 1974).

Apart from their antibody-forming activities, the free-floating cells in lymph from a stimulated node play a part in propagating the immune response throughout the body and in establishing an immunological memory. Free-floating lymphoid cells are able to initiate immune reactions in other areas of lymphoid tissue in the absence of antigen by acting as immunologically conditioned messenger cells. They may express this function by colonizing other lymph nodes and lymphoid organs where they proliferate and differentiate to give rise to many more antibody-forming cells or by transferring information to other effector cells (Hall, Morris, Moreno and Bessis, 1967).

After an initial antigenic challenge a widespread secondary reactivity exists throughout the body in lymph nodes that have had no direct experience of the antigen previously. It is suggested that such secondary reactivity is due to free-floating circulating cells which possess a memory fashioned by previous experience in the lymph node in which the antigen was initially localized (Smith, Cunningham, Lafferty and Morris, 1970). These circulating cells are recruited to a lymph node on subsequent exposures to the antigen.
(b) EXPERIMENTAL

The main purpose of the experimental investigations reported in this thesis was to examine various metabolic parameters of lymphocytes before and after antigenic challenge. The principal antigen used for the experiments was freeze-dried *Salmonella* organisms. In consequence the characteristics of the response of the popliteal lymph node of the sheep were first examined in some detail to establish the nature of the cellular changes occurring in the efferent lymph from the stimulated nodes.

Primary and secondary immune responses to *Salmonella muenchen* were investigated. The primary response was induced by the subcutaneous injection of 2 mg of freeze-dried organisms in 0.2 ml of saline into the lateral side of the lower hind leg of the sheep. The popliteal efferent lymph was collected over 12-hourly periods and the total and large cells counts estimated with Coulter counter. Differential cell counts were done on smears stained with Leishman's stain and the haemagglutinating antibody titres in the lymph plasma measured.

The secondary response was induced by priming the sheep with 2 mg of freeze-dried *Salmonella muenchen* organisms and injecting a second dose of the same antigen 6 weeks later. The popliteal efferent lymphatic was cannulated a few days before the second injection of antigen.

(c) THE CELLULAR RESPONSE IN THE POPLITEAL LYMPH TO *SALMONELLA MUECHEN* ORGANISMS

Following injection of the antigen there was always an initial drop in the cell output in the efferent lymph irrespective of whether the response was a primary or a secondary one (Figures
4.1, 4.2). The cell output "shutdown" in the first 12 hr after antigen to about 30% of the pre-injection level. This was followed by an increase in the cell output over the following 3-4 days.

In a primary response to *Salmonella* organisms the total cell output reached a maximum on day 4 after the antigen was injected (Figure 4.1) and then gradually decreased over the next 3 or 4 days to near the pre-injection level. The maximum total cell output was around $150 \times 10^6$ cells per hr. The number of large cells (Figure 4.3c) in the efferent lymph increased significantly on the third day reaching a maximum on day 4 of the response. At this time they accounted for about 18.0% of the total cells. By day 6 or 7, the number of large cells in the lymph had returned to pre-challenge levels.

Secondary responses differed from primary responses in that they were usually shorter in duration and more vigorous in terms of the total cell output and the percentage of large cells. The difference between the two types of responses with this antigen however was not very dramatic. The maximum total cell output and blast cell output occurred on day 3 after challenge (Figure 4.2). The maximum total cell output was about $180 \times 10^6$ cells per hr. At the height of the response the blast cells amounted to about 22% of the total.

In some cases, particularly in primary responses, two peaks of cell output were observed (Figures 4.1, 4.2). The second peak always coincided with the maximum output of large cells.

Virtually all the cells in the lymph were small and medium lymphocytes before challenge (Figure 4.3a). Occasionally polymorphonuclear leucocytes-neutrophils and eosinophils were also present, but these never amounted to more than 1% of the
FIGURE 4.1: The primary response to Salmonella muenchen organisms - the cellular response.

The upper part of the figure shows the total cell output per hr and the blast cell output per hr (shaded area) over the response. The lower part shows the percentage of blast cells in the total white cell population.
Cell output per hour ($\times 10^6$)

- Total cells
- Blasts

% Blasts

Hours after challenge
FIGURE 4.2: The secondary response to Salmonella muenchen organisms - the cellular response.

The upper part of the figure shows the total cell output per hr and the blast cell output per hr (shaded area) over the response. The lower part shows the percentage of blast cells in the total white cell population.
FIGURE 4.3: Cells in popliteal efferent lymph collected at various times during the immune response to Salmonella muenchen organisms (Leishman's stain).

(a) cells collected before antigenic challenge.
(b) cells collected on day 1 after challenge.
(c) cells collected at the height of the blast cell response.
total. Immediately after antigen injection there was always a transient appearance of large numbers of neutrophil polymorphs in the lymph (Figure 4.3b). These cells could account for a very high percentage of the total cell population in some experiments. In the experiment depicted in Figure 4.1, 29.2% of the total cells in lymph collected from 0 hr to 12 hr and 15.6% from 12 hr to 24 hr after injection of antigen, were polymorphs. Such a burst of neutrophils usually lasted for one day after challenge; by day 2, they had usually disappeared and remained absent from the lymph throughout the response. Eosinophils were present throughout the response but they never exceeded 0.5% of the total cells in any circumstances.

(d) THE ANTIBODY TITRE IN LYMPH DURING THE IMMUNE RESPONSE TO SALMONELLA MUENCHEN ORGANISMS

Haemagglutination titres in the popliteal efferent lymph in the primary response to *Salmonella muenchchen* gave a peak of IgM antibody activity on days 5 and 6 after injection of the antigen. IgG with haemagglutinating activity was present in much lower amounts than IgM haemagglutinating antibody but it too, reached a peak on around days 5 and 6 (Table 4.1).

The haemagglutination titres of lymph collected during the secondary response were in general similar to those in the primary response with most of the activity again being ascribed to IgM (Table 4.2). There was some IgM and IgG activity in the lymph prior to the second injection of the antigen. The IgG antibody activity reached a peak around days 5 and 6 of the response at the same time as IgM.
## TABLE 4.1: The primary antibody response to Salmonella muenchen organisms in the popliteal efferent lymph collected at various times following antigenic challenge.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM + IgG</td>
<td>1:24</td>
<td>1:60</td>
<td>1:120</td>
<td>1:1016</td>
<td>1:32760</td>
<td>1:16380</td>
<td>1:8188</td>
<td>1:1020</td>
</tr>
<tr>
<td>IgG (2-mercapto-ethanol resistant)</td>
<td>1:8</td>
<td>1:32</td>
<td>1:28</td>
<td>1:64</td>
<td>1:128</td>
<td>1:512</td>
<td>1:128</td>
<td>1:128</td>
</tr>
<tr>
<td>Day</td>
<td>IgM + IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>1:124</td>
<td>1:124</td>
<td>1:1016</td>
<td>1:2046</td>
<td>1:4094</td>
<td>1:2048</td>
<td>1:512</td>
<td>1:512</td>
</tr>
<tr>
<td>IgG (2-mercapto-ethanol resistant)</td>
<td>1:64</td>
<td>1:128</td>
<td>1:128</td>
<td>1:256</td>
<td>1:1024</td>
<td>1:1024</td>
<td>1:256</td>
<td>1:256</td>
</tr>
</tbody>
</table>

**TABLE 4.2:** The secondary antibody response to Salmonella muenchen organisms in the popliteal efferent lymph collected at various times following antigenic challenge.
The $pO_2$ of popliteal efferent lymph was measured in three sheep before and after the popliteal node was challenged with killed *Salmonella* minnesota organisms. The method of measurement is discussed in detail in Chapters 2 and 3. $pO_2$ measurements usually started 2 days after operation and continued for 2-3 days before the animal was challenged. After antigenic challenge, $pO_2$ measurements were made at regular time intervals as shown in the figures in the text.

SECTION C

THE OXYGEN TENSION OF POPLITEAL EFFERENT LYMPH DURING AN IMMUNE RESPONSE

Figure 4.4 shows the changes in $pO_2$ of popliteal efferent lymph during a primary immune response to killed *Salmonella* organisms. The figure also shows the cellular response that occurred in the lymph and the rate of flow of lymph at the time the $pO_2$ measurements were made. The $pO_2$ of the lymph during the response ranged from a highest value of 98.6 mm Hg at 8 hr after injection of the antigen to a lowest value of 31.8 mm Hg at the peak of the response 26 hr after challenge. The overall average $pO_2$ value throughout the response was 46.4 ± 8.4 mm Hg.

Figure 4.6 shows the changes in $pO_2$ that occurred in the lymph during a secondary response to *Salmonella*. The $pO_2$ values changed significantly over the response, ranging from a highest value of 160.2 mm Hg at 9 hr after challenge to a lowest value of 33.6 mm Hg at the peak of the cellular response 76 hr after challenge. The overall average $pO_2$ was 62.3 ± 7.4 mm Hg.
(a) **EXPERIMENTAL**

The $P_{O_2}$ of popliteal efferent lymph was measured in three sheep before and after the popliteal node was challenged with killed **Salmonella muenchen** organisms. The method of measurement is discussed in detail in Chapters 2 and 3. $P_{O_2}$ measurements usually started 2 days after operation and continued for 2-3 days before the animal was challenged. After antigenic challenge, $P_{O_2}$ measurements were made at regular time intervals as shown in the figures in the text.

(b) **THE OXYGEN TENSION OF POPLITEAL EFFERENT LYMPH DURING AN IMMUNE RESPONSE**

Figure 4.4 shows the change in $P_{O_2}$ of popliteal efferent lymph during a primary immune response to killed **Salmonella** organisms. The figure also shows the cellular response that occurred in the lymph and the rate of flow of lymph at the time the $P_{O_2}$ measurements were made. The values for the $P_{O_2}$ of the lymph during the response ranged from a highest value of 58.5 mm Hg at 8 hr after injection of the antigen to a lowest value of 31.6 mm Hg at the peak of the response 80 hr after challenge. The overall average $P_{O_2}$ value throughout the response was $45.1 \pm 6.4$ mm Hg.

Figure 4.6 shows the changes in $P_{O_2}$ that occurred in the lymph during a secondary response to **Salmonella**. The $P_{O_2}$ values changed significantly over the response, ranging from a highest value of 56.7 mm Hg at 9 hr after challenge to a lowest value of 33.0 mm Hg at the peak of the cellular response 75 hr after challenge. The overall average $P_{O_2}$ was $42.0 \pm 7.4$ mm Hg.
(c) **THE CORRELATION BETWEEN THE OXYGEN TENSION OF LYMPH AND THE CONCENTRATION OF CELLS IN LYMPH**

The results reported above were subjected to regression analysis to see if there was any correlation between the different parameters monitored. It was found that no statistically significant correlation existed between the $P_{O_2}$ values and the rate of flow of lymph in any of the experiments. However, a statistically significant negative linear correlation existed between the $P_{O_2}$ and the concentration of cells in both the primary and secondary responses (Figures 4.5, 4.7). The slope of the regression relating $P_{O_2}$ to cell concentration was steeper for the secondary response than for the primary response.
FIGURE 4.4: The primary response to Salmonella muenchen organisms - $P_{O_2}$ of popliteal efferent lymph determined at various times during the response.

The upper part of the figure shows the change in cell concentration (□) and per cent blast cells (◇) in the lymph collected during the response. The lower part shows the change in oxygen tension (○) and the rate of flow of lymph (△). The arrow indicates the time of antigen administration. For oxygen tension, the standard deviations for triplicate measurements are shown as vertical bars on the time points. For those points without the bars, the standard deviations are smaller than the vertical dimension of the symbols.
FIGURE 4.5: The regression of the $P_{O_2}$ of popliteal efferent lymph on the concentration of cells in the lymph during a primary response to Salmonella muenchen organisms.

The linear regression relating $P_{O_2}$ of the lymph to cell concentration is given by the equation $P_{O_2} = 51.302 - 0.306$ (cell concentration). The intercept on the $y$-axis is shown as an open circle on the diagram. The $t$ ratio (slope/standard error) is 2.660 ($p<0.02$) and the coefficient of correlation ($r$) is -0.611 ($p<0.02$).
FIGURE 4.6: The secondary response to Salmonella muenchen organisms - $\frac{P_{O_2}}{PO_2}$ of popliteal efferent lymph determined at various times during the response.

The upper part of the figure shows the change in cell concentration (□) and per cent blast cells (◊) in the lymph collected during the response. The lower part shows the change in oxygen tension (○) and rate of flow of lymph (△). The arrow indicates the time of antigen administration. For oxygen tension, the standard deviations for triplicate measurements are shown on the graph as vertical bars on the time points. For those points without the bars, the standard deviations are smaller than the dimension of the symbols.
CELL CONCENTRATION (CELLS x 10^9/mL)

OXYGEN TENSION (mm Hg)

FLOW RATE (ML/HR)

% BLASTS

HOURS AFTER CHALLENGE
FIGURE 4.7: The regression of the $\text{PO}_2$ of popliteal efferent lymph on the concentration of cells in the lymph during a secondary response to *Salmonella muenchen* organisms.

The linear regression relating $\text{PO}_2$ of the lymph to cell concentration is given by the equation $\text{PO}_2 = 52.578 - 0.639$ (cell concentration). The intercept on the $y$-axis is shown as an open circle on the diagram. The $t$ ratio (slope/standard error) is 2.947 ($p<0.02$) and the coefficient of correlation ($r$) is -0.720 ($p<0.02$).
OXYGEN TENSION (mm Hg)

CELL CONCENTRATION (CELLS×10^6/ML)
SECTION D

THE EFFECT OF CONTAMINATING ERYTHROCYTES AND POLYMORPHONUCLEAR LEUCOCYTES ON THE MEASUREMENT OF THE ENERGY METABOLISM OF LYMPHOCYTES

...
(a) INTRODUCTION

The cell population of normal popliteal efferent lymph is entirely lymphocytes. Other cell types are present in the lymph to a variable extent at different times after antigenic challenge. Before biochemical studies were done on the energy metabolism of lymphocytes, allowance had to be made for the effects of contaminating erythrocytes and polymorphonuclear leucocytes on the results obtained. Erythrocytes (RBC) while having no protein or nucleic acid synthetic activities do consume glucose (London, 1960-61; Ling and Kay, 1975). Polymorphonuclear (PMN) leucocytes have a very active carbohydrate metabolism. Barron and Harrop (1929) observed that PMN leucocytes had a glycolytic activity at least 5 times as great as that of lymphocytes although no significant differences were observed in O$_2$ consumption between the two types of cells. Rauch, Loomis, Johnson and Favour (1961) found that lymphocytes had a rate of O$_2$ consumption twice that of PMN leucocytes but had a lower glycolytic rate. Frei, Borel, Horvath, Cullity and Vannotti (1961) observed that the rates of glycolysis and respiration in PMN leucocytes were higher than in lymphocytes. Hedeskov and Esmann (1966) estimated that human PMN leucocytes at a concentration of 40 x 10$^6$ cells/ml had rates of glucose uptake and lactate production that were 7 times higher than those of lymphocytes. Hedeskov and Esmann (1966) and Rabinowitz (1964) reported that PMN leucocytes and lymphocytes had a more or less equal respiratory activity as measured by "Direct Manometry". In view of these variable findings (summarized in Table 4.3), attempts were made to remove RBC and PMN leucocytes from lymphocyte suspensions whenever possible to establish their metabolic contribution in mixed populations.
<table>
<thead>
<tr>
<th>Activity</th>
<th>Reference (species)</th>
<th>PMN leucocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (human)</td>
<td>66.4</td>
<td>21.7</td>
</tr>
<tr>
<td></td>
<td>2 (human)</td>
<td>117</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>3 (human)</td>
<td>160.7</td>
<td>183.0</td>
</tr>
<tr>
<td></td>
<td>4 (human)</td>
<td>96</td>
<td>155</td>
</tr>
<tr>
<td>Glucose uptake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (human)</td>
<td>437</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>4 (human)</td>
<td>208</td>
<td>71</td>
</tr>
<tr>
<td>Lactate production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (human)</td>
<td>1050</td>
<td>672</td>
</tr>
<tr>
<td></td>
<td>2 (human)</td>
<td>640</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>4 (human)</td>
<td>403</td>
<td>175</td>
</tr>
</tbody>
</table>

**TABLE 4.3:** Metabolic activities of PMN leucocytes compared to lymphocytes.

Activities in µmole/hr/10^10 cells calculated from reported data.

Reference: 1. Frei et al., 1961
2. Hedekov and Esmann, 1966
3. Rabinowitz, 1964
4. Rauch et al., 1961
The number of RBC present in popliteal efferent lymph was rather variable ranging from practically zero to up to 25% of the total cells. The mean was around 10% and the percentage usually rose immediately after operation and after injection of antigen. There were two ways by which the problem of RBC contamination could be tackled; either to remove them from the cell suspension by lysing them, or to evaluate their metabolic contribution by doing control experiments with pure suspension of RBC. Preliminary experiments were done with RBC suspensions and it was found that at the concentrations of these cells in lymph they had no detectable glucose consumption as monitored by enzymatic assays and radiorespirometry. This observation was contradictory to some of the results mentioned above but agreed with the results of Hedeskov and Esmann (1966). It was subsequently decided to remove the red cells from the lymphocyte preparations by treating them with ACT solution (Tris-buffered isotonic ammonium chloride solution).

During the first day following antigenic challenge there was always a steep rise in the energy metabolism of the cell population in lymph as measured by Warburg manometry, enzymatic determination of glucose uptake and lactate production, and $^{14}$CO$_2$ release from labelled glucose. These parameters returned to near prechallenge levels during the second day. During this period the lymphocytes appearing in lymph showed no obvious signs of transformation or of proliferation. There were however many PMN leucocytes in the lymph during this period.

The percentage of PMN leucocytes in the lymph varied from injection to injection; in some experiments it was as high as 30% of the total cell output. It was not always feasible to remove PMN leucocytes from the lymph because during the first
day after challenge the cell output was usually low. Removal of PMN leucocytes at this time often meant that there were not enough cells left to do biochemical studies. The experiments on day 1 were done as on other days of a response, keeping in mind that the assays carried out on cells collected at this time related to a population of lymphocytes heavily contaminated by PMN leucocytes, mainly neutrophils.

The effect of PMN cells on the results was evaluated as described below.

(b) THE REMOVAL OF ERYTHROCYTES FROM LYMPHOCYTE PREPARATIONS

To establish that the ACT treatment of RBC was not detrimental to the viability of lymphocytes, the release of $^{14}$CO$_2$ as measured by radiorespirometry was compared between cell suspensions before and following treatment with ACT solution. The results are shown in Table 4.4 and in Figure 4.8. In these experiments the effect of another lysing solution, the sodium citrate lysing solution suggested by Pachman (1967) was also included for comparison. It was found that neither the ACT nor the sodium citrate solutions were detrimental to the viability of lymphocytes (Table 4.4). The ACT solution was found to have less effect than the citrate solution on the release of $^{14}$CO$_2$ from labelled glucose (Figure 4.8) and it was concluded that the metabolic vitality of lymphocytes was unharmed by the treatment. ACT solution was also more effective as a lysing agent for RBC than the citrate solution, as shown in Table 4.5.

(c) THE RESPIRATION OF POLYMORPHONUCLEAR LEUCOCYTE CONTAMINATED LYMPHOCYTE POPULATIONS

In order to verify the contribution to the total energy metabolism of PMN leucocytes present in the cell population
Sample number & No treatment & Viability, % & ACT & Sodium citrate
--- & --- & --- & --- & ---
1 & 89.8 & 89.7 & - & -
2 & 95.1 & 94.3 & - & -
3 & 97.8 & 91.9 & - & -
4 & 93.9 & 83.6 & 87.9 & 84.6
5 & 87.7 & 87.1 & 84.6 & 84.6

TABLE 4.4: Viability of lymphocytes after lysing treatment with lysing solution.

No treatment: the cells were washed with Krebs & de Gasquet buffer three times.

ACT: the cells were washed with ACT solution once, followed by another two washes with Krebs & de Gasquet buffer.

Sodium citrate: the cells were washed with sodium citrate lysing solution once, followed by another two washes with Krebs & de Gasquet buffer.

The viability of the cells after treatment was determined by the trypan blue exclusion test.
FIGURE 4.8: The release of $^{14}$CO$_2$ from D-[U-$^{14}$C] glucose by different lymphocyte preparations.

Cells collected from popliteal efferent lymph were washed three times with Krebs & de Gasquet buffer ($\triangle$), or washed with ACT solution once, followed by another two washes with Krebs & de Gasquet buffer ($\bigcirc$), or washed with sodium citrate lysing solution once, followed by another two washes with Krebs & de Gasquet buffer ($\square$). These cells were then incubated with D-[U-$^{14}$C]glucose in the radiorespirometer and the release of $^{14}$CO$_2$ from the substrate was followed.
**TABLE 4.5: The effectiveness of RBC lysing solutions.**

Cells collected from lymph were counted to determine the percentage of RBC. They were then either washed three times with Krebs & de Gasquet buffer, or washed with ACT solution once, followed by another two washes with Krebs & de Gasquet buffer, or washed with sodium citrate lysing solution once, followed by another two washes with Krebs & de Gasquet buffer. The cell preparations were then re-counted to determine the number of RBC remaining.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>No treatment</th>
<th>ACT</th>
<th>Sodium citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.62</td>
<td>6.30</td>
<td>10.46</td>
</tr>
<tr>
<td>2</td>
<td>18.66</td>
<td>0</td>
<td>3.96</td>
</tr>
<tr>
<td>3</td>
<td>13.43</td>
<td>3.89</td>
<td>11.33</td>
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<tr>
<td>4</td>
<td>26.96</td>
<td>0.19</td>
<td>11.33</td>
</tr>
<tr>
<td>5</td>
<td>10.37</td>
<td>0.85</td>
<td>11.33</td>
</tr>
<tr>
<td>6</td>
<td>8.38</td>
<td>0.23</td>
<td>11.33</td>
</tr>
<tr>
<td>7</td>
<td>7.20</td>
<td>0.93</td>
<td>11.33</td>
</tr>
<tr>
<td>8</td>
<td>5.24</td>
<td>0</td>
<td>11.33</td>
</tr>
<tr>
<td>9</td>
<td>3.98</td>
<td>0</td>
<td>11.33</td>
</tr>
<tr>
<td>10</td>
<td>0.64</td>
<td>0</td>
<td>11.33</td>
</tr>
</tbody>
</table>

Mean | 1.24 |
in lymph on day 1 following challenge, experiments were done to separate the lymphocytes collected at this time from the PMN leucocytes by means of a glass bead column using the method described by Rabinowitz (1964). The metabolic activities of the cell suspensions before and after column fractionation were compared in terms of D-[1-\textsuperscript{14}C] glucose radiorespirometry. This measurement was chosen because it was sensitive when the number of cells used was small and because during day 1 after challenge there was a large increase in the release of \textsuperscript{14}CO\textsubscript{2} from D-[1-\textsuperscript{14}C] glucose (see Chapter 5).

The experiments were set up in series, beginning with an investigation into the effectiveness of the glass bead column for the separation and recovery of cells from the column. The results are summarized in Table 4.6. The column used was very effective in removing PMN leucocytes from cell populations and it provided essentially perfect recoveries of the small and medium lymphocytes, but poor recoveries of the blast cells. The poor recovery of blast cells was unimportant because these were usually not present in significant numbers in lymph collected on day 1 after challenge when the separation experiments were done. The cells were also investigated further for their metabolic activity in terms of \textsuperscript{14}CO\textsubscript{2} release from D-[1-\textsuperscript{14}C] glucose. These results are shown in Table 4.6 and analysed below.

Cumulative radioactivity of \textsuperscript{14}CO\textsubscript{2} released by cells not subjected to column fractionation = 27164 c.p.m. per 10\textsuperscript{6} viable cells per hr,
Cumulative radioactivity of $^{14}$CO$_2$ released by cells after passing through column = 5135 c.p.m. per $10^6$ viable cells per hr.

This number of counts (5135 c.p.m.) was comparable to the counts obtained with cells collected before antigenic challenge.

Assuming:

1. Small and medium lymphocytes had the same metabolic rate of 
   "p" c.p.m. per cell per hr,
2. PMN leucocytes (neutrophils) had a metabolic rate of "q" 
   c.p.m. per cell per hr,
3. Per cent viability was similar among all cell types.

Before column separation,

\[
\left(10^6 \times \frac{16.4}{100}\right) + \left(10^6 \times \frac{83.6}{100}\right) = 27164 \quad \text{(1)}
\]

After column separation, neglecting the contribution from the few blast cells and neutrophils,

\[
\left(10^6 \times \frac{98.3}{100}\right) = 5135 \quad \text{(2)}
\]

Solving the simultaneous equations gives

\[p = 52.24 \times 10^{-4}, \quad q = 314.16 \times 10^{-4}\]

Or \[q = 6.0\] p

Thus PMN leucocytes had a metabolic rate as measured by $^{14}$CO$_2$ release from D-[1-$^{14}$C]glucose, of about 6.0 times higher than lymphocytes. In another experiment on cells collected from another sheep, the value of q was found to be 6.5 times that of p. A further experiment was also done using lymphocyte suspension containing undetectable numbers of PMN leucocytes to see if passing the cells through a glass bead column would affect their metabolic rate in terms of $^{14}$CO$_2$ release from
<table>
<thead>
<tr>
<th></th>
<th>Before column</th>
<th>After column</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of cells</td>
<td>68.2 x 10^5</td>
<td>10.5 x 10^5</td>
<td>15.4</td>
</tr>
<tr>
<td>Small and medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.1% = 10.3 x 10^5</td>
<td>98.3% = 10.3 x 10^5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Blasts</td>
<td>1.3% = 0.9 x 10^5</td>
<td>0.9% = 0.1 x 10^5</td>
<td>11.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>83.6% = 57.0 x 10^5</td>
<td>0.8% = 0.08 x 10^5</td>
<td>0.1</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Cumulative radioactivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of 14CO₂</td>
<td>27164</td>
<td>5135</td>
<td></td>
</tr>
<tr>
<td>(c.p.m./hr/10^6 viable cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 4.6: Separation of PMN leucocytes from lymphocytes on a glass bead column.**

Cells collected from popliteal efferent lymph on day 1 after antigen injection were divided into two portions. One portion was run through a glass bead column. The cells eluted from the column, as well as those in the portion not passing through the column were analyzed for their composition and for their ability to release 14CO₂ from D-[1-14C]glucose. The details of the experiment are described in the text.
D-[1-\textsuperscript{14}C]glucose. The metabolic rate of the cells was found to be unaffected by this procedure.

The PMN leucocytes retained by the glass beads were eluted from the column and subjected to radiorespirometric assay. It was found that pure suspensions of PMN leucocytes metabolized D-[1-\textsuperscript{14}C]glucose much more rapidly than did lymphocytes.
SECTION E

THE RATE OF RESPIRATION OF LYMPHOCyTES IN POPLITEAL EFFERENT LYMPH MEASURED BY WARBURG MANOMETRY AND BY THE CLARK OXYGEN ELECTRODE METHOD
(a) **EXPERIMENTAL**

Gaseous exchange by lymphocyte suspensions was measured with a Warburg constant volume respirometer and carried out according to the techniques described by Umbreit et al. (1974). Both "Direct" and "Indirect" methods of Warburg manometry were used for measuring O\textsubscript{2} and CO\textsubscript{2} exchange. The differences in these methods have been mentioned in detail in Chapter 2. One important point concerning these two methods is that in the "Direct Method" O\textsubscript{2} uptake is measured with the cells respiring in an atmosphere free of CO\textsubscript{2}. There is the possibility that such measurements are not valid, for the rate of respiration and of decarboxylation reactions might be altered in the absence of CO\textsubscript{2}. Thus the "Indirect Method" which measures O\textsubscript{2} uptake in the presence of CO\textsubscript{2} was also done when measuring gaseous exchanges during an immune response. It was found that the two methods did give different results when used with the same batch of cells (Figure 4.9). The "Direct Method" gave more linear gaseous exchange rates during the 2 hr incubation period than the "Indirect Method". This could have been due to the accumulation of CO\textsubscript{2} in the Warburg flask in the "Indirect Method" which could have been deleterious to the respiring cells. Thus only the initial linear rate of gaseous exchange was accepted in calculating the O\textsubscript{2} uptake and CO\textsubscript{2} production.

The absorption of CO\textsubscript{2} from the gas phase in the "Direct Method" is usually done by placing small rolls of filter paper in the alkali cup as they provide a large surface for the absorption of CO\textsubscript{2}. Since the paper might react with the concentrated solution of KOH and result in an erroneous O\textsubscript{2} uptake, this method was not used here. It was found that
FIGURE 4.9: Comparison between the "Direct" and "Indirect" methods of Warburg manometry.

\[ \text{O}_2 \text{ uptake (▲) and CO}_2 \text{ production (△)} \]
determined by the "Direct Method" of Warburg manometry. \[ \text{O}_2 \text{ uptake (●) and CO}_2 \text{ production (○)} \]
determined by the "Indirect Method" of Warburg manometry.
The rate of O₂ consumption by lymphocytes was also measured by the Clark oxygen electrode. For these experiments, a lymphocyte suspension was adjusted to a concentration of 1.0–2.0 x 10⁸ cells per ml and 0.5 ml of the cell suspension was introduced into the reaction vessel of the electrode, which contained 1.6 ml of saturated buffer (Ridsdale et al., 1958). The pH was maintained at 7.4 by the addition of 0.1 N NaOH. The maximal rate of O₂ consumption was determined from the slope of the linear portion of the O₂ gradient, as indicated by a pen recorder (Figure 6). This technique was found to be reliable and valid for calculating the rate of O₂ consumption by lymphocytes. The rates obtained with cellulose membrane and 0.2 µm pore size membranes by lymphocytes collected from popliteal and afferent lymph before antigenic challenge are shown in Table 4.7. The "Direct Method" and the "Indirect Method" of Warburg were used for the determination and calculation of the O₂ exchange rates. The Respiratory Quotient of normal sheep lymphocytes determined by the "Indirect Method" of Warburg was 1.000 and that determined by the "Direct Method" was 0.776. The variation in the gaseous exchange rates measured was about ± 10% of the mean for both methods.
results obtained with omission of the filter paper were not significantly different from the results obtained when filter papers were used.

In all the manometric experiments $2 \times 10^8$ cells were placed in each flask. This number of cells was found in preliminary experiments to have a sufficient respiration rate for accurate measurement.

The rate of $O_2$ consumption by lymphocytes was also measured by the Clark oxygen electrode. For these experiments, the lymphocyte suspension was adjusted to a concentration of $4 \times 10^8$ cells per ml and 0.5 ml of the cell suspension introduced into the reaction vessel of the electrode which contained 1.5 ml air-saturated buffer (Krebs & de Gasquet buffer, pH 7.4 containing 5.56 mM glucose). The maximum rate ($V_{max}$) of $O_2$ consumption was determined from the slope of the linear portion of the curve recorded by the pen recorder (Figure 4.10a). The method for calculating the results has been detailed in Chapter 2.

(b) OXYGEN UPTAKE AND CARBON DIOXIDE PRODUCTION BY NORMAL LYMPHOCYTES

The normal rates of $O_2$ uptake and $CO_2$ production by lymphocytes collected from popliteal efferent lymph before antigenic challenge are shown in Table 4.7. The "Direct Method" and the "Indirect Method" of Warburg manometry gave different values in terms of the mean and the range of gaseous exchange rates. The Respiratory Quotient of normal sheep lymphocytes determined by the "Indirect Method" of Warburg manometry was 1.000 and that determined by the "Direct Method" was 0.776. The variation in the gaseous exchange rates measured was about ± 10% of the mean for both methods.
FIGURE 4.10: Respiration characteristics of sheep lymphocytes determined by Clark oxygen electrode polarography.

(a) original trace recorded. The solution was initially saturated with $O_2$ at atmospheric pressure and finally it was $O_2$ free. The arrow indicates the time when the cell suspension was added to the reaction vessel of the electrode. The region x of curve was amplified 10 times on (b) as y to z.

(b) the change in the rate of respiration at low $O_2$ concentration.
1. line drawn to touch curve, slope of line half maximum slope of experimental curve.
2. $K_m$, the concentration of $O_2$ at which the cells respired at half their maximum rate.
3. $[O_2]_{crit}$, the concentration of $O_2$ at which the respiration rate of the cells had just begun to fall.

(c) a plot of $(S_0-S)/t$ (y, the ordinate) against $1/t.\ln(S/S_0)$ (x, the abscissa). The explanation of the symbols is in the text. The straight line is the line of best fit for the points on the graph. The slope gives the value of $K_m$. 
TABLE 4.7: The rate of gas phase exchange of normal sheep lymphocytes postulated from polarographic technic.

<table>
<thead>
<tr>
<th>Method</th>
<th>Number</th>
<th>Rate of gas phase exchange of sheep lymphocytes ( \times 10^{-6} \mu l / (ml/min) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
<td>50</td>
<td>0.49 ( \pm ) 0.0166x</td>
</tr>
<tr>
<td>Indirect</td>
<td>40</td>
<td>0.36 ( \pm ) 0.0244x</td>
</tr>
</tbody>
</table>

The figures show the mean standard deviation and the variation of \( Y \) and \( X \) as calculated by the polarigraphic analysis of sheep lymphocytes postulated from polarographic technic. The diagram represents the gas phase exchange of sheep lymphocytes before antigenic challenge.
<table>
<thead>
<tr>
<th>Method of determination</th>
<th>Number of sheep studied</th>
<th>Rate of gaseous exchange ($\mu$ moles/hr/2 x $10^8$ cells ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$O_2$ uptake</td>
</tr>
<tr>
<td>&quot;Direct&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warburg</td>
<td>5</td>
<td>1.254 ± 0.204</td>
</tr>
<tr>
<td>&quot;Indirect&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warburg</td>
<td>8</td>
<td>2.476 ± 0.476</td>
</tr>
<tr>
<td>Clark oxygen electrode</td>
<td>5</td>
<td>1.699 ± 0.226</td>
</tr>
</tbody>
</table>

**TABLE 4.7:** The rate of gaseous exchange of normal sheep lymphocytes collected from popliteal efferent lymph.

The table shows the mean value ± standard deviation and the range of variation (in brackets) of the rates of $O_2$ uptake and $CO_2$ production of lymphocytes collected from popliteal efferent lymph of sheep before antigenic challenge. The number of sheep studied by "Direct" and "Indirect" Warburg manometry and by Clark oxygen electrode is also given.
The maximum rate of $O_2$ consumption ($V_{\text{max}}$) of normal lymphocytes before antigenic stimulation determined by the Clark oxygen electrode method in 5 sheep was $1.699 \pm 0.226 \ \mu\text{moles/hr/}2 \times 10^8 \ \text{cells}$ with a range of $1.330 - 1.940 \ \mu\text{moles/hr/}2 \times 10^8 \ \text{cells}$. These rates were close to those determined by Warburg manometry and in between the values obtained from the "Direct" and "Indirect Methods" (Table 4.7).

(c) GASEOUS EXCHANGE OF LYMPHOCYTES COLLECTED DURING THE IMMUNE RESPONSE TO SALMONELLA MUECHEN ORGANISMS

The rates of gaseous exchange in lymphocytes collected during the primary response to *Salmonella muenchen* organisms were measured by Warburg manometry in 3 sheep. The first day after the injection of antigen was usually marked by a peak of gaseous exchange which dropped back to prechallenge levels on day 2. The gaseous exchange increased on day 3 and reached a second peak on day 4 after challenge. The pattern of change is illustrated in Figure 4.11. The magnitude of the peak on day 1 after challenge was very variable, ranging from 100-145% of the pre-challenge rate of gaseous exchange. The peak on day 4 was from 125-231% above the pre-challenge level with a mean of $161 \pm 41\%$.

The changes in the rates of gaseous exchange during secondary responses to *Salmonella* were followed in 3 sheep. The pattern of change was similar to that observed in the primary responses except that the second peak occurred on day 3, coinciding with the peak of the cellular response. A typical example of the pattern of gaseous exchange during a secondary response to *Salmonella* is depicted in the experiment
FIGURE 4.11: The primary response to Salmonella muenchen organisms - the rate of gaseous exchange in sheep lymphocytes determined by Warburg manometry throughout the immune response.

- ■ the rate of gaseous exchange determined by the "Indirect Method" of Warburg manometry (rates of O₂ consumption and CO₂ production were indistinguishable).
- ▲ the rate of O₂ consumption determined by the "Direct Method" of Warburg manometry.
- △ the rate of CO₂ production determined by the "Direct Method" of Warburg manometry.

The arrow indicates the time of antigen administration.
shown in Figure 4.12. The average magnitude of the peak rates of gaseous exchange of lymphocytes from the 3 sheep studied was 14.9 ± 40.6% (range: 10.2 ± 31.9%) of the pre-challenge level for day 1 and 150.7 ± 188.6% (range: 119.4 ± 221.5%) for day 3. The results obtained with both methods of counting counts agreed well with each other.

The rate of oxygen consumption studied by means of Clark oxygen electrodes was found (Figures 4.10 and 4.11) to be the same as observed by Warburg manometry. There was an early peak on day 1 corresponding to the appearance of the number of polymorphonuclear cells in the lymph, followed by a second peak on day 4 when the output of blast cells was maximum. The rate of respiration at peak of the cellular response was approximately that of pre-challenge and close to the rate measured by Warburg manometry.
shown in Figure 4.12. The average magnitude of the peak rates of gaseous exchange of lymphocytes from the 3 sheep studied was 147.9 ± 40.6% (range: 106.5 - 213.9%) of the pre-challenge level for day 1 and 158.2 ± 14.6% (range: 142.1 - 181.1%) for day 3. The results obtained with both methods of Warburg manometry agreed well with each other.

When the rate of $O_2$ consumption was studied by means of the Clark oxygen electrode, it was found (Figure 4.13a, b) that the change in the rate of $O_2$ uptake ($V_{max}$) in the primary response was the same as observed by Warburg manometry. There was an early peak on day 1 corresponding to the appearance of large numbers of polymorphonuclear cells in the lymph followed by a second peak on day 4 when the output of blast cells was maximum. The rate of respiration at the peak of the cellular response was about twice that of the pre-challenge rate and was close to the rate observed by Warburg manometry.
FIGURE 4.12: The secondary response to Salmonella muenchen organisms - the rate of gaseous exchange of sheep lymphocytes determined by Warburg manometry throughout the immune response.

(■) the rate of gaseous exchange determined by the "Indirect Method" of Warburg manometry (rates of O₂ consumption and CO₂ production were indistinguishable).

(▲) the rate of O₂ consumption determined by the "Direct Method" of Warburg manometry.

(▲) the rate of CO₂ production determined by the "Direct Method" of Warburg manometry.

The arrow indicates the time of antigen administration.
GASEOUS EXCHANGE (μMOLES / HOUR / 2 x 10^8 CELLS)

DAYS AFTER CHALLENGE
FIGURE 4.13: The primary response to Salmonella muenchen organisms - the respiration characteristics, including Vmax, Km and \([O_2]_{\text{crit}}\) of lymphocytes collected at various times during the response.

(a) the cellular response, showing the percentages of neutrophils (-----) and blast cells (——). The arrow indicates the time of antigen administration.

(b) the change in Km (○) and Vmax (△) values during the immune response.

(c) the change in value of \([O_2]_{\text{crit}}\) (□) over the response.

The standard deviations for triplicate measurements are shown on the graphs as vertical bars on the time points. For those points without the bars, the standard deviations are smaller than the vertical dimension of the symbols.
SECTION F

THE RESPIRATION CHARACTERISTICS OF LYMPHOCYTES

A similar a kinetic approach had been applied to the relationship between the specific growth rate of cells in culture and the concentration of a limiting nutrient, such as dissolved O₂ (Ruttan, 1964; Butter and Garver, 1964; Haapala, 1966; Brown, 1970), sugar (Vincent, 1947; 1950; 1958; glycerol (Harbert, Elsworth and Telling, 1950), and amino acids (Davis, Karsch and Rada, 1963).
(a) INTRODUCTION

According to Hill (1948), Warburg was the first to investigate the effect of $P_{O_2}$ on cellular respiration under conditions of optimal gas diffusion. Warburg and Kubowitz (1931) found that the respiration rates of sea-urchin eggs at $20^\circ C$ was the same at $P_{O_2}$ of 33 mm and 132 mm Hg. Nucleated red cells respired at the same rate at $P_{O_2}$ varying between 5 mm and 75 mm Hg, while Micrococcus candidans respiring at $1^\circ C$ had the same respiration rate at $10^{-5}$ atmosphere partial pressure as in air. Thus an "all-or-nothing" law of cellular respiration was accepted which assumed that the rate of respiration of cells was independent of the $P_{O_2}$.

This assumption was later challenged by the findings of other workers (Tang, 1933; Kempner, 1937, 1939; Winzler, 1941; Longmuir, 1954; Baender and Kiesse, 1955; Longmuir, 1957).

Longmuir (1954, 1957) not only studied the effect of $P_{O_2}$ on cellular respiration, but also tried to determine the relation between respiration rate and $P_{O_2}$ in terms of the Michaelis-Menten relationship. The present investigations into the effect of $P_{O_2}$ on the rate of respiration of sheep lymphocytes collected from popliteal efferent lymph followed the methodology of Longmuir (1954; 1957) and that of McCabe and Gilbert (1965) and Brown (1970).

A similar kinetic approach had been applied to the relationship between the specific growth rate of cells in culture and the concentration of a limiting nutrient, such as dissolved $O_2$ (Button, 1964; Button and Garver, 1966; Hospodka, 1966; Brown, 1970), sugar (Monod, 1942; 1949; 1950), glycerol (Herbert, Elsworth and Telling, 1956), and amino acids (Davis, Karush and Rudd, 1965).
(b) EXPERIMENTAL

The Rank oxygen electrode was used to study the critical \( O_2 \) concentration \( ([O_2]_{crit}) \) and the apparent Michaelis constant \( (K_m) \) of lymphocytes responding to Salmonella muenchen organisms.

The cell suspensions and the electrode were prepared as for the experiments in which the rate of \( O_2 \) consumption \( (V_{max}) \) was studied. Initial calibration points using air-saturated \( P_{O_2} \) and zero \( P_{O_2} \) were first obtained. More air was introduced into the solution by raising and then lowering the perspex disc and the disappearance of the introduced \( O_2 \) was recorded at a higher instrumental sensitivity and faster chart speed. This procedure was repeated three times.

It was found that \( O_2 \) in the reaction medium was first used up at a constant rate and later more slowly until none remained. The curve on the chart at this time was horizontal (Figure 4.10a, b). This observation led to the idea that the relationship of lymphocytes to \( O_2 \) was similar to the relationship of an enzyme to its substrate. As a consequence the respiration rate of the cells, as a function of \( O_2 \) concentration, could be described by the Michaelis-Menten equation, as reported for bacteria and for rat-liver cells (Longmuir, 1954; 1957).

The integral form of the Michaelis-Menten equation is given by

\[
\frac{S_0 - S}{t} = V_{max} \int_{0}^{t} \left( \frac{K_m - P_{O_2}}{S_0} \right) dt
\]

Where \( S_0 = \) the initial \( O_2 \) concentration,
\( S = \) the \( O_2 \) concentration at time \( t \),
\( V_{max} = \) the maximum rate of \( O_2 \) consumption,
\( K_m = \) the Michaelis constant (i.e. the \( O_2 \) concentration at which the rate of \( O_2 \) consumption is half maximum).
In order to test the validity of the above concept experimentally, (So-S)/t was plotted against 1/t x ln(S/So) - the Eadie-Hofstee plot. If a straight line relationship existed with this plot it would provide further support to the concept. As shown in Figure 4.10c, a straight line relationship was always obtained. Thus the respiration rate of lymphocytes was related to the O₂ concentration by the Michaelis-Menten equation.

More information could be obtained by inspection of the whole respiration curve. With reference to the integral equation above and the plot shown in Figure 4.10c values for maximum respiration rates (V_max) could be read off from the intercept on the y-axis and Km values from the slope. The critical O₂ concentration (the concentration of O₂ at which the respiration rate just began to fall) could be read off from the original curve on the chart (Figure 4.10b). Km values could also be determined by drawing a line with a slope half the maximum of the experimental curve, to touch the original curve; the distance between the point of contact and the bottom of the curve (zero O₂) was a measure of Km (Figure 4.10b). This method of evaluating Km is not as good as the Eadie-Hofstee plot because it considers one point only instead of the slope of a line. It was found however, that the two different methods gave Km values very close to each other. The Km values reported here were all determined from Eadie-Hofstee plots.

The relation between Km and V_max was also studied in the present investigations.

(c) THE CHANGE IN Km AND [O₂]_crit DURING THE IMMUNE RESPONSE TO SALMONELLA MUENCHEN ORGANISMS

The respiration characteristics, including Km and [O₂]_crit of sheep lymphocytes were studied in 5 sheep challenged with
Salmonella muenchen organisms. It was found that no significant changes occurred in the values of $[O_2]_{crit}$ before or after antigenic stimulation. Mean values of $1.998 \pm 0.197 \mu M$ (1.477 ± 0.144 mm Hg) with a range of 1.756 - 2.396 µM (1.299 - 1.773 mm Hg) were obtained with cells from one of the sheep studied as shown in Figure 4.13c. The values for the cells from the other sheep were similar.

The Km values changed significantly over the period of the immune response, as shown in Figure 4.13b. Before antigenic stimulation, the Km value of the lymphocytes from the same sheep as in Figure 4.13c was $0.462 \pm 0.108 \mu M$ with a range of $0.342 - 0.628 \mu M$. This value was about 25% of the critical $O_2$ concentration. After primary antigenic challenge, the Km value remained more or less unaltered during the first 2 days but rose sharply on day 3 to reach a maximum on day 4 when the percentage of blast cells in the lymph was at its peak. The Km value declined over the next few days. The Km value at the peak of the response was about 2.5 - 3.0 times that of the prechallenge level (Figure 4.13b).

A positive linear correlation between Km and Vmax was observed when the former was plotted against the latter, as shown in Figure 4.14. The equation that described this relationship was $Km = -0.106 + 0.288 Vmax$. 
FIGURE 4.14: The correlation between the parameters $K_m$ and $V_{max}$ determined for sheep lymphocyte respiration.

The linear regression relating $K_m$ to $V_{max}$ of sheep lymphocyte respiration determined in a primary response to *Salmonella muenchen* organisms is given by the equation $K_m = -0.106 + 0.288 \times V_{max}$. The t ratio (slope/standard error) is 7.38 ($p<0.001$).
V_{\text{max}} \ (\mu\text{MOLES/HR/}2 \times 10^8 \text{CELLS})

K_m \ (\mu\text{M})
SECTION G

THE CHANGES IN THE RATE OF RESPIRATION OF LYMPHOCYTES RESPONDING TO ALLOGENEIC STIMULATION

In order to compare the metabolic activities of sheep lymphocytes stimulated by Salmonella paratyphi organisms with lymphocytes responding to another antigenic stimulus, the respiration of lymphocytes was studied during a response to allogeneic cells. Allogeneic lymphocytes were obtained from the peripheral blood of an unrelated sheep. The number of cells used for challenge was $2 \times 10^6$ and these were injected subcutaneously into the lower leg of the responding sheep, as in the case for Salmonella organisms. The second peak of the stimulating allogeneic lymphocytes was determined in vitro in mixed lymphocyte cultures.

The rate of respiration was measured by Warburg manometry. The metabolic activity of lymphocytes stimulated in vivo by allogeneic lymphocytes is lower than that of lymphocytes responding to Salmonella organisms. A first peak of $O_2$ uptake and $CO_2$ production, measured by "indirect" Warburg manometry was observed on day 2 after challenge and lasted for 24 hr. The second peak of gaseous exchange occurred much later than in the Salmonella response on day 9 after the injection of the allogeneic cells, this coincided with the peak of the blast cell response in the lymph.
(a) **EXPERIMENTAL**

In order to compare the metabolic activities of sheep lymphocytes stimulated by *Salmonella muenchen* organisms with lymphocytes responding to another antigenic stimulus, the respiration of lymphocytes was studied during a response to allogeneic cells. Allogeneic lymphocytes were obtained from the peripheral blood of an unrelated sheep. The number of cells used for challenge was $2 \times 10^8$ and these were injected subcutaneously into the lower leg of the responding sheep as in the case for *Salmonella* organisms. The antigenicity of the stimulating allogeneic lymphocytes was determined in vitro in mixed lymphocyte culture (MLC).

(b) **THE RATE OF RESPIRATION OF LYMPHOCYTES RESPONDING TO ALLOGENIC CELLS**

The result of one of these experiments is shown in Figure 4.15. The blast cell response in the lymph was similar to that observed by Grant and Cameron (1975) and Cahill, Frost and Trnka (1976). The metabolic activity of lymphocytes stimulated *in vivo* by allogeneic lymphocytes in terms of their respiration was found to be similar to that observed in lymphocytes responding to *Salmonella* organisms. A first peak of $O_2$ uptake and $CO_2$ production, measured by "Indirect" Warburg manometry was observed on day 1 after challenge and lasted for 24 hr. The second peak of gaseous exchange occurred much later than in the *Salmonella* response on day 9 after the injection of the allogeneic cells, this coincided with the peak of the blast cell response in the lymph.
FIGURE 4.15: The primary response to allogeneic lymphocytes - the rate of gaseous exchange of lymphocytes collected from the efferent lymph of the popliteal node at various times during the allogeneic response.

The upper part of the figure shows the percentage of blast cells in lymph. The lower part shows the changes in the rate of gaseous exchange in lymphocytes throughout the response as determined by the "Indirect Method" of Warburg manometry. The arrow indicates the time of antigen administration.
(a) THE RESPONSE OF THE POPITAEAL LYMPH NODE TO SALMONELLA PREVIOUS ORGANISMS

The immune response to Salmonella organisms is reflected in changes to the cellular and antibody content of the efferent lymph. The general sequence of events was similar to that described by English (1974) for lipopolysaccharide isolated from the same organisms and to that described by Hall and Harris (1965) for killed Salmonella organisms.

In the present study, only two classes of immunoglobulin, IgM and IgG, were detected in the popliteal efferent lymph. This result is similar to that reported by Cunningham et al. (1966), Hay (1970) and English (1974), except that in primary responses Hay (1970) and English (1974) observed a peak of IgG activity on around day 12 of the response. In the present investigation, IgM antibody was detected on around day 5 and 6, at the same time as the IgG peak. The observed similarity between primary and secondary responses is probably due to the fact that all the sheep used had probably been exposed previously to Salmonella antigens. The antibody activity that was present in the lymph prior to the second injection of Salmonella can be attributed to both IgM and IgG antibody produced late in the primary response.

(b) THE CHANGE IN OXYGEN TENSION OF THE POPITAEAL EFFERENT LYMPH DURING AN IMMUNE RESPONSE

In Section B of this chapter, it was reported that the O2% of popliteal efferent lymph changed during an immune response to killed Salmonella organisms. The highest value for O2% was recorded during the "shutdown" period of the response when
(a) THE RESPONSE OF THE POPLITEAL LYMPH NODE TO SALMONELLA MUENCHEN ORGANISMS

The immune response to *Salmonella muenchen* organisms in the popliteal node of sheep is reflected in changes in the cellular and antibody content of the efferent lymph. The general sequence of events was similar to that described by English (1974) for lipopolysaccharide isolated from the same organisms and to that described by Hall and Morris (1963) for killed *Salmonella typhi* organisms.

In the present study, only two classes of immunoglobulin, IgG and IgM were detected in the popliteal efferent lymph. This result is similar to that reported by Cunningham et al. (1966), Hay (1970) and English (1974), except that in primary responses Hay (1970) and English (1974) observed a late peak of IgG activity on around day 12 of the response. In the present investigations, a peak of IgG antibody was detected on around day 5 and 6, at the same time as the IgM peak. The observed similarity between primary and secondary responses is probably due to the fact that all the sheep used had probably been exposed previously to *Salmonella* antigens. The antibody activity that was present in the lymph prior to the second injection of *Salmonella* can be attributed to both IgM and IgG antibody produced late in the primary response.

(b) THE CHANGE IN OXYGEN TENSION OF THE POPLITEAL EF FERENT LYMPH DURING AN IMMUNE RESPONSE

In Section C of this chapter, it was reported that the $P_{O_2}$ of popliteal efferent lymph changed during an immune response to killed *Salmonella* organisms. The highest value for $P_{O_2}$ was recorded during the "shutdown" period of the response when
the concentration of cells in the lymph was lowest while
the lowest value of $P_{O_2}$ was recorded at the peak of the
cellular response when the number of blast cells and the
total number of cells in the lymph were maximum. This
observation led to the subsequent studies on the relationship
between the $P_{O_2}$ value and the concentration of cells present
in the lymph. There was a statistically significant negative
correlation between the $P_{O_2}$ and the concentration of cells
present in the lymph. This relationship was observed in the
different sheep studied. Calculation of the $r^2$ values to assess
the coefficient of determination showed that the amount of
variation in $P_{O_2}$ of lymph which had been accounted for by the
regression relating $P_{O_2}$ to cell concentration was about 45% of
the total variance. This significant correlation suggested
either that the respiratory activity of the cells determines
at least in part, the $P_{O_2}$ of the lymph in which they exist,
or that the correlation was due to the presence of the cells
affecting the functioning of the electrode, i.e. it was an
artefact introduced by the cells in contact with the electrode
surface. Since practically no effect of cell concentration
on electrode performance was ever observed with various kinds
of cells (Hagihara, personal communication), the first
explanation was more probable. As a proportion of the total
popliteal lymph is formed within the popliteal node and as the
afferent lymph to the popliteal node would mix with this to
provide the milieu in which the cells in the node respire it
was reasonable to assume that the gaseous tensions in the
lymph reflected closely the gaseous tension within the node.
This was a further example of the closeness with which events
in the efferent lymph reflect events occurring within the lymph node.

It was shown that the slope of the regression relating $P_{O_2}$ of popliteal lymph to cell concentration was steeper for the secondary response than for the primary response. This suggested that cells in secondary responses consumed $O_2$ more avidly and metabolized $O_2$ more actively than cells in a primary response. This was considered to be due to the presence of greater numbers of blast cells.

(c) CONTAMINATION OF LYMPHOCYTE PREPARATIONS BY ERYTHROCYTES AND POLYMORPHONUCLEAR LEUCOCYTES

Studies of energy metabolism of lymphocyte populations are invalid unless the contributions of contaminating RBC and PMN leucocytes are taken into account. The removal of RBC by lysis with ACT solution was unharmful to lymphocytes and so simple and efficient to do that this was done routinely to all preparations of cells used for metabolic studies. The results shown in Section D indicated that pure populations of lymphocytes collected on the first day following antigenic challenge had metabolic activities similar to those of normal lymphocytes, thus it was safe to attribute the sudden increases in metabolism observed at this stage of the immune response to the transient appearance of PMN leucocytes in the lymph. This assumption was supported by the high levels of energy metabolism recorded for these cells in Section D(c).

(d) RATES OF GASEOUS EXCHANGE OF LYMPHOCYTES

Before antigenic challenge, it was found that lymphocytes consumed $O_2$ at a rate of $1.254 \pm 0.204 \mu$moles/hr/2 x $10^8$
cells as determined by the "Direct" Warburg manometry. This value was lower than that calculated from the data of other workers using the same method but studying lymphocytes of other species. The values obtained by other workers (Table 4.8) were comparable to those obtained by "Indirect" manometry, namely $2.476 \pm 0.476$ µmoles/hr/2 $\times 10^8$ cells. The $O_2$ consumption rates determined by the Clark oxygen electrode, $1.699 \pm 0.226$ µmoles/hr/2 $\times 10^8$ cells were higher than the rates listed on Table 4.8, 1.06 and 1.23 µmoles/hr/2 $\times 10^8$ cells.

The difference between the values of $O_2$ consumption determined by the "Direct" and "Indirect Method" could be attributed to the fact that the cells studied were respiring under different gaseous atmospheres in the two different methods. Examples of difference in results obtained from the two different methods when applied to the same batch of cells have been reported by Umbreit et al. (1974). Very few observations on the gaseous metabolism of free-floating lymphoid cells have been reported in the literature.

Respiratory quotients (R.Q.) determined by the "Direct" and "Indirect Method" were also found to be different (Section E). The respiratory quotient, a term first coined by Pflueger (cf. Soskin and Levine, 1952) is the relationship expressed in volumes, between the amount of $O_2$ consumed and the $CO_2$ given off ($CO_2/O_2$). The theoretical R.Q. for the complete oxidation of glucose is 1.00, for fat it is 0.707 and for protein is 0.809. The physiological interpretation of the R.Q. of a whole animal or a single tissue is very difficult in practice because of the fact that the total R.Q. is always a composite of many possible R.Q.'s arising through the various
Table 4.8: Results reported in the literature for the rate of oxygen consumption by lymphocytes.

Reference: 1. Rauch et al., 1961
2. Hedeskov and Esmann, 1966
3. Culvenor and Weidemann, 1976
4. Pachman, 1967
5. Roos and Loos, 1973

Method of determination:

WD: "Direct Method" of Warburg manometry

CO: Clark-type oxygen electrode

O₂ consumption rate: Calculated from data reported in the literature.
metabolic pathways. Thus when carbohydrate is transformed into fat, an R.Q. of about 8.0 can be obtained. The theoretical R.Q. for the conversion of protein to carbohydrate is 0.613. The R.Q. for gluconeogenesis from fat is about 0.28 and that for ketogenesis from fat may be calculated to range from 0.00 - 0.65 (Soskin and Levine, 1952). The value of the R.Q, by its very nature, depends on the starting material and the end-products of the series of metabolic reactions and gives no indication of the intermediate reactions. Also the interpretation of the R.Q. determined in vitro, when applied to in vivo conditions is subject to a further complication in that the three main metabolic substrates, protein, fat and carbohydrate, or their breakdown products are constantly available and may be metabolized simultaneously.

In view of this, the R.Q. of 1.0 for lymphocytes determined by the "Indirect Method" in the present investigations indicates only a theoretically exclusive carbohydrate oxidation under the experimental conditions. It is possible that this is an accurate estimate of the substrate being metabolized as glucose was the only exogeneous substrate available to the cells during the incubation period. Whether this R.Q. value can be applied to physiological conditions is doubtful since there is good evidence that many cells derive a large part of their energy from fatty acids. The lower R.Q. value obtained in the "Direct Method" may be a result of the effect of the absence of CO₂ from the gas phase of the incubation flask, which caused the cells to shift their metabolic activities.
The increase in respiration rates observed after antigenic challenge coincided with the appearance of large numbers of blast cells in the lymph. Since the blast cells were undergoing transformation it would be expected that they would require increased supplies of energy for the processes involved in transformation and migration. This extra energy requirement would be supplied by an increase in aerobic respiration associated with an increased supply of energy in the form of ATP and other substances essential for their transformation.

Since the same phenomenon was observed when allogeneic cells were used to stimulate lymphocytes, there is a basis for saying that blast cell transformation occurred with stimulated aerobic respiratory activity irrespective of whether the transformation leads to humoral or cell mediated immunity.

(e) THE EFFECT OF OXYGEN TENSION ON THE RESPIRATION RATE OF LYMPHOCYTES AND THE AFFINITY OF LYMPHOCYTES TO OXYGEN

It was found that sheep lymphocytes respired at rates that were related to the $O_2$ concentration (or partial oxygen tension, $P_{O_2}$) of the medium according to the Michaelis-Menten equation. Deviations from the straight line relationship were frequently observed at high and very low $O_2$ concentrations. At high concentrations this was due to the fact that the respiration rate approached the theoretical maximum rate ($V_{max}$) asymptotically, i.e. there was no "critical $O_2$ concentration" at which the respiration rate just began to fall. Experimentally there was always a true "critical $O_2$ concentration" (Figure 4.10b), thus the respiration rate approached the maximum rate at a lower
O₂ concentration than predicted by the equation, and this led to deviation from the straight line relationship at high O₂ concentrations. Longmuir (1957) provided two possible explanations for this deviation:

1. A reduction of the concentration of O₂ in the reaction medium eventually results in anoxia for those mitochondria furthest from the surface of the cell.

2. A component of the succinic oxidase system becomes limiting at above 80% saturation of the respiratory enzyme.

Deviation at very low O₂ concentrations might be due to fluctuations in the current output from the measuring electrode (McCabe and Gilbert, 1965).

From the studies reported in Section F of this chapter, the critical O₂ concentration of lymphocytes was found to be low ($1.477 \pm 0.144$ mm Hg O₂ pressure). These values were close to those determined by Hill (1948) on frog's muscle and by Longmuir (1957) on rat liver cells but much lower than those reported by Brown (1970) for bacteria. Because of the high $P_{O_2}$ of lymph it is certain that sheep lymphocytes exist in the lymph in vivo in an environment that is amply saturated with O₂ and the rates of respiration of these cells would be unaffected by the normal fluctuations in $P_{O_2}$ that occur in lymph.

However, the Km values of lymphocytes in regard to O₂ changed during a response. Maximum values of Km were observed when the percentage of blast cells in the lymph collected was highest. This indicated that blast cells had reduced affinity for O₂ when compared to small lymphocytes. This was due either
to a difference in the affinity of the respiratory enzymes in blast cells for $O_2$ or to an increased barrier between the $O_2$ in the medium and the enzymes in the mitochondria of the blast cells. Longmuir (1954) and Longmuir, Milesi and Bourke (1960) who studied the respiration rates of bacteria of different sizes as a function of $O_2$ concentration, found that large bacteria had larger $K_m$ values than small bacteria. The effect of cell size on $K_m$ values could be related by the equation $K_m = A.d^x$ where $A =$ proportionality factor, $d =$ the cell diameter, and $x =$ a constant which is a function of cell shape.

Longmuir (1954) conducted experiments on cell-free preparations obtained from different sized bacteria and found that the $K_m$'s of these preparations were approximately the same. The affinity for $O_2$ of the respiratory enzymes from different sized bacteria was thus essentially identical.

The different $K_m$ values measured in intact cell preparations were not true values as their measurement was affected by the $O_2$ gradient between the medium and the interior of the cell. In cells of large size, the distance between the interface of the suspending medium and the respiratory enzymes in the mitochondria is proportionately greater than in cells of smaller size. Because of this the diffusion gradient between the interfacial layer of the medium adjacent to the cell membrane and the mitochondrial membrane is greater in larger cells. In order to sustain the same level of $O_2$ concentration around the enzymes, a higher $P_{O_2}$ is required in the suspending medium. Since experimentally $K_m$ is a measure of the concentration of $O_2$ in the suspending medium at which the rate of respiration is half maximum, this value,
measured by the oxygen electrode in contact with the suspending medium would be higher for cells of larger diameter. The true Km value however might not be different in cells of different sizes.

This explanation is considered to relate to the present investigation since blast cells are known to have a much bigger cell volume than unstimulated lymphocytes (Figure 4.3a, c). Electron micrographs of lymphocytes and blast cells (Figure 4.16a, b) clearly show that in small lymphocytes, the amount of cytoplasm is sparse and thus the mitochondria are usually close to the cell membrane. In blast cells the cytoplasm is much greater in volume and many of the mitochondria are located deep to the cell membrane and separated from the bulk of the suspending fluid by a layer of cytoplasm across which the O$_2$ in the medium must diffuse. This would affect the rate of diffusion of O$_2$ through the cytoplasm into the mitochondria, and thus affect the apparent Km value.

The fact that the critical O$_2$ concentration did not change in accordance with the apparent Michaelis constant during the immune response was a paradoxical result as the existence of a diffusion gradient would require that the critical O$_2$ concentration increased with increasing cell size. McCabe and Gilbert (1965) thus proposed the existence of an active transport mechanism for O$_2$ within the cell. In the present investigations this aspect of O$_2$ transport was not studied.

According to Winzler (1941), [O$_2$]$_{crit}$ values change with temperature and Longmuir (1957) found that Km values were also affected. The effect of temperature on these values for
FIGURE 4.16: Electron microscope picture of lymphocytes in popliteal efferent lymph.

(a) a small lymphocyte.
(b) a blast cell.
\( n = \text{nucleus} \)
\( m = \text{mitochondria} \)

Micrographs are provided by Professor Bede Morris.
lymphocytes was not studied in the present investigations. In sheep the body temperature is constant, so effects of temperature on $[O_2]_{crit}$ and $K_m$ were not considered to be important in understanding the physiological respiration of lymphocytes in vivo.
CHAPTER 5

SECTION A

THE ENERGY METABOLISM OF LYMPHOCYTES IN POPLITEAL EFFERENT LYMPH DURING THE IMMUNE RESPONSE TO SALMONELLA MUECHEN ORGANISMS
Nearly all previous studies on the energy metabolism of lymphocytes stimulated by various mitogens have been done on cells isolated from tissues such as Peyer’s and lymph nodes. Whilst these studies have given information concerning the changes that occur in energy metabolism during lymphocyte transformation in vitro, the results may not apply to physiological situations such as when lymphocytes are stimulated in vivo by antigen. This chapter describes investigations into the energy metabolism of lymphocytes collected from different lymph of the popliteal node after it was challenged with killed histologically mycobacterium organisms.

It has been shown by other workers that energy glycolysis is active in lymphocytes in vitro. In the present investigations the uptake of glucose and the production of lactate by lymphocytes with the cells suspended in a medium containing with atmospheric air. These studies together with the respiratory studies reported in Chapter 4 were done to provide information on the means in which glucose was metabolized by lymphocytes.

Another important alternative route of glucose metabolism, the pentose phosphate pathway (pentose phosphate shunt) was also studied by radiorespirometry. In these experiments the Incorporation of C-1- and C-6-labeled glucose carbon atoms into HCO₃⁻ was recorded. Data from radiorespirometric studies provide further information on the nature of the metabolic pathways, the rate of biochemical reactions and the relative contribution of other oxidative reactions to the energy metabolism of lymphocytes.
Nearly all previous studies on the energy metabolism of lymphocytes stimulated by various mitogens have been done on cells isolated from tissues such as spleen and lymph nodes. Whilst these studies have given information concerning the changes that occur in energy metabolism during lymphocyte transformation in vitro, the results may not apply to physiological situations such as when lymphocytes are stimulated in vivo by antigen. This chapter describes investigations into the energy metabolism of lymphocytes collected from efferent lymph of the popliteal node after it was challenged with killed Salmonella muenchen organisms.

It has been shown by other workers that aerobic glycolysis is active in lymphocytes in vitro. In the present investigations the uptake of glucose and the production of lactate by lymphocytes was studied with the cells suspended in a medium equilibrated with atmospheric air. These studies together with the respiratory studies reported in Chapter 4 were done to provide information on the ways in which glucose was metabolized by lymphocytes.

Another important alternative route of glucose metabolism, the pentose phosphate pathway (hexose monophosphate shunt) was also studied by radiorespirometry. In these experiments the incorporation of C-1- and C-6-labelled glucose carbon atoms into $^{14}$CO$_2$ was recorded. Data from radiorespirometric studies provided further information on the nature of the metabolic pathways, the rate of biochemical reactions and the relative contribution of other oxidative reactions to the energy metabolism of lymphocytes.
Since the $P_{O_2}$ values of popliteal efferent lymph had previously been determined (Chapters 3 and 4) the effect of different levels of $P_{O_2}$ on these metabolic activities of lymphocytes was also studied, so that a better evaluation of the metabolic data could be made in physiological terms.

The level of glucose in the popliteal efferent lymph was also determined to see if changes occurred in glucose concentration during the immune response.
GLUCOSE UPTAKE AND LACTATE PRODUCTION
BY CELLS IN POPLITEAL LYMPH DURING AN
IMMUNE RESPONSE TO SALMONELLA MUNCHEN
ORGANISMS

SECTION B

These two sections of preinfiltrating lymphocytes in the
incubation medium on rates of glucose uptake and lactate
production was also investigated. It was found that there
was no significant correlation between rate of lactate
production and the concentration of lymphocytes. On the other
hand, the rate of glucose uptake was found to be related to the
concentration of cells present in the assay (Figure 6.1). This
experiment was done on lymphocytes collected from a single sheep.
At concentrations below 70 x 10^6 cells/ml, there was a close
increase in the rate of glucose uptake and values between
1.0 - 2.5 nmol/h/mg/2 x 10^7 cells were observed. At higher
concentrations of lymphocytes—the effect of cell concentration
on the rate of glucose uptake was not critical and between
the range 80 - 170 x 10^6 cells/ml there was no significant
change in the rate of uptake. Thus in all experiments in which
glucose and lactate were assayed, a standard cell concentration
Glucose and lactate concentrations were measured according to the methods described in Sigma Technical Bulletins No. 510 and No. 826 with the following modifications:

1. Samples for glucose determinations were deproteinized with HClO₄ instead of Ba(OH)₂/ZnSO₄ solution as recommended by the Bulletin.
2. 2.0 ml of the Combined Enzyme Color Solution was used instead of 5.0 ml in glucose assays and 1.4 ml of the Reagent Mixture Solution was used instead of 2.8 ml in lactate assays.

These two modifications were justified by the results of preliminary experiments.

The effect of the concentration of lymphocytes in the incubation medium on rates of glucose uptake and lactate production was also investigated. It was found that there was no significant correlation between rate of lactate production and the concentration of lymphocytes. On the other hand, the rate of glucose uptake was found to be related to the concentration of cells present in the assay (Figure 5.1). This experiment was done on lymphocytes collected from a single sheep. At concentrations below 70 x 10⁶ cells/ml, there was a steep increase in the rate of glucose uptake and values between 1.0 - 5.6 µmoles/hr/2 x 10⁶ cells were observed. At higher concentrations of lymphocytes the effect of cell concentration on the rate of glucose uptake was not critical and between the range 80 - 170 x 10⁶ cells/ml there was no significant change in the rate of uptake. Thus in all experiments in which glucose and lactate were assayed, a standard cell concentration
FIGURE 5.1: The effect of cell concentration on the uptake of glucose by sheep lymphocytes.

Lymphocytes collected from popliteal efferent lymph were assayed for their rate of glucose uptake at different final cell concentrations.
of 68.2 x 10^6 cells/ml (= 4 x 10^6 cells/6 ml incubation medium) was used to eliminate any concentration effect.

In the glucose and lactate assays as well as in the respiration studies mentioned previously, the lymphocytes were suspended in Krebs-Bruceton buffer containing 9.56% glucose. This concentration of glucose was chosen such that it would provide the cells with sufficient glucose to meet their metabolic requirements. The glucose concentration was determined to be the preliminary experiment shown in Figure 1, in which the rate of lactate production was determined when the cells were incubated with various labeled glucose concentrations. It can be seen that normal and stimulated lymphocytes showed similar rates of glucose uptake, as measured by the rate of lactate production.

A concentration of 3.0 - 4.0 mmol/L of glucose was chosen in subsequent experiments to provide a sufficient glucose source for the study of energy metabolism, except in the case of energy metabolism, when the lower concentrations were used. 

In normal sheep, the level of glucose in the popliteal lymph varied from 3.20 to 6.20 mmol/L with a mean and standard deviation of 4.61 ± 0.33 mmol/L; the level of glucose in popliteal venous blood varied from 3.00 to 4.70 mmol/L with a mean and standard deviation of 4.20 ± 0.31 mmol/L. The daily fluctuations in the glucose levels were small (Table 3.1).
of $66.7 \times 10^6$ cells/ml ($= 4 \times 10^8$ cells/6 ml incubation medium) was used to eliminate any concentration effect.

In the glucose and lactate assays as well as in the respiration studies mentioned previously, the lymphocytes were suspended in Krebs & de Gasquet buffer containing 5.56 mM glucose. This concentration of glucose was chosen on the basis that this would provide the cells with sufficient glucose to saturate their metabolic requirements. The concentration was determined in the preliminary experiment shown in Figure 5.2 in which the rate of lactate production by lymphocytes was determined when the cells were incubated at various initial glucose concentrations. It can be seen that in normal and stimulated lymphocytes the cells were saturated with glucose, as measured by the rate of glycolysis, at around a concentration of 3.0 - 4.0 mM. A concentration of 5.56 mM was chosen in subsequent experiments to provide a glucose excess for the studies on energy metabolism, except when radiorespirometric methods were used.

(b) **GLUCOSE LEVELS IN LYMPH AND BLOOD SERUM**

The level of glucose in lymph and in peripheral venous blood serum collected at various times during primary and secondary immune responses was determined in a number of sheep to see if any change occurred in the glucose concentration after antigenic challenge.

In normal sheep, the level of glucose in the popliteal lymph varied from 3.52 to 4.70 mM with a mean and standard deviation of $4.01 \pm 0.33$ mM; the level of glucose in jugular venous blood varied from 3.86 to 4.77 mM with a mean and standard deviation of $4.20 \pm 0.31$ mM. The daily fluctuations in the glucose level were small (Table 5.1).
FIGURE 5.2: The relationship between the rate of lactate production by sheep lymphocytes and the initial concentration of glucose in the incubation medium.

Lymphocytes collected before antigenic challenge (○), lymphocytes collected at the peak of the cellular response (13.7% blasts) following antigenic challenge (△). The cells were incubated in medium containing different initial concentrations of glucose. The rate of lactate production at these concentrations is plotted against the initial glucose concentration.
TABLE 1. The average low values for the glucose concentration of pooled lymph and jugular venous blood serum collected from a sheep on successive days.

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>Lymph glucose (mM)</th>
<th>Blood serum glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.37</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>4.10</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>4.25</td>
<td>1.01</td>
</tr>
</tbody>
</table>

![Graph showing lactate production versus glucose concentration](image)
TABLE 5.1: The daily variation in the level of glucose in the popliteal efferent lymph and in the jugular venous blood serum.

The numbers in each column are the values for the glucose concentration of popliteal lymph or jugular venous blood serum collected from a sheep on successive days.

<table>
<thead>
<tr>
<th>Sheep Number</th>
<th>Lymph glucose (mM)</th>
<th>Blood serum glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.97</td>
<td>3.97</td>
</tr>
<tr>
<td></td>
<td>4.10</td>
<td>3.90</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>4.01</td>
</tr>
<tr>
<td></td>
<td>4.26</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.88</td>
<td></td>
</tr>
</tbody>
</table>

Notes: Observations were made on successive days during the first 24 hours of antigenic challenge in 9 sheep. The daily variation in these two estimates in a single sheep was about 10-20% of the mean.
In the days following the injection of antigen no significant change was observed in the level of glucose in either the lymph or in the jugular venous blood except during the first few hours after antigenic challenge irrespective of whether the response was primary or secondary. Following challenge there was an obvious fall in the glucose level in the lymph. This phenomenon was observed in 7 out of 10 sheep investigated. Figure 5.3 illustrates one example where such a transient fall in the glucose level of the popliteal efferent lymph was recorded. The level of glucose in the jugular venous blood serum immediately after antigenic challenge varied from sheep to sheep and did not seem to be related to the fall in the level of glucose in the lymph.

(c) GLUCOSE UPTAKE AND LACTATE PRODUCTION BY LYMPHOCYTES DURING THE PRIMARY RESPONSE TO SALMONELLA MUENCHEN ORGANISMS

Lymphocytes in popliteal efferent lymph collected from normal sheep before antigenic challenge had a rate of glucose uptake which varied from 0.712 to 1.432 µmoles/hr/2 x 10^8 cells with a mean and standard deviation of 0.970 ± 0.290. The rate of lactate production varied from 0.781 to 1.946 µmoles/hr/2 x 10^8 cells with a mean and standard deviation of 1.306 ± 0.368. The daily variation in these two estimates in a single sheep was about ±10-20% of the mean.

Changes in the rates of glucose uptake and lactate production by lymphocytes collected from popliteal efferent lymph during the primary response to killed Salmonella muenchen organisms were studied in 5 sheep. There was a significant rise in both glucose uptake and lactate production (Figure 5.4).
FIGURE 5.3: The level of glucose in the popliteal efferent lymph determined at various times during a response to Salmonella muenchen organisms.

The arrow indicates the time of antigen administration.
FIGURE 5.4: The primary response to Salmonella muenchen organisms - the rate of glucose uptake and lactate production by lymphocytes collected at various times during the response.

The upper part of the figure shows the percentages of neutrophils (-----) and blast cells (-----) in lymph. The lower part shows the changes in rates of glucose uptake (●) and lactate production (▲) throughout the response. The arrow indicates the time of antigen administration.
DAYs AFTER CHALLENGE
by lymphocytes on the day immediately following the injection of antigen. This early stimulation of metabolic activity was observed in 4 out of the 5 sheep studied. By day 2 the rates had fallen back to prechallenge levels but they rose again on day 3 and reached a maximum on day 4 after challenge. The rates then declined over the next 3 or 4 days to the prechallenge levels. The magnitude of the first metabolic peak was variable ranging from 108 - 190% of the prechallenge level with a mean of 141 ± 34% for glucose uptake and from 92 - 210% of prechallenge level with a mean of 138 ± 51% for lactate production. The peak on day 4 of the immune response was significantly greater ranging from 127 - 326% of prechallenge level with a mean of 196 ± 86% for glucose uptake and from 120 - 200% with a mean of 144 ± 39% for lactate production. 

(d) GLUCOSE UPTAKE AND LACTATE PRODUCTION BY LYMPHOCYTES DURING THE SECONDARY RESPONSE TO SALMONELLA MUENCHEN ORGANISMS

The changes in the rates of glucose uptake and lactate production by lymphocytes in popliteal efferent lymph during the secondary response to killed Salmonella muenchen organisms was followed in 3 sheep. Again on day 1 after challenge there was a significant peak in glucose uptake and lactate production (Figure 5.5). The magnitude of the increase ranged from 130 - 174% of the prechallenge level, with a mean of 158 ± 24% for glucose uptake and from 102 - 220% with a mean of 170 ± 61% for lactate production. This peak lasted for one day. The metabolic rates returned to near prechallenge level on day 2 but rose again to reach a maximum on day 3. From day 4 onwards
FIGURE 5.5: The secondary response to Salmonella muenchen organisms - the rate of glucose uptake and lactate production by lymphocytes collected at various times during the response.

The upper part of the figure shows the percentages of neutrophils (-----) and blast cells (——) in lymph. The lower part shows the changes in rates of glucose uptake (●) and lactate production (▲) throughout the response. The arrow indicates the time of antigen administration.
The rates declined and returned quickly to prechallenge levels. The magnitude of the peak on Day 3 ranged from 120 - 160% of the prechallenge level with a mean of 140 ± 13% for glucose uptake and from 130 - 190% with a mean of 160 ± 12% for lactate production.
the rates declined and returned quickly to prechallenge levels. The magnitude of the peak on day 3 ranged from $120 - 166\%$ of the prechallenge level with a mean of $144 \pm 25\%$ for glucose uptake and from $134 - 195\%$ with a mean of $156 \pm 33\%$ for lactate production.
SECTION C

RADIOESPIROMETRIC STUDIES ON LYMPHOCYTES COLLECTED FROM POPLITEAL EFFERENT LYMPH DURING AN IMMUNE RESPONSE TO SALMONELLA MUNCHEN ORGANISMS
Radiorespirometry is a technique which enables information to be obtained on the kinetic aspects of metabolic pathways (Wang, Gregg, Forbusch, Christensen and Cheldelin, 1956; Wang, Stern, Gilmour, Klungsoyr, Reed, Bialy, Christensen and Cheldelin, 1958). The method involves measurement of the production of radioactive CO$_2$ by cells metabolizing radioactive substrates.

Although the respiratory activity of biological systems can be studied with either radiorespirometric or manometric methods, the latter method is concerned with the measurement of O$_2$ uptake and CO$_2$ production by the material under investigation, while the former method is concerned with the rate and extent to which $^{14}$C-labelled substrate (e.g. specifically labelled glucose) is converted to respiratory CO$_2$. With manometric methods, measurements can be made only when the biological material is incubated in a medium in which the carbonaceous substrate under study is the sole carbon source. This imposes an "unphysiological" restriction on the material under study. With radiorespirometric methods measurement of the production of respiratory CO$_2$ from a $^{14}$C-labelled substrate can be made in a much more complete medium containing all the necessary nutritional requirements. Respiratory CO$_2$ data obtained from manometric studies represent the total CO$_2$ production from all metabolizable substrates. There is no possible way of identifying the net contribution of individual carbon atoms of a particular substrate to the total CO$_2$ output. In radiorespirometric studies, by using $^{14}$C-specifically labelled
substrates, it is possible to determine the rate and extent of conversion of the individual carbon atoms of the substrate to respiratory $^{14}\text{CO}_2$, and from these data, determine the metabolic pathways through which the catabolism of the substrates has taken place (Wang, 1967; 1972).

The design of radiorespirometric apparatus varies depending on the type of biological system under study, the aim of the experiment, the availability of equipment and the ingenuity of the investigator (Wang et al., 1958; Wang and Krackov, 1962; Wang, 1967; Saba and Di Luzio, 1966; Wang, 1972; Duncombe, 1974; Tsan et al., 1976). The components of any such system are a respiration chamber, a system to enable a stream of gas to be passed through the respiration chamber at a prescribed rate directly into a CO$_2$ trap or an ion chamber and a CO$_2$ trap properly designed to absorb the CO$_2$ in the stream of gas coming from the respiration chamber. In the present investigations the design of Duncombe (1974) was adopted (see Chapter 2).

The original radiorespirometric experiments described by Wang et al. (1956; 1958) were studies in which cells or microorganisms were incubated with comparatively large amounts of labelled substrates for long periods of time (up to 15 hr). CO$_2$ was sampled at intervals of 0.5-1hr. With this experimental approach the quantitative evaluation of the results is complicated by the fact that labelled metabolites will be recycled in the system, the viability of the cells may deteriorate during incubation, the cell population may change and contamination may occur with bacteria and inhibitory products may accumulate in the medium. The experimental system designed by Duncombe, on the
other hand, allowed a small amount of labelled substrate to be metabolized rapidly while the appearance of any resulting $^{14}$CO$_2$ could be followed closely. With this experimental approach there is effectively a pulse of labelled material passing along the metabolic pathways, rather than a continual flow from a large exogenous supply.

A number of requirements have to be fulfilled for the experimental design to fulfil this proposition. The penetration of the labelled substrate into the biological system should be rapid. The labelled substrate should be present in a low enough concentration, and the amount of biological material large enough for the substrate to be substantially metabolized during the course of a short incubation period. Any $^{14}$CO$_2$ formed should be released rapidly from the medium and it should be possible to follow the changes that occur in the radioactivity in the effluent gas stream accurately and rapidly. The above requirements were more or less fulfilled in the present investigations.

The rate of carbohydrate metabolism in sheep lymphocytes was relatively slow and the number of cells available in the lymph from a single lymph node was limited. Thus it can be seen from Figure 5.6 that the complete release of radioactive CO$_2$ from substrates did not occur until the incubation was carried out for 8 hr at a cell concentration of $100 \times 10^6$ cells/ml and a labelled substrate concentration of 8.5 µM. This number of cells was about the limit available per day in the popliteal lymph from a single node. An 8 hr incubation period was not desirable for the reasons mentioned above.
FIGURE 5.6: Oxidation of glucose labelled at different carbon atoms by sheep lymphocytes.

The upper part of the figure shows the time-course of oxidation of the C-1 (▲) and C-6 (△) atoms of glucose. The incubation temperature was 37°C. The glucose concentration was 8.5 µM, and 1 µCi of radioactivity was used for each experiment. The lower part shows the ratio of C-1/C-6 glucose carbon atoms oxidized, calculated from the results of the upper figure. Each point represents the ratio of the cumulative values for C-1 and C-6 atoms oxidized to that time.
In order to gain an undisturbed picture of the metabolism of different substrates, the duration of the corresponding experiments was restricted to short time periods, and the sampling continued until there was a complete utilization of the test substances in the reaction mixture. Cumulative count-rate activity in the media, collected in these periods, was used as an indication of the rate and extent of metabolism of the various substrates through specific metabolic pathways. The qualitative evidence of the metabolic pathways was obtained in the experiments, during which was constant the pigment of the cells. In addition, it was observed that the early stages of utilization of the labelled substrates were characterized by oxidation that was not statistically different. Furthermore, experiments with $\text{C}_4$-labeled glucose showed that the experiments with $\text{C}_6$-labeled glucose are not significantly different and that the amount of cells that were not metabolically active, or that already utilized these differently labeled substrates, was found to be about 1% regardless of the pathway. Nevertheless, time periods were chosen on the basis of the fact that the samples had a sufficiently high radioactivity. In the samples, the activity could be outlined with a cell density of 1 x $10^5$ cells per ml of inoculation medium. The sampling frequencies were checked to avoid contamination.
In order to gain an undistorted picture of the metabolism of the different substrates, the duration of the radiorespirometric experiments was restricted to short periods without waiting until there was a complete release of $^{14}\text{C} \text{CO}_2$ from the reaction mixture. Cumulative counts of radioactivity in the $^{14}\text{C} \text{CO}_2$ collected in these periods were used as an indication of the rate and extent of metabolism of the various substrates through specific metabolic pathways. Since only a qualitative account of the metabolic pathways was sought in the present investigations, this experimental approach was considered justifiable. Axelrod (1967) emphasized that the most significant information was obtained during the early stages of oxidation of the labelled substrates. It is obvious in studies of C-1- and C-6-labelled glucose oxidation that if oxidation was followed to completion, the C-1/C-6 ratio would be 1 regardless of the pathway. Thus it was decided that experiments with D-[1-$^{14}$C]glucose should be carried on for ½ hr and experiments with D-[6-$^{14}$C] glucose for 2 hr. These time periods were chosen on the basis that they were relatively short and that the amount of radioactive CO$_2$ derived from these 2 differently labelled glucose molecules was more or less logarithmically linear with time. The success of the experiments depended on a sufficiently high radioactivity in $^{14}\text{CO}_2$ samples. This could be obtained with a cell concentration of $50 \times 10^6$ cells/ml ($= 1 \times 10^8$ cells/2 ml incubation medium) and sampling frequencies
of 15 min for C-1 and 30 min for C-6-labelled glucose.

In the present investigations, the comparative yields of $^{14}\text{CO}_2$ from C-1- and C-6-labelled glucose were studied to provide information about the relative significance of the Embden-Meyerhof-Parnas glycolytic pathway plus the Krebs cycle and the pentose phosphate pathway in the oxidation of glucose by lymphocytes. The glycolytic sequence and the Krebs cycle yielded $^{14}\text{CO}_2$ from both types of labelled glucose at equal initial rates whereas the pentose phosphate pathway initially yielded $^{14}\text{CO}_2$ only from C-1-labelled glucose (Axelrod, 1967; Duncombe, 1974; Lehninger, 1975). In Figure 5.6 the cumulative C-1/C-6 ratio in $^{14}\text{CO}_2$ has been plotted against time. The ratio which was more than 30 during the first 30 min of incubation came down rapidly to 2.76 over a period of 2 hr suggesting a comparatively small contribution from the pathway preferentially oxidizing C-1 of glucose - the pentose phosphate pathway. Thus the cumulative radioactivity of $^{14}\text{CO}_2$ collected from D-[1-$^{14}$C]glucose was used as an index of glucose oxidation via the pentose phosphate pathway while the cumulative radioactivity from D-[6-$^{14}$C]glucose was used as an index of glucose oxidation via glycolysis and the Krebs cycle. Attempts were made to make use of cumulative $^{14}\text{CO}_2$ yields from C-1- and C-6-labelled glucose, measured at the time the substrate was exhausted (about 8 hr incubation), to estimate the fraction of the labelled glucose that was being catabolized by way of the glycolytic pathway and the pentose phosphate pathway (Wang, 1972). These attempts were unsuccessful and large variations were observed in the experimental results.
In order to assess the efficiency of $^{14}\text{CO}_2$ absorption by Carbo-Sorb II and to make sure that the respiratory $\text{CO}_2$ was completely trapped when the rate of air-flow was 200 ml/min, 2 trains of 3 absorption vials were made up in series connected by glass tubing sealed through air-tight caps. The effluent gas from an incubation flask containing the respiring cells in a medium with the usual amount of D-[1-$^{14}\text{C}$]glucose was bubbled through each absorber in turn. Absorption was continued in each vial for 15 min at a gas flow rate of 200 ml/min. The result of this experiment showed that 99.5% of the $^{14}\text{CO}_2$ was absorbed in the first vial (Table 5.2). The counts obtained in the second and the third vials were not significantly above background. Thus it was concluded that a single vial would be adequate for $^{14}\text{CO}_2$ absorption and this was used in all subsequent experiments.

The cells used in the radiorespirometric studies were washed and incubated in a buffer solution free of "cold" glucose. This was found to be important otherwise the $^{14}\text{C}$-labelled glucose (approximately 8.50 μM) which was introduced subsequently in the experiment became too diluted, making the collection of a sufficiently high number of counts over short periods of incubation very difficult. The dilution effect is illustrated by the results of the experiment shown in Figure 5.7 which was done to follow the release of $^{14}\text{CO}_2$ from D-[1-$^{14}\text{C}$]glucose in two separate incubation flasks, one with 5.56 mM of added "cold" glucose and the other without any added cold glucose.
<table>
<thead>
<tr>
<th>Time after cell addition (min)</th>
<th>c.p.m./vial</th>
<th>% of total $^{14}$CO$_2$ absorbed by vial 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>12936</td>
<td>47</td>
</tr>
<tr>
<td>30</td>
<td>26782</td>
<td>48</td>
</tr>
<tr>
<td>45</td>
<td>23018</td>
<td>45</td>
</tr>
<tr>
<td>60</td>
<td>17490</td>
<td>41</td>
</tr>
</tbody>
</table>

TABLE 5.2: The efficiency of $^{14}$CO$_2$ absorption by Carbo-Sorb II.

Trains of 3 absorption vials, each containing 9 ml of Carbo-Sorb II were made up in series connected by glass tubing sealed through airtight screw caps. The effluent gas from an incubation flask with cells respiring in a medium containing 1 µCi D-[1-$^{14}$C]glucose was bubbled through each absorber in turn. Absorption into each vial was continued for 15 min at a gas flow rate of 200 ml/min. The radioactivity of the $^{14}$CO$_2$ collected in each vial was then determined.
FIGURE 5.7: The dilution effect of added "cold" glucose in radiorespirometric measurements.

D-[1-¹⁴C]glucose (1 μCi) was used in each experiment at a concentration of 8.5 μM. A total of 10⁸ cells were incubated in Krebs & de Gasquet buffer with (◊) or without (♦) "cold" glucose of 5.56 mM.
The concentration effect that was demonstrated for spread lymphocytes in the studies on glucose uptake was also observed in the radiometric studies shown in Figure 5.6. In these experiments lymphocyte suspensions were prepared at different cell concentrations and allowed to respire in incubation flasks containing the same quantity of $\text{D-[1-14C]}$glucose for the same period. At varying cell concentrations below $15 \times 10^5$ cells/ml, there was a sharp rise in the $\text{CO}_2$ production rate and values between 2.5 - 17.75 c.p.m./10^6 cells were observed with concentrations higher than $40 \times 10^5$ cells/ml. However, the effect of cell concentration on the rate of $\text{CO}_2$ production was not critical. It was decided that the cell concentration of 50 $\times 10^5$ cells/10^6 ml for subsequent experiments involving radiometric studies was optimal. The chapter briefly explained that for cooperativity the observed production rates (c.p.m.) were uncorrected at any one time point in the experiment depicted in Table 5.3 in which sample pairs with different normal standardization and their respective efficiencies were compared. The results of the experiment showed that the assumptions were valid.

The release of $\text{CO}_2$, $\text{OH}^-$, and $\text{H}_2\text{O}$ was measured in response to $\text{D}-[\text{1-14C}]$glucose. The cumulative $\text{CO}_2$ activity produced by lymphocytes incubated with $\text{D-[1-14C]}$glucose and $\text{D-[16-14C]}$glucose before antigenic challenge was within the range of 15 - $25 \times 10^3$ c.p.m./0.5 hr/10^6 cells for C-3 and 5 - 30 x $10^3$ c.p.m./hr/10^6 cells for C-65. On day 1 following either primary or secondary antigenic challenge...
The concentration effect that was demonstrated for sheep lymphocytes in the studies on glucose uptake was also observed in the radiorespirometric studies shown in Figure 5.8. In these experiments lymphocyte suspensions were prepared at different cell concentrations and allowed to respiration in incubation flasks containing the same quantity of D-[1-\(^{14}\)C]glucose for the same period of time. At cell concentrations below 15 \times 10^6 \text{ cells/ml}, there was a steep rise in the \(^{14}\text{CO}_2\) production rate and values between 2.00 - 17.75 \times 10^2 \text{ c.p.m./10^6 cells were observed. At cell concentrations higher than 40 \times 10^6 \text{ cells/ml}, the effect of cell concentration on the rate of \(^{14}\text{CO}_2\) production was not so critical. It was decided to use a cell concentration of 50 \times 10^6 \text{ cells/ml for all subsequent experiments involving radiorespirometric studies.}

In Chapter 2 it was explained that for comparative purposes the observed counting rates (c.p.m.) were used uncorrected as quenching was almost identical in all samples. This assumption was tested in the experiment depicted in Table 5.3 in which samples with different counting rates were subject to internal standardization and their respective efficiencies were compared. The result of the experiment showed that the assumption was valid.

(b) \text{THE RELEASE OF} \(^{14}\text{CO}_2\) \text{ FROM C-1- AND C-6-LABELLED GLUCOSE DURING AN IMMUNE RESPONSE TO SALMONELLA MUENCHEN ORGANISMS}

The cumulative \(^{14}\text{CO}_2\) radioactivity produced by lymphocytes incubated with D-[1-\(^{14}\)C]glucose and D-[6-\(^{14}\)C]glucose before antigenic challenge was within the range of 15 - 25 \times 10^3 \text{ c.p.m./0.5 hr/10}^8 \text{ cells for C-1 and 5 - 20 \times 10^3 \text{ c.p.m./2hr/10}^8 \text{ cells for C-6. On day 1 following either primary or secondary antigenic challenge...}
FIGURE 5.8: The effect of cell concentration on the release of $^{14}\text{CO}_2$ from labelled glucose by sheep lymphocytes.

Lymphocytes collected from popliteal efferent lymph were incubated with D-[1-14C]glucose (1 µCi, 8.5 µM) at various cell concentrations.
The data in Table 5.2 represent the radioactivity levels measured in a series of samples obtained from radioreceptor experiments. Each sample was exposed to a standard concentration of labeled hormone, and the radioactivity was determined using a scintillation counter. The data were then used to calculate the binding affinity of the receptor for the hormone.

The graph illustrates the relationship between cell concentration and radioactivity. The x-axis represents the cell concentration (cells x 10^6/ml), while the y-axis represents the radioactivity (CPM x 10^2/20 min/10^6 cells). The data points show a clear decrease in radioactivity as the cell concentration increases, indicating a saturable binding capacity of the receptor.

The table at the bottom of the page lists the sample number, cell concentration, and the corresponding radioactivity values. The mean ± S.D. for the radioactivity is provided, indicating the variability in the measurements.
<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Sample c.p.m.</th>
<th>(Int. standard + sample) c.p.m.</th>
<th>% efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3605</td>
<td>14024</td>
<td>24.0</td>
</tr>
<tr>
<td>2</td>
<td>3787</td>
<td>12160</td>
<td>19.3</td>
</tr>
<tr>
<td>3</td>
<td>5226</td>
<td>14703</td>
<td>21.8</td>
</tr>
<tr>
<td>4</td>
<td>6522</td>
<td>15089</td>
<td>19.7</td>
</tr>
<tr>
<td>5</td>
<td>8277</td>
<td>17584</td>
<td>21.4</td>
</tr>
<tr>
<td>6</td>
<td>12646</td>
<td>20420</td>
<td>17.9</td>
</tr>
<tr>
<td>7</td>
<td>13778</td>
<td>23543</td>
<td>22.5</td>
</tr>
<tr>
<td>8</td>
<td>20269</td>
<td>29662</td>
<td>21.6</td>
</tr>
<tr>
<td>9</td>
<td>20318</td>
<td>28918</td>
<td>19.8</td>
</tr>
<tr>
<td>10</td>
<td>24344</td>
<td>33804</td>
<td>21.8</td>
</tr>
</tbody>
</table>

**Mean ± S.D. = 21.0 ± 1.8**

**TABLE 5.3:** The internal standardization of samples obtained from radiorespirometric experiments.

Random samples obtained from radiorespirometric experiments were subjected to internal standardization. The samples were first counted to determine their respective c.p.m. A fixed amount of internal standard (50 µl of $^{14}$C-hexadecane at 8.68 x $10^5$ d.p.m./ml) was added to each sample which was then recounted. The table shows the counting efficiency for each sample.
(Figure 5.9, Figure 5.10) there was always a large increase in the production of $^{14}$CO$_2$ from D-[1-$^{14}$C]glucose. The radioactivity produced from the metabolism of D-[6-$^{14}$C]glucose changed very little during this time. The pattern of $^{14}$CO$_2$ production in the following days of the responses was very similar to that observed in the experiments on glucose uptake, lactate production and gaseous exchange. Maximum rates of $^{14}$CO$_2$ production from C-1- and C-6-labelled glucose occurred on around day 4 of the primary response and on around day 3 of the secondary response and this always coincided with the peak of the blast cell response in the lymph. The rates of $^{14}$CO$_2$ production then decreased as the immune response died away over the next few days.
FIGURE 5.9: The primary response to Salmonella muenchen organisms - the rate of production of $^{14}$CO$_2$ from labelled glucose by lymphocytes collected at various times during the response.

The upper part of the figure shows the percentages of neutrophils (-----) and blast cells (-----) in the lymph. The lower part shows the production of $^{14}$CO$_2$ from D-[1-$^{14}$C]glucose (●) and from D-[6-$^{14}$C]glucose (■) over the response. The arrow indicates the time of antigen administration.
FIGURE 5.10: The secondary response to Salmonella muenchen organisms - the rate of production of $^{14}\text{CO}_2$ from labelled glucose by lymphocytes collected at various times during the response.

The upper part of the figure shows the percentages of neutrophils (- - -) and blast cells (-----) in the lymph. The lower part shows the production of $^{14}\text{CO}_2$ from D-[1-$^{14}$C]glucose (●) and from D-[6-$^{14}$C]glucose (■) over the response. The arrow indicates the time of antigen administration.
RADIOACTIVITY (CPM × 10³/hr/10⁸ CELLS)

% NEUTROPHILS

% BLASTS

DAYS AFTER CHALLENGE

THE EFFECT OF STRESS TENSION ON THE METAMORPHOSIS OF LEPHYESHES
**SECTION D**

**THE EFFECT OF OXYGEN TENSION ON THE ENERGY METABOLISM OF LYMPHOCYTES**

The effect of \( \text{PO}_2 \) on the rates of energy metabolism as indicated by glucose uptake, lactate production and release of \(^{14} \text{CO}_2\) from \(^{14} \text{C}\)-labeled glucose was studied with lymphocytes collected from pooled, different lymph before antigenic challenge and at the peak of the blast cell response. The gas mixtures used to establish different \( \text{PO}_2 \) in the incubation medium were: 0\% \( \text{O}_2/96\% \text{N}_2 \), 12\% \( \text{O}_2/88\% \text{N}_2 \), 72\% \( \text{O}_2/28\% \text{N}_2 \), 72\% \( \text{O}_2/96\% \text{N}_2 \), and 100\% \( \text{O}_2 \).

In the studies on glucose uptake and lactate production, the cells were placed in Warburg flasks attached to their manometers. The cells were incubated at 37°C with gentle shaking for 15 min after which the 2-way stopcock of the manometer and the stopper of the sidearm of the Warburg flask were closed so that the system was air-tight and the cells metabolizing under the \( \text{PO}_2 \) of the specific gas mixture. It was established in preliminary experiments that flushing the flasks with the gas mixtures for 15 min gave identical results to flushing for the whole period of 2 hr. Exactly 1 hr after the first sample was taken the reaction was stopped and a second sample obtained.

The rates of glucose uptake and lactate production by cells metabolizing under different \( \text{PO}_2 \) levels were then evaluated.

**THE EFFECT OF OXYGEN TENSION ON THE UPTAKE OF GLUCOSE AND THE PRODUCTION OF LACTATE BY LYMPHOCYTES**

Table 8.4b summarizes the rates of glucose uptake and lactate production under different \( \text{PO}_2 \) conditions by lymphocytes collected from the pooled, different lymph before antigenic challenge and at the peak of the blast cell response following...
(a) **EXPERIMENTAL**

The effect of $P_{O_2}$ on the rates of energy metabolism as indicated by glucose uptake, lactate production and release of $^{14}$CO$_2$ from C-1- and C-6-labelled glucose was studied with lymphocytes collected from popliteal efferent lymph before antigenic challenge and at the peak of the blast cell response. The gas mixtures used to establish different $P_{O_2}$ in the incubation medium were air, 12% O$_2$/88% N$_2$, 7% O$_2$/93% N$_2$, 2% O$_2$/98% N$_2$ and 100% N$_2$.

In the studies on glucose uptake and lactate production, the cells were placed in Warburg flasks attached to their manometers. The cells were incubated at 37°C with gentle shaking for 10 min before the first samples were taken. Gas mixtures of specific O$_2$ content were then passed through the system for 15 min after which the 3-way stopcock of the manometer and the stopper of the sidearm of the Warburg flask were closed so that the system was air-tight and the cells metabolizing under the $P_{O_2}$ of the specific gas mixture. It was established in preliminary experiments that flushing the flasks with the gas mixtures for 15 min gave identical results to flushing for the whole period of 2 hr. Exactly 2 hr after the first sample was taken the reaction was stopped and a second sample obtained. The rates of glucose uptake and lactate production by cells metabolizing under different $P_{O_2}$ levels were then evaluated.

(b) **THE EFFECT OF OXYGEN TENSION ON THE UPTAKE OF GLUCOSE AND THE PRODUCTION OF LACTATE BY LYMPHOCYTES**

Table 5.4a summarizes the rates of glucose uptake and lactate production under different $P_{O_2}$ conditions by lymphocytes collected from the popliteal efferent lymph before antigenic challenge and at the peak of the blast cell response following
TABLE 5.4: The effect of oxygen tension on the metabolism of glucose in sheep lymphocytes.

Lymphocytes were collected from popliteal efferent lymph before antigenic challenge or at the peak of the blast cell response following challenge. The rates of (a) glucose uptake and lactate production and (b) the release of $^{14}$CO$_2$ from C-1- and C-6-labelled glucose were determined with the cells incubated under different gas mixtures. The mean value ± standard deviation of 5 determinations is given for glucose uptake and lactate production. For radiorespirometric studies, the mean value ± standard deviation was calculated from the results of triplicate measurements.
<table>
<thead>
<tr>
<th>Gas Mixture</th>
<th>Glucose uptake (µmoles/hr/2 x 10^8 cells)</th>
<th>Lactate production (µmoles/hr/2 x 10^8 cells)</th>
<th>Radiorespirometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before challenge</td>
<td>After challenge</td>
<td>Before challenge</td>
</tr>
<tr>
<td>Air</td>
<td>0.949 ± 0.349</td>
<td>1.544 ± 0.092</td>
<td>1.336 ± 0.238</td>
</tr>
<tr>
<td>12% O₂/88% N₂</td>
<td>1.370 ± 0.242</td>
<td>1.562 ± 0.098</td>
<td>1.567 ± 0.165</td>
</tr>
<tr>
<td>7% O₂/93% N₂</td>
<td>1.109 ± 0.398</td>
<td>1.629 ± 0.251</td>
<td>1.527 ± 0.342</td>
</tr>
<tr>
<td>2% O₂/98% N₂</td>
<td>1.561 ± 0.199</td>
<td>1.876 ± 0.020</td>
<td>1.833 ± 0.221</td>
</tr>
<tr>
<td>100% N₂</td>
<td>2.660 ± 0.400</td>
<td>3.408 ± 0.002</td>
<td>4.641 ± 0.188</td>
</tr>
</tbody>
</table>

(a) Glycolysis

(b) Radiorespirometry

[1-¹⁴C]glucose (c.p.m./0.5hr/10^8 cells)
[6-¹⁴C]glucose (c.p.m./2hr/10^8 cells)
challenge. The rates of glucose uptake and lactate production by normal lymphocytes increased as $P_{O_2}$ of the atmosphere under which the cells were metabolizing decreased from 21% $O_2$ down to 0% $O_2$. The increases in the rates of glucose uptake and lactate production occurred gradually over the range of 21% to 2% $O_2$ but rapidly from 2% to 0% $O_2$. The cells collected at the peak of the blast cell response reacted to the different $P_{O_2}$ levels in the same manner as the normal unstimulated cells. The rates of glucose uptake and lactate production by these cells however were higher than those of normal lymphocytes at all $P_{O_2}$ values.

(c) THE EFFECT OF OXYGEN TENSION ON THE RELEASE OF $^{14}C O_2$ FROM LABELLED GLUCOSE BY LYMPHOCYTES

As shown in Table 5.4b, the effect of $P_{O_2}$ on the release of $^{14}C O_2$ from C-1- and C-6-labelled glucose by lymphocytes was the reverse to that observed in the glucose and lactate studies, i.e. the amount of $^{14}C O_2$ released from labelled glucose decreased with decreasing $P_{O_2}$. Again the effect was most dramatic at zero $P_{O_2}$. The production of radioactive $CO_2$ was significantly higher in populations containing blast cells at all $P_{O_2}$ values.
SECTION E

DISCUSSION AND CONCLUSIONS

(a) THE CONCENTRATION OF GLUCOSE IN LYMPH AND IN THE INCUBATION MEDIA

It was determined that at concentrations of 2.0 to 4.0 mM glucose, the availability of glucose was not limiting for lymphocytes. Netto et al. (1981) reported a similar finding. The concentration of glucose in popliteal efferent lymph which varied between 3.67 - 4.70 mM was sufficient to ensure that lymphocytes existed in vivo in an environment that was saturated with respect to glucose as a substrate for glycolysis. It was considered that the rates of glucose metabolism determined in vitro were maximal and not dependent on the concentration of this substrate.

The production of lactate by lymphocytes in the absence of added glucose was determined. This finding suggested that lymphocytes do not contain appreciable amounts of endogenous glucose and do not convert any significant amounts of glycogen to glucose-6-phosphate.

The fall in glucose concentration in the lymph which occurred shortly after challenge was correlated with the transient appearance of a large number of neutrophils in the lymph which have a high rate of consumption of glucose. There was no change in the glucose concentration in peripheral blood at this time.

(b) THE GLYCOLYTIC ACTIVITY OF LYMPHOCYTES RESPONDING TO SALMONELLA SPLEEN ORGANISMS

From the rates of glucose uptake and lactate production it could be calculated that a large proportion (about 57%) of the glucose taken up by lymphocytes was converted into lactate. These results are compared to those calculated from experiments by others (Table 1). The high degree of glycolysis by lymphocytes...
(a) THE CONCENTRATION OF GLUCOSE IN LYMPH AND IN THE INCUBATION MEDIA

It was determined that at concentrations of 3.0 - 4.0 mM glucose, the availability of glucose was not limiting for lymphocytes. Rauch et al. (1961) reported a similar finding. The concentration of glucose in popliteal efferent lymph which varied between 3.52 - 4.70 mM was sufficient to ensure that lymphocytes existed in vivo in an environment that was saturated with respect to glucose as a substrate for glycolysis. It was considered that the rates of glucose metabolism determined in vitro were maximal and independent of the concentration of this substrate.

The production of lactate by lymphocytes in the absence of added glucose was found to be close to zero. This finding suggested that lymphocytes do not contain appreciable amounts of endogenous glucose and do not convert any significant amounts of glycogen to glucose-6-phosphate.

The fall in glucose concentration in the lymph which occurred shortly after challenge was correlated with the transient appearance of large numbers of neutrophils in the lymph which have a high rate of consumption of glucose. There was no change in the glucose concentration in peripheral blood at this time.

(b) THE GLYCOLYTIC ACTIVITY OF LYMPHOCYTES RESPONDING TO SALMONELLA MUECHEN ORGANISMS

From the rates of glucose uptake and lactate production it could be calculated that a large proportion (about 67%) of the glucose taken up by lymphocytes was converted into lactate. These results are compared to those calculated from experiments by others (Table 5.5). The high degree of glycolysis by lymphocytes
<table>
<thead>
<tr>
<th>Reference</th>
<th>Source of lymphocytes</th>
<th>Glucose uptake (µmoles/hr/2 x 10^8 cells)</th>
<th>Lactate production (µmoles/hr/2 x 10^8 cells)</th>
<th>Lactate/(2 x glucose) x 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human venous blood</td>
<td>-</td>
<td>13.44</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Human venous blood</td>
<td>1.416</td>
<td>3.500</td>
<td>124%</td>
</tr>
<tr>
<td>3</td>
<td>Human venous blood</td>
<td>1.240</td>
<td>1.900</td>
<td>77%</td>
</tr>
<tr>
<td>4</td>
<td>Horse venous blood</td>
<td>2.244</td>
<td>2.760</td>
<td>62%</td>
</tr>
<tr>
<td>5</td>
<td>Human venous blood</td>
<td>-</td>
<td>0.828</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Rat thymus</td>
<td>0.278</td>
<td>0.220</td>
<td>40%</td>
</tr>
<tr>
<td>7</td>
<td>Sheep popliteal lymph</td>
<td>0.970</td>
<td>1.306</td>
<td>67%</td>
</tr>
</tbody>
</table>

**TABLE 5.5:** The rates of glucose uptake and lactate production of lymphocytes isolated from various sources.

The rates on the table are calculated from data reported in the literature.

Reference: 1. Frei et al., 1961
2. Rauch et al., 1961
3. Hedeskov and Esmann, 1966
4. Pachman, 1967
5. Roos and Loos, 1973
6. Culvenor and Weidemann, 1976
7. Present investigations
in popliteal lymph would assist these cells to survive under conditions of low \(P_02\) that might occur in lymph under certain conditions, particularly in peripheral limb tissues. It was observed that both the rates of uptake of glucose and the production of lactate were stimulated after antigenic challenge. Maximum rates were observed at the peak of the blast cell response in the lymph and this indicated that glycolysis in blast cells was significantly greater than in small lymphocytes, a finding which agreed with the results of other in vitro studies (Chapter 1). This finding suggested that aerobic glycolysis was an important energy yielding reaction in lymphocytes undergoing transformation and proliferation. The observations were made on cells metabolizing in medium equilibrated with air, but the conclusions from these studies is applicable to physiological conditions where the \(P_02\) was around 7\% \(O_2\) (Chapter 3 and 4) as judged from the results reported in Section D of this chapter.

The present investigations also demonstrated that the uptake and oxidation of glucose decreased with increasing cell concentrations, a phenomenon that has been observed previously by other workers (Barron and Harrop, 1929; Hedeskov and Esmann, 1966; Pachman, 1967; Sand, Condie and Rosenberg, 1977). This effect, together with the limitation in cell output from popliteal efferent lymph, determined the number of lymphocytes that could be used in the present experiments. The effect of cell concentration on metabolism has been claimed to be due to the release of humoral factors which regulate the metabolic rates of the cells (Sand et al. 1977).
The radiorespirometric experiments were carried out for short periods of not more than 2 hr, so as to obtain a qualitative assessment of the metabolic pathways through which glucose was oxidized. In these studies the requirement for substrate saturation was not met and the assumption was made that the relative rates of $^{14}$CO$_2$ release from the labelled substrates indicated the relative rates of oxidation through the different pathways. The reproducibility of the experimental results and the good agreement of these results with those reported by other workers using other systems seemed to justify the above assumption.

Much higher initial rates of $^{14}$CO$_2$ production from D-[1-$^{14}$C]glucose occurred in comparison to $^{14}$CO$_2$ production from D-[6-$^{14}$C]glucose and this suggested that the pentose phosphate pathway, as well as the glycolytic pathway plus the Krebs cycle were active in lymphocytes. If only glycolysis and the Krebs cycle were operating in concert, the initial rate of evolution of $^{14}$CO$_2$ from the 2 isotopes would be the same. This finding was in agreement with that of MacHaffie and Wang (1964) and Tsan et al. (1976). Both these two metabolic pathways were stimulated and operated at enhanced rates in lymphocytes undergoing transformation in vivo since maximal rates of production of $^{14}$CO$_2$ from labelled glucose were observed in cell populations containing large numbers of blast cells. This was consistent with in vitro findings (Sagone et al., 1974).
Since the rate of $^{14}\text{CO}_2$ production was affected by the 
$\text{PO}_2$ of the gas phase with which the incubation medium was 
equilibrated, the magnitude of the $^{14}\text{CO}_2$ production rates 
observed with air would overestimate the physiological rates 
that would pertain in the lymph where the $\text{PO}_2$ is around 7% $\text{O}_2$.

In qualitative terms the metabolic response of blast cells to 
different $\text{PO}_2$ levels was similar to small lymphocytes so the 
conclusions from radiorespirometric studies carried out under 
air were considered relevant to physiological conditions.

(d) THE EFFECT OF OXYGEN TENSION ON THE ENERGY METABOLISM
OF LYMPHOCYTES

The capacity of lymphocytes to respond with increased 
glycolysis in an anaerobic environment (Pasteur effect) has 
been studied by many workers (Barron and Harrop, 1929; Helmreich 
and Eisen, 1959; Frei et al., 1961; Hedeshkov and Esmann, 1966; 
Pachman, 1967; Suter and Weidemann, 1975). Thomas, Neptune 
and Sudduth (1963) studied in detail the effect of $\text{O}_2$ on the 
metabolism of D-glucose by dispersed rat brain tissue using $\text{O}_2$ 
pressures of zero, 1 and 5 atmospheres. Haugaard, Hess and 
Itskovitz (1957) also studied glucose oxidation by rat heart 
homogenate at different concentrations of $\text{O}_2$, including 4%, 
7.4%, 21% (air) and 100%. They reported the rates of $\text{O}_2$ uptake 
by the cells at these $P_{\text{O}_2}$ values but mentioned nothing about the 
respective rates of glucose uptake or lactate production. The 
Pasteur effect was studied in the present investigations both on 
normal and stimulated cells and was found to occur in both $P_{\text{O}_2}$ 
populations of cells. Studies of the effect of different $P_{\text{O}_2}$ 
values from 0% to 21% $\text{O}_2$ on glycolysis and the oxidation of
C-1- and C-6-labelled glucose showed that each of these parameters was affected by changing the \( P_{O_2} \) of the gas phase under which the cells were metabolizing. The effect was not quantal. Bergofsky et al. (1962) claimed that cells continue to operate aerobically as long as the ambient \( P_{O_2} \) exceeds 1 mm Hg. In the present experiments there was a close relationship between the rate of each metabolic activity of the cell and the \( P_{O_2} \).

The glycolytic pathway plus the Krebs cycle were affected more by decreasing \( P_{O_2} \) than was the pentose phosphate pathway (Table 5.4). Cells incubated under a gas phase of \( N_2 \) produced about 10\% of the amount of \(^{14}\text{C}O_2 \) from C-6-labelled glucose as did cells under air while for C-1-labelled glucose the amount was around 40\%. Thus when the \( P_{O_2} \) of the incubation medium decreased, the cells changed their metabolic activity so that their energy was derived to a much larger extent from glycolysis than from oxidative phosphorylation. This was evident when the proportion of glucose being converted to lactate under different \( P_{O_2} \) levels was calculated from the data shown in Table 5.4a. For cells collected at the height of the response the proportion of available glucose converted to lactate increased with decreasing \( P_{O_2} \) from 65\% at 21\% \( O_2 \) to 77\% at 0\% \( O_2 \).

The Pasteur effect is generally accounted for in the regulatory properties of the glycolytic enzyme phosphofructokinase (White, Handler and Smith, 1973). This enzyme controls the committed step of the glycolytic sequence - the phosphorylation of fructose-6-phosphate with the formation of fructose-1,6-diphosphate. The inhibition of this enzyme by ATP, citrate, etc. reflects the operative
effects of oxidative phosphorylation, whereas the stimulation by ADP, AMP and inorganic phosphate reflects the situation when the O₂ supply is deficient. Behm (1974) studying the metabolism of the tape worm Moniezia expansa asserted that PFK did not appear to play a role in limiting the flux of substrate through the glycolytic pathway under aerobic or anaerobic conditions. The explanation that PFK activity regulates the Pasteur effect accords well with the results obtained in present investigations on the effect of PO₂ on energy metabolism of lymphocytes, particularly in relation to glycolysis and Krebs cycle activity.

The above argument explained the observation of increased glycolysis and decreased ¹⁴CO₂ release from C-6-labelled glucose with decreasing PO₂. The effect of PO₂ on the production of ¹⁴CO₂ from C-1-labelled glucose was difficult to explain. Thomas et al. (1963) have studied the effect of O₂ at high pressure on the oxidation of D-[U-¹⁴C]glucose, but nothing is known about the effects of PO₂ on the oxidation of D-[l-¹⁴C]glucose. The operation of the pentose phosphate pathway serves as a source of NADPH, which plays an important role in biosynthetic processes within the cell. The pathway also provides for the synthesis and utilization of pentoses. It cannot be visualized as the consecutive transformation of glucose to 6 molecules of CO₂ and the pathway is usually not related to the supply of energy to the cell - once glucose-6-phosphate is formed there is no further requirement in the pathway for ATP nor is ATP generated. The observation that the operation of the pentose phosphate pathway was dependent on PO₂ cannot be explained in terms of the energy requirements of the cells, as for glycolysis and Krebs cycle activities.
The phenomenon may have to do with the following reactions. Ribose can be produced from the pentose phosphate pathway through the action of phosphoriboisomerase on ribulose-5-phosphate produced by the irreversible oxidative portion of the pathway:

\[
\text{glucose-6-phosphate} \rightarrow \text{ glucono-δ-lactone-6-phosphate} \rightarrow \text{ 6-phosphogluconic acid} \rightarrow \text{ ribulose-5-phosphate} + \text{ CO}_2, \text{ or through the formation of ribose-5-phosphate from fructose-6-phosphate and glyceraldehyde-3-phosphate by way of the reversible nonoxidative reactions catalyzed by transketolase and transaldolase.}
\]

Thus, a cell which can metabolize glucose through the pentose phosphate pathway has the option of producing ribose-5-phosphate by an oxidative route or by a nonoxidative route. Under conditions of reduced \(O_2\) supply, the nonoxidative route would be favoured over the oxidative route. Since the nonoxidative formation of ribose does not involve the reaction catalyzed by 6-phosphogluconic dehydrogenase, no \(CO_2\) is released from C-1-labelled glucose substrate. This may explain the observation that when the \(P_{O_2}\) of the gas phase was reduced, less \(^{14}CO_2\) was collected during the radiorespirometric assay of C-1-labelled glucose metabolism.

If this explanation is correct, then under low \(P_{O_2}/P_{O_2}\) conditions such as occurs in popliteal efferent lymph, the pentose phosphate pathway would be directed towards the formation of ribose through the nonoxidative route. The net amount of ribose formed was not affected but since the oxidative route was inhibited, the amount of NADPH would be decreased. This would certainly affect the synthetic activities of the cell, especially the synthesis of substances such as long-chain fatty acids.
The yield of $^{14}$CO$_2$ from specifically labelled glucose has commonly been used to estimate the contributions of the oxidative route of the pentose phosphate pathway and of the glycolytic pathway and Krebs cycle to glucose metabolism (Wang et al., 1958; Beck, 1958; Wang and Krackov, 1962; Katz and Wood, 1963; Wood, Katz and Landau, 1963; Katz, Landau and Bartsch, 1966; Wang, 1972; Lengle et al., 1978). It was not possible to make this estimate in the present investigations.

The rate of $^{14}$CO$_2$ production from labelled glucose depends on the $P_{O_2}$ of the gas phase used in the experiment and C-1-C-6-labelled glucose metabolism was affected by different $P_{O_2}$ levels to a different extent. Also because of the possible changes that may occur in the pentose phosphate pathway at low $P_{O_2}$ values, the results of estimates of the contribution of these metabolic pathways reported by other workers, using atmospheric air as the gas phase, are likely to be wrong and inapplicable to physiological situations where the $P_{O_2}$ is much lower.
CHAPTER 6

STUDIES ON THE SYNTHETIC ACTIVITIES AND MOTILITY
OF LYMPHOCYTES IN POPLITEAL EFFERENT LYMPH
DURING AN IMMUNE RESPONSE
The process of blastogenesis which is a feature of the response of lymphocytes to antigenic challenge involved the enlargement of the cell and an increase in its protein content and in the mean dry mass. These changes would require an increase in protein synthesis in the transforming cells. The degree of cytoplasmic basophilia increases in lymphocytes during their transformation into blast cells, and this suggests that there is an increase in the cytoplasmic RNA of these cells. As many of the blast cells are frequently observed in mitosis it is also certain that there would be an increase in DNA synthesis in the cells at this stage of their developmental cycle.

Increases in protein synthesis, DNA and RNA synthesis would require an additional supply of energy and of metabolite intermediates. These syntheses were studied in lymphocytes collected during the immune response, in view of their correlation with the changes in energy metabolism reported in previous chapters. The synthesis of protein, DNA and RNA was studied by measuring the extent to which labelled precursor molecules were incorporated into these substances. The distribution of the isotopes between different cell types and within the cells was studied by autoradiography.

A further energy-demanding activity of the cells relates to their migratory capacity within tissues. The extent of locomotion of the different cell types involved in the immune response in lymph was also studied by examining the motility of the different cell types under phase-contrast microscopy.
The process of blastogenesis which is a feature of the response of lymphocytes to antigenic challenge involves the enlargement of the cell and an increase in its protein content and in the mean dry mass. These changes would require an increase in protein synthesis in the transforming cells. The degree of cytoplasmic basophilia increases in lymphocytes during their transformation into blast cells, and this suggests that there is an increase in the cytoplasmic RNA of these cells. As many of the blast cells are frequently observed in mitosis it is also certain that there would be an increase in DNA synthesis in the cells as they replicated their chromosomal DNA.

Increases in protein synthesis, DNA and RNA synthesis would require an additional supply of energy and of metabolic intermediates. These synthetic activities were studied in lymphocytes collected during the immune response to see if they could be correlated with the changes in energy metabolism reported in previous chapters. The synthesis of protein, DNA and RNA was studied by measuring the extent to which tritiated precursor molecules were incorporated into these substances. The distribution of the isotopes between the different cell types and within the cells was studied by autoradiography.

A further energy demanding activity of the cells relates to their migratory capacity within tissues. The extent of locomotion of the different cell types involved in the immune response in lymph was also studied by examining the motility of the different cell types under phase contrast microscopy.
THE INCORPORATION OF $^3$H-THYMIDINE, $^3$H-URIDINE AND $^3$H-LEUCINE INTO LYMPHOCYTES

SECTION B

THE INCORPORATION OF $^3$H-THYMIDINE, $^3$H-URIDINE AND $^3$H-LEUCINE INTO LYMPHOCYTES
DNA synthesis was assayed in lymphocytes collected during immune responses to *Salmonella muenchen* organisms by studying the incorporation of \([\text{methyl-}^3\text{H}]\)thymidine \((^3\text{H-thymidine})\) into nuclear DNA. RNA synthesis was assayed by studying the extent of incorporation of \([5-^3\text{H}]\)uridine \((^3\text{H-uridine})\) into cytoplasmic and nuclear RNA. Protein synthesis was studied by the measurement of \(L-[4, 5-^3\text{H}]\)leucine \((^3\text{H-leucine})\) incorporation into cell proteins.

The incorporation of \(^3\text{H-thymidine}\) into cells is a satisfactory way of determining whether or not lymphocytes are involved in DNA synthesis. Thymidine has been reported to break down slowly on storage and rapidly on incubation with leucocytes at \(37^\circ\text{C}\), to thymine and dihydrothymine. Neither of these breakdown products are utilized as precursors for DNA synthesis (Cooper and Milton, 1964; Milton, Cooper and Hale-Pannenko, 1965). An experiment was done to verify these findings. Lymphocytes were incubated with \(^3\text{H-thymidine}, ^3\text{H-uridine}\) and \(^3\text{H-leucine}\) and harvested at different times after addition of the isotopes. The results of this experiment are shown in Figure 6.1. The incorporation of \(^3\text{H-uridine}\) and \(^3\text{H-leucine}\) was essentially linear throughout the incubation period, at least up to 24 hr. In the case of \(^3\text{H-thymidine}\), incorporation was linear only for the first 4 hr. After this time the rate of incorporation slowed down. This result had been reported previously by Bain (1970). The decreased rate of incorporation, according to Bain, is probably due in part to degradation of the isotope and in part to radiation damage inflicted on the nucleus of the cell. Thus it was preferable to add the isotope to the cell cultures for as short a time as possible. In all subsequent studies into the
FIGURE 6.1: The effect of the time of exposure of cells to $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine on the uptake of these isotopes in 2-day cultures of sheep lymphocytes.

Lymphocytes ($10^6$ cells) from popliteal efferent lymph were incubated with 1.0 µCi of $^3$H-thymidine (○), $^3$H-uridine (△) or $^3$H-leucine (□) under 10% CO$_2$, 7% O$_2$ and balance N$_2$ at 37°C. The cells were harvested after different periods of incubation with the isotopes and the radioactivity incorporated into the cells was determined. The standard deviations for triplicate determinations are shown on the graph as vertical bars on the time points. For those points without the bars, the standard deviations are smaller than the vertical dimension of the symbols.
incorporation of $^{3}H$-thymidine, $^{3}H$-uridine and $^{3}H$-leucine the cells were incubated with the isotope for 4 hr and then harvested.

The $^{3}H$ label in the 5-position of $^{3}H$-thymidine, if subjected to hydrolysis would be lost when incorporated subsequently into DNA (Dayhoff and Ungar, 1969). There is also the possibility that thymidine could be converted to cytidine nucleotides which are incorporated into both DNA and RNA. Given these alternative metabolic possibilities, it is believed that the percentage of $^{3}H$ label in thymidine was lost (unknown). In an unlabelled thymidine or uracil added and in these circumstances not exposed to a concentration of these precursors, the incorporation of $^{3}H$-thymidine or $^{3}H$-uridine into DNA and RNA by different cell populations will only indicate relative rates of DNA and RNA synthesis respectively if the proportion of nucleosides entering the nucleic acids derived from the labelled precursors is constant. This assumption seems to hold (King and Key, 1972).

The F-15 medium used for the cell culture was changed instead of F-15 because it contained less leucine. After 24 hr leucine was added to F-15 the ratio between radioactive leucine (5 uCi) and non-radioactive leucine (500 uCi) would allow for more radioactive counts to be taken up by the cells.
incorporation of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine the cells were incubated with the isotopes for 4 hr and then harvested.

The $^3$H label in the 5-position of [5-$^3$H]uridine, if subjected to methylation would be lost when incorporated subsequently into DNA (Hayhoe and Quaglino, 1965). There is also the possibility that uridine could be converted to cytidine nucleotides which are incorporated into both DNA and RNA (Comings, 1966). In this case the label would be retained and appear in DNA. Given these alternative metabolic possibilities, it was assumed that the percentage of the $^3$H label in $^3$H-uridine that would enter DNA would be very small.

The final concentration of $^3$H-thymidine and $^3$H-uridine in the cultures was 1µmole/ml. No unlabelled thymidine or uridine was added and in these circumstances, the cells were not exposed to a saturation concentration of these precursors. Most of the thymidine or uridine nucleotides entering the nucleic acids would be expected to be derived from endogenous pathways. The rates of $^3$H-thymidine incorporation into DNA and $^3$H-uridine into RNA by different cell populations will only indicate relative rates of DNA and RNA synthesis respectively if the proportion of nucleotides entering the nucleic acids derived from the labelled precursors is constant. This assumption seems to hold (Ling and Kay, 1975).

The F-15 medium used for the cell cultures was chosen instead of H-16 because it contained less leucine. When $^3$H-leucine was added to F-15 the ratio between radioactive leucine (5 µM) and non-radioactive leucine (400 µM) would allow for more radioactive counts to be taken up by the cells.
The concentration of leucine in sheep popliteal lymph is around 0.525 \( \pm \) 1.312 mg/100 ml (= 40-100 nmoles/ml) (English, 1974). Cells incubated in F-15 medium would thus have sufficient exogenous leucine (400 \( \mu \)M = 5.25 mg/100 ml) for protein synthesis.

After 4 hr incubation with the isotopes the cells were harvested and washed with distilled water. Trichloroacetic acid precipitation was unnecessary as the amounts of isotope incorporated were small compared to the amount in the nucleic acids and proteins in the cells. This was verified from results of preliminary experiments.

For all the experiments with isotopes the samples of cells were collected from the lymph under sterile conditions. The preparation of the cells and the incubation procedures were done immediately after collection. It was found that if lymph samples were left on the bench for 12 hr before cultures were set up, the incorporation of \(^3\)H-thymidine, \(^3\)H-uridine and \(^3\)H-leucine was significantly less compared to that of cultures of freshly collected cells.

(b) THE UPTAKE OF \(^3\)H-THYMIDINE, \(^3\)H-URIDINE and \(^3\)H-LEUCINE BY LYMPHOCYTES IN POPLITEAL EFFERENT LYMPH DURING THE PRIMARY RESPONSE TO SALMONELLA MUECHEN ORGANISMS

The synthesis of DNA, RNA and protein by lymphocytes responding to a primary challenge with Salmonella muenchen organisms was studied in 3 sheep. The PMN leucocytes collected in the first 24 hr after challenge did not take up the isotopes to any extent and no peaks of isotope incorporation were observed at this time. When blast cells began to appear in the lymph the uptake of \(^3\)H-thymidine, \(^3\)H-uridine and \(^3\)H-leucine increased
dramatically and reached maximum levels at around 96 hr after challenge when the numbers of blast cells in the lymph reached their peak. After this time, the incorporation of each of the labelled precursors declined as the cellular response subsided and returned to near pre-stimulation levels by about day 8-9 after antigen challenge (Figure 6.2). It was observed that the rate of $^3$H-thymidine uptake by cells collected at around 96 hr after challenge was $16.9 \pm 4.3$ times greater than in unstimulated cells. The uptake of $^3$H-uridine increased by $5.7 \pm 0.7$ times while the uptake of $^3$H-leucine increased by $3.1 \pm 0.7$ times.

In two of the sheep studied, the efferent lymphatic of the contralateral popliteal node was also cannulated and the cells collected and studied as a control. There was no significant change in the lymphocyte output, in the level of blast cells or in the incorporation of isotopes by cells from the contralateral node (Figure 6.3).

(c)  **THE UPTAKE OF $^3$H-THYMIDINE, $^3$H-URIDINE AND $^3$H-LEUCINE BY LYMPHOCYTES IN POPLITEAL EFFERENT LYMPH DURING THE SECONDARY RESPONSE TO SALMONELLA MUENCHEN ORGANISMS**

The synthetic activities of lymphocytes collected during secondary responses to *Salmonella muenchen* organisms were studied in 3 sheep. The uptake of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine by lymphocytes was similar to that observed in the primary response except that the peaks of uptake occurred at around 72-84 hr after challenge. In one sheep studied, the number of blast cells in the lymph showed 2 peaks, one at 72 hr and a second at 120 hr. In this sheep the incorporation of isotopes followed closely the number of blast cells in the lymph and showed 2 peaks coinciding with the blast cells. Figure 6.4 shows the pattern of
FIGURE 6.2: The primary response to Salmonella muenchen organisms - the uptake of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine by lymphocytes collected at various times during the response.

The upper part of the figure shows the percentages of neutrophils (-----) and blast cells (-----) in the lymph. The lower part shows the radioactivity (c.p.m.) incorporated by the cells following incubation with $^3$H-thymidine (○), $^3$H-uridine (△) and $^3$H-leucine (□). The cultures contained $10^6$ cells and 1 µCi of the tritiated isotopes and were set up in triplicate for 4 hr under a gas phase of 10% CO$_2$, 7% O$_2$ and balance N$_2$. The vertical bars at the individual time points represent 1 standard deviation. For those points without the bars, the standard deviations are smaller than the vertical dimension of the symbols. The arrow indicates the time of antigen administration.
FIGURE 6.3: The uptake of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine by cells collected from the popliteal efferent lymph following a primary challenge in the contralateral node with Salmonella muenchen organisms.

The upper part of the figure shows the percentage of blast cells in the lymph from the unchallenged node. The lower part of the figure shows the $^3$H-thymidine (○), $^3$H-uridine (△) and $^3$H-leucine (□) incorporated by these cells.
HOURS AFTER CHALLENGE OF CONTRALATERAL NODE

RADIOACTIVITY

( CPM x 10^2 : 3H-Leu, CPM x 10^3 : 3H-UR ) % BLASTS

( CPM x 10^3 : 3H-TdR )
FIGURE 6.4: The secondary response to Salmonella muenchen organisms - the uptake of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine by lymphocytes collected at various times during the response.

The upper part of the figure shows the percentages of neutrophils (-----) and blast cells (-----) in the lymph. The lower part shows the radioactivity (c.p.m.) incorporated by the cells following incubation with $^3$H-thymidine (○), $^3$H-uridine (△) and $^3$H-leucine (□). The cultures contained $10^6$ cells and 1 µCi of the tritiated isotopes and were set up in triplicate for 4 hr under a gas phase of 10% CO$_2$, 7% O$_2$ and balance N$_2$. The vertical bars at the individual time points represent 1 standard deviation. For those points without the bars, the standard deviations are smaller than the vertical dimension of the symbols. The arrow indicates the time of antigen administration.
isotope incorporation into the cells collected from the popliteal efferent lymph of a sheep during a secondary response to *Salmonella muenchen* organisms. It was observed that the rate of $^3$H-thymidine uptake by cells collected at around 72 hr after challenge was $18.5 \pm 5.4$ times greater than in cells collected before the challenge. The uptake of $^3$H-uridine increased by $6.8 \pm 0.8$ times while the uptake of $^3$H-leucine increased by $4.1 \pm 1.4$ times.

The contralateral popliteal efferent duct was again cannulated in 2 of the sheep and the incorporation of isotopes into the cells collected from this node was used as a control. As observed in the primary response, no significant change occurred in the control cell population in regard to the incorporation of any of the 3 isotopes (Figure 6.5).
FIGURE 6.5: The uptake of $^{3}$H-thymidine, $^{3}$H-uridine and $^{3}$H-leucine by cells collected from the popliteal efferent lymph following a secondary challenge in the contralateral node with Salmonella muenchen organisms.

The upper part of the figure shows the percentage of blast cells in the lymph from the unchallenged node. The lower part of the figure shows the $^{3}$H-thymidine (○), $^{3}$H-uridine (△) and $^{3}$H-leucine (□) incorporated by these cells.
RADIOACTIVITY

( CPM×10^{-2} \cdot {^{3}}H-Leu ; CPM×10^{3} \cdot {^{3}}H-UR )

\% BLASTS

HOURS AFTER CHALLENGE OF CONTRALATERAL NODE

( CPM×10^{3} \cdot {^{3}}H-TdR )
SECTION C

AUTORADIOGRAPHIC STUDIES OF NUCLEIC ACID AND PROTEIN METABOLISM IN LYMPHOCYTES

(a) EXPERIMENTAL

The distribution of "H-thymidine, "$\beta$-uridine and "$\gamma$-leucine in lymphocytes collected from popliteal effluent lymph during the immune response to salmonella muenchen argenties was investigated by autoradiography. The cells were prepared as for the previous experiments in which the rate of incorporation of these isotopes was studied. Hanks's culture medium plus Youss calf serum was used instead of RPMI medium and the cells were incubated at 37°C for 1 hr in a gas phase of air.

(b) DISTRIBUTION OF "$\beta$-URIDINE, "$\gamma$-URIDINE AND "$\gamma$-LEUCINE IN LYMPHOCYTES FOLLOWING RESPONSE TO SALMONELLA MUECHEN PROTEINS

Before antigenic challenge, most of the small and medium size lymphocytes incorporated "$\beta$-thymidine (Figure 6.6a, b1, c1). Macrophages present in the lymph on day 7 did not incorporate any of the isotopes in significant amounts (Figure 6.6a, b2, c2).

At around the peak of the immune response, the large number of blast cells in the lymph were all found to label intensely with the isotopes (Figure 6.6b, b3, c3). Some of the small and medium size lymphocytes incorporated "$\beta$-uridine and "$\gamma$-leucine to a greater extent than their counterparts collected before antigenic challenge. The cell population collected later in the response showed a great reduction in the number of blast cells and few cells were labelled heavily (Figure 6.6a, c4, c6). In the blast cells, the "$\gamma$-leucine was distributed throughout the cytoplasm. "$\beta$-uridine was found to label principally the nucleus, although the cytoplasm was also labelled. "$\gamma$-thymidine labelled only the nucleus.
(a) EXPERIMENTAL

The distribution of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine in lymphocytes collected from popliteal efferent lymph during the immune response to Salmonella muenchen organisms was investigated by autoradiography. The cells were prepared as for the previous experiments in which the rates of incorporation of these isotopes were studied. Hank's culture medium plus foetal calf serum was used instead of F-15 medium and the cells were incubated at $37^\circ$C for 1 hr in a gas phase of air.

(b) DISTRIBUTION OF $^3$H-THYMIDINE, $^3$H-URIDINE AND $^3$H-LEUCINE IN LYMPHOCYTES DURING THE IMMUNE RESPONSE TO SALMONELLA MUENCHEN ORGANISMS

Before antigenic challenge, most of the small and medium size lymphocytes showed some incorporation of $^3$H-leucine and $^3$H-uridine but practically no incorporation of $^3$H-thymidine (Figure 6.6a1, b1, c1). Neutrophils present in the lymph on day 1 did not incorporate any of the isotopes in significant amounts (Figure 6.6a2, b2, c2).

At around the peak of the immune response, the large number of blast cells in the lymph were all found to label intensely with the isotopes (Figure 6.6a3, b3, c3). Some of the small and medium size lymphocytes incorporated $^3$H-uridine and $^3$H-leucine to a greater extent than their counterparts collected before antigenic challenge. The cell population collected later in the response showed a great reduction in the number of blast cells and few cells were labelled heavily (Figure 6.6a4, b4, c4). In the blast cells, the $^3$H-leucine was distributed throughout the cytoplasm. $^3$H-uridine was found to label principally the nucleus, although the cytoplasm was also labelled. $^3$H-thymidine labelled only the nucleus.
FIGURE 6.6a: Autoradiographs of cells collected from the popliteal efferent lymph at various times during an immune response to Salmonella muenchen organisms and incubated with $^3$H-thymidine in vitro.

(a1) cells collected before antigenic challenge.

(a2) cells collected on day 1 after challenge.

(a3) cells collected at the height of the blast cell response (day 4 after challenge).

(a4) cells collected late in the response (day 7 after challenge).
FIGURE 6.6b: Autoradiographs of cells collected from the popliteal efferent lymph at various times during an immune response to Salmonella muenchen organisms and incubated with $^3$H-uridine in vitro.

(b1) cells collected before antigenic challenge.
(b2) cells collected on day 1 after challenge.
(b3) cells collected at the height of the blast cell response (day 4 after challenge).
(b4) cells collected late in the response (day 7 after challenge).
FIGURE 6.6.c: Autoradiographs of cells collected from the popliteal efferent lymph at various times during an immune response to Salmonella muenchen organisms and incubated with ³H-leucine in vitro.

(c1) cells collected before antigenic challenge.
(c2) cells collected on day 1 after challenge.
(c3) cells collected at the height of the blast cell response (day 4 after challenge).
(c4) cells collected late in the response (day 7 after challenge).
SECTION D

CHANGES IN THE MOTILITY OF LYMPHOCYTES DURING AN IMMUNE RESPONSE

(a) EXPERIMENTAL

Lymphocytes obtained in their living state under phase contrast microscopy showed active locomotion. Mobile lymphocytes had a characteristic appearance of a "hand mirror" in which the forward part of the cell was rounded and contained the nucleus; the rear part of the cell contained the centrosome surrounded by mitochondria.

The number of lymphocytes showing motility was counted in cell populations collected throughout the immune response. For photography, the centrifuge of the cell preparation was compressed slightly to allow better definition of the cells. Transformed blast cells looked cytoplasmic with fringes and processes. They manifested very active movements, but did not always have the "hand mirror" appearance.

(b) IMMUNE RESPONSE ORGANISATION

The motility of lymphocytes responding to killed Salmonella bacteria organisms was studied in several sheep. Before antigenic challenge around 5-10% of the cells, predominantly normal small lymphocytes showed evidence of motility at the time the preparation was examined. During the early phase of the response there was no change in the percentage of motile small and normal lymphocytes and this held throughout the response. The blast cells were very motile and usually more than 30% of them were observed to be motile at any one time. At the peak of the response up to 70% of the blast cells were actively motile (Figure 6.3). The neotropism which appeared in the lymphocytes 48 hours after challenge were also very actively motile.
(a) EXPERIMENTAL

Lymphocytes examined in their living state under phase contrast microscopy showed active locomotion. Motile lymphocytes had a characteristic appearance of a "hand mirror" in which the forward part of the cell was rounded and contained the nucleus; the rear part of the cell contained the centrosome surrounded by mitochondria.

The number of lymphocytes showing motility was counted in cell populations collected throughout the immune response. For photography, the coverslip of the cell preparation was compressed slightly to allow better definition of the cells. Transformed blast cells had thin cytoplasm with fringes and processes. They manifested very active movement, but did not always have the "hand mirror" appearance.

(b) MOTILITY OF LYMPHOCYTES RESPONDING TO SALMONELLA MUECHEN ORGANISMS

The motility of lymphocytes responding to killed Salmonella muenchen organisms was studied in several sheep. Before antigenic challenge around 5-10% of the cells, predominantly normal small lymphocytes showed evidence of motility at the time the preparation was examined. During the early phase of the response there was no change in the percentage of motile small and medium lymphocytes and this held throughout the response. The blast cells were very motile and usually more than 30% of them were observed to be motile at any one time. At the peak of the response up to 50% of the blast cells were actively motile (Figure 6.7). The neutrophils which appeared in the lymph on day 1 after challenge were also very actively motile.
FIGURE 6.7: Phase contrast pictures of lymphocytes in popliteal efferent lymph.

(a), (b) lymphocytes showing "hand mirror" appearance.

(c), (d) motile blast cells.
SECTION E

DISCUSSION AND CONCLUSIONS

The incorporation of 

by lymphocytes

It was observed that incorporation of radioactivity into lymphocytes coincided with the appearance of large numbers of blast cells. The extent of incorporation coincided with the maximum percentage of blast cells in the lymph node. The changes in the extent of incorporation of the isotopes were considered to be independent of the nature of the response. The rate of incorporation was related to the number of blast cells present, in secondary lymphocytes, when blast cells appeared to increase, the rate of incorporation was higher than in primary responses. The rate of incorporation was increased after the injection of the isotopes, and it was observed that, although the leucocytes were shown to have a high level of energy metabolism, their additional requirements were not concerned with the synthesis of protein and nucleic acids as was the case for lymphocytes. This agreed with the findings of Cooper (1963) and Penfield et al. (1968) and was an expected finding. In view of the fact that the leucocytes are monophasic cells incapable of division (Penfield et al. 1968).
(a) **THE INCORPORATION OF H-THYMIDINE, H-URIDINE and H-LEUCINE BY LYMPHOCYTES**

It was observed that increases in the rates of incorporation of the H-thymidine, H-uridine and H-leucine into DNA, RNA and protein respectively occurred in the cell population of lymph coincident with the appearance of large numbers of blast cells. The maximum rates of isotope incorporation coincided with the maximum percentage of blast cells in the lymph in both the primary and the secondary immune response. The changes in the extent of incorporation of the isotopes were considered to be independent of the nature of the response. The rates of isotope incorporation were related to the number of blast cells present; in secondary responses, when blast cells appeared in greater numbers, the rates of incorporation of the isotopes were higher than in primary responses.

No measurable increases occurred in the incorporation rates of the isotopes on day 1 after challenge when there were large numbers of neutrophils present. This implied that, although PMN leucocytes were shown to have a high level of energy metabolism (Chapters 4 and 5), their energy requirements were not concerned with the synthesis of protein and nucleic acids as was the case for lymphocytes. This agreed with the findings of Cooper (1961) and Pogo et al. (1966), and was an expected finding in view of the fact that PMN leucocytes are end-stage cells incapable of division (Rauch et al., 1961).

(b) **AUTORADIOGRAPHIC STUDIES ON NUCLEIC ACID AND PROTEIN METABOLISM IN LYMPHOCYTES**

The autoradiographic studies reported in Section C of this chapter showed that it was the blast cells that were most
active in the incorporation of the labelled isotopes. The fact that some of the small and medium lymphocytes collected after antigenic challenge incorporated greater amounts of $^3$H-uridine and $^3$H-leucine without showing evidence of morphological change suggested that RNA and protein metabolism was activated in these cells at an early stage following stimulation with antigen. The actively dividing blast cells would be expected to have incorporated $^3$H-thymidine as an accompaniment to DNA synthesis during replication of the chromosomal DNA (Smith, 1967; Hall et al., 1967). As a consequence, the labelling pattern was confined to the nuclear region of the cells. As no appreciable incorporation of $^3$H-thymidine was observed in small and medium lymphocytes, it seemed that dedifferentiation of small lymphocytes to blast cells was a necessary prerequisite for lymphocyte proliferation. Blast cells were the only cells in short-term cultures which synthesized DNA (Bain, Vas and Lowenstein, 1964).

Leucine is an indispensable amino acid for protein biosynthesis and the increased incorporation of $^3$H-leucine into blast cells indicated that these cells had a much higher rate of protein synthesis than did small lymphocytes. The whole machinery of protein synthesis, involving the different species of RNA molecules, the ribosomes and a number of enzymes and cofactors occurs in the cytoplasm and as a consequence the incorporated leucine was found within the cytoplasm of the cells.

Most of the labelling with uridine was found in the nuclear region of the blast cells, indicating that the nucleus was the most active site of RNA synthesis in these cells. It is the nucleolus that contains the enzymes and genes for ribosomal
RNA biosynthesis. The enzymes responsible for the synthesis of messenger and transfer RNA's are also localized in the nucleoplasm (Conn and Stumpf, 1976). The few grains found in the cytoplasm were most probably due to the migration of newly synthesized RNA into the cytoplasm. This assumption is supported by observations that in lymphoid blast cells exposed to a short pulse of $^3$H-uridine, there was a decrease in labelled nuclear RNA and a rise in labelled cytoplasmic RNA with time (Mitchell, 1964; Hayhoe and Quaglino, 1965; Mitchell, Bard, L'Anglais and Kaplan, 1978). Some biosynthesis of RNA might occur in the cytoplasm in organelles such as the mitochondria (Conn and Stumpf, 1976).

(c) MOTILITY OF LYMPHOCYTES

The increase in the number of motile blast cells during the immune response to Salmonella suggested that these cells would have a higher energy requirement for this activity than would the small lymphocytes. The highly motile state of these cells would enable them to migrate from their sites of origin in the node into the lymph stream and so facilitate their role in the dissemination of the response and the establishment of systemic immunity (Morris, 1966; Hall et al., 1967).
GENERAL DISCUSSION AND SUMMARY
SECTION A

GENERAL DISCUSSION

A novel achievement reported in this thesis was the direct determination of the PO₂ in the popliteal efferent lymph of conscious sheep. The results of these experiments made it possible to assess the physiological significance of the metabolic studies done on lymphocytes in vitro.

It was found that the PO₂ of popliteal efferent lymph as it left the node was in a range of 5.3 - 8.2 kPa, with a mean and standard deviation of 7.17 ± 0.65 kPa. Maximum PO₂ values of around 9.0 kPa were recorded during the first 24 hr after antigenic challenge while values around 5.0 kPa were recorded 72-96 hr after challenge as part of cellular response. The overall average PO₂ throughout the immune response was around 7.0 kPa. The PO₂ of the lymph varied inversely with the cell concentration. From these results, it was established that lymphocytes in vivo would be metabolizing under a PO₂ of about 45 mm Hg, while the concentration of cells in the lymph was about 10.6 x 10⁶ cells/µl. At higher cell concentrations, particularly during an immune response, the PO₂ in the lymph would be significantly lower.

The effect of O₂ on the metabolism of cells is usually studied at extremes of O₂ concentration, either at zero pressure to determine the Ficoll effect or at a pressure of several atmospheres to determine O₂ toxicity (reviewed by Haugland, 1986). The present investigations were concerned with studying the effects of physiological ranges of PO₂ on lymphocyte metabolism.
A novel achievement reported in this thesis was the direct determination of the $P_{O_2}$ in the popliteal efferent lymph of conscious sheep. The results of these experiments made it possible to assess the physiological significance of the metabolic studies done on lymphocytes in vitro.

It was found that the $P_{O_2}$ of popliteal efferent lymph as it left the node was in a range of 6.3 - 8.2% $O_2$ with a mean and standard deviation of 7.1 ± 0.8% $O_2$. Maximum $P_{O_2}$ values of around 9.0% $O_2$ were recorded during the first 24 hr after antigenic challenge while values around 5.0% were recorded 72-96 hr after challenge at the peak of cellular response. The overall average $P_{O_2}$ in popliteal lymph throughout the immune response was around 7.0% $O_2$. The $P_{O_2}$ of the lymph varied inversely with the cell concentration. From these results it was established that lymphocytes in vivo would be metabolizing under a $P_{O_2}$ of about 45 mm Hg, when the concentration of cells in the lymph was about $18.0 \times 10^6$ cells/ml. At higher cell concentrations, particularly during an immune response, the $P_{O_2}$ in the lymph would be significantly lower.

The effect of $O_2$ on the metabolism of cells is usually studied at extremes of $O_2$ concentration, either at zero pressure to determine the Pasteur effect or at a pressure of several atmospheres to determine $O_2$ toxicity (reviewed by Haugaard, 1968). The present investigations were concerned with studying the effects of physiological ranges of $P_{O_2}$ on lymphocyte metabolism.
It was determined that the rate of respiration of lymphocytes depended on the concentration of \( \text{O}_2 \) in the incubation medium and this dependence was described by the Michaelis-Menten equation. The concentration of \( \text{O}_2 \) at which the respiration rate of lymphocytes first began to fall (the \([\text{O}_2]_{\text{crit}}\)) was found to be around 1.5 mm Hg. This was very low compared to the \( \text{PO}_2 \) of the lymph and consequently it could be asserted that the rate of respiration of lymphocytes in vivo is maximal and is not affected by the fluctuations observed in the \( \text{PO}_2 \) of lymph, before or after antigenic challenge. In view of this the respiration rates determined when the cells are respiring under atmospheric air would be similar to physiological respiration rates.

The rates of glycolysis and the release of \( ^{14}\text{CO}_2 \) from C-1- and C-6-labelled glucose as determined under the \( \text{PO}_2 \) of atmospheric air were not similar to those which occurred at physiological \( \text{PO}_2 \) values. It was found that the rate of glycolysis was affected by the \( \text{PO}_2 \) of the atmosphere under which the cells were metabolizing. With decreasing \( \text{PO}_2 \) values glycolysis was enhanced while the production of \( ^{14}\text{CO}_2 \) from C-1- and C-6-labelled glucose was inhibited. This effect was most marked at zero \( \text{PO}_2 \), i.e. under anaerobic conditions. The glycolytic activity of lymphocytes in culture medium equilibrated with air was lower than occurred at physiological \( \text{PO}_2 \), while the results from radiorespirometric studies, made at the \( \text{PO}_2 \) of air overestimated the physiological rates of production of \( ^{14}\text{CO}_2 \) from C-1- and C-6-labelled glucose. It was determined that the energy metabolism of stimulated lymphocytes responded to different \( \text{PO}_2 \) levels in the same manner as normal lymphocytes. Because of this it was
considered that the overall picture of lymphocyte metabolism described in this thesis would apply to physiological conditions.

No investigations were done on the effect of changes in $\frac{P_{O_2}}{P_{O_2}}$ on the incorporation of $^3$H-thymidine, $^3$H-uridine and $^3$H-leucine into lymphocytes. It is generally believed that growth and nucleic acid synthesis are inhibited in mammalian cells at $P_{O_2}$ greater than 30% $O_2$ (Haugaard, 1968). Anderson, Hellung-Larsen and Sorensen (1968) showed that human lymphocytes stimulated with PHA had optimal rates of incorporation of $^3$H-thymidine in 68 hr cultures at $O_2$ levels between 5-20%. Hofbert (1974) found that there was an acute effect of high $P_{O_2}$ (90% $O_2$ - 10% $CO_2$) on nucleic acid metabolism in thymocytes which was manifested by a decline in $^3$H-deoxycytidine incorporation into DNA. The uptake of $^3$H-thymidine was not affected.

In the present investigations the cells were incubated under a gas phase of 7% $O_2$ so that the overall pattern of synthetic activities in lymphocytes responding to antigen could be related to the physiological conditions.

(b) ENERGY METABOLISM OF LYMPHOCYTES IN RELATION TO THE SYNTHETIC AND LOCOMOTIVE ACTIVITIES OF THESE CELLS DURING RESPONSES TO SALMONELLA MUECHEN ORGANISMS

The free-floating cells in lymph play a role in the production of antibody, the propagation of the immune response throughout the body, and in the establishment of immunological memory. The cells responsible for these activities are lymphocytes which have undergone dedifferentiation and have
transformed into proliferating blast cells.

It was observed in the present investigations that before antigenic challenge the small and medium sized lymphocytes collected from the popliteal efferent lymph incorporated appreciable amounts of $^3$H-leucine. This suggested that these cells are actively synthesizing proteins in the absence of any antigenic stimulus. After antigenic challenge the blast cells were found to incorporate $^3$H-leucine to a much greater extent than did the small and medium lymphocytes. This increased protein synthesis would be related partly to cell growth and partly to antibody synthesis (English et al., 1976).

The synthesis of protein involves translation of genetic information contained in mRNA molecules from which the order of insertion of the specific amino acids into the protein molecule is directed. In this process other species of RNA such as tRNA and probably rRNA are also involved. Thus RNA is important in the control of stimulated growth. In theory the overall rate of protein synthesis could be controlled by the availability of rRNA or tRNA, while the synthesis of an individual protein could be limited by the availability of the specific mRNA's (Ling and Kay, 1975).

The $^3$H-uridine taken up by small and medium lymphocytes may indicate a constant turnover of the ribonucleic acids of these cells as a necessary requirement for protein production to maintain viability. The increased rate of RNA synthesis that occurs in blast cells could be explained in terms of an increased requirement of RNA for increased protein synthesis (Jagus-Smith and Kay, 1976). The good correlation between the change in rates of incorporation of $^3$H-leucine and $^3$H-
uridine observed over the immune responses supports this argument.

The increased synthesis of DNA by the blast cells would be essential for the replication of chromosomes. It was observed in the present investigations that the blast cells were the only cells which incorporated significant amounts of $^3$H-thymidine into their DNA.

The temporal correlation between DNA, RNA and protein synthesis and the blast cell response to antigen does not necessarily imply that each of these three synthetic processes started at the same time inside the transforming cells, because the cells collected from the popliteal efferent lymph were not synchronized in their mitotic cycle. It has been found that in PHA-stimulated lymphocytes in vitro DNA synthesis did not begin until after an increase in RNA and protein synthesis was evident (Chapter 1). It is certain though that following antigenic challenge in vivo, each of these three processes was enhanced in the blast cells rather than in the small and medium lymphocytes.

The increased synthetic activities and increased motility of the blast cells require an increased energy production to enable these cells to carry out their immune functions. Since carbohydrates are a major source of energy for cells, changes would be expected to occur in the metabolism of carbohydrates. It has been shown in vitro that the suppression of glycolysis and Krebs cycle activity inhibits DNA synthesis in lymphocytes (Polgar et al., 1968; Lindahl-Kiessling and Mattsson, 1971; Roos and Loos, 1973) while Mendelsohn, Nordeen and Young (1977)
have established that both glycolytic and oxidative ATP are required for the maintenance of maximal rates of protein synthesis in lymphocytes.

The observed increases in the uptake of glucose and in the production of lactate indicate that, in transforming blast cells, glycolysis plays an important role in supplying energy in the form of ATP. Aerobic oxidation of glucose would also be an important energy yielding reaction because the rates of $O_2$ consumption and of $^{14}CO_2$ release from C-6-labelled glucose by blast lymphoid cells were high when compared to unstimulated small lymphocytes. The aerobic oxidation of glucose provides more energy than anaerobic oxidative processes because the glycolytic conversion of 1 mole of glucose to 2 moles of lactate forms only 2 high-energy phosphate bonds in the form of ATP. On the other hand, when 1 mole of glucose is converted to pyruvate and further oxidized in the Krebs cycle, a total of 36 molecules of ATP would be formed in the complete oxidation process (Conn and Stumpf, 1976). Roos and Loos (1970) estimated from in vitro studies that as much as 85% of the ATP synthesized in lymphocytes might be formed as a result of oxidative phosphorylation even though only 25-30% of the glucose taken up by the cells was oxidized aerobically and 60-70% was oxidized anaerobically. In the present investigations it was found that in unstimulated lymphocytes, about 67% of the glucose consumed was converted to lactate, a result very close to that obtained by Roos and Loos (1970). As lymphocytes produced lactate at a mean rate of 1.306 $\mu$moles/hr/2 x 10^8 cells and consumed $O_2$ at a rate of 1.810 $\mu$moles/
hr/2 x 10^8 cells (average of mean values obtained from Warburg manometry and Clark oxygen electrode determinations) it can be calculated that theoretically 68.4% (very close to 67% mentioned above) of the consumed glucose was converted to lactate while 31.6% was completely oxidized to CO₂ and H₂O and from these figures it can be estimated that 15.7% of the ATP was formed during glycolysis and 84.3% formed during oxidative phosphorylation (Roos and Loos, 1973; Conn and Stumpf, 1976). Again this agreed very well with the percentages determined by Roos and Loos (1973) and Lengle et al. (1978). The above calculations are based on data obtained from cells collected before antigenic challenge and incubated in vitro in air. Under a physiological PO₂ of around 7% O₂, the rate of lactate production would be higher than that under air while the rate of O₂ consumption would be similar. It would be expected that more ATP would be derived from glycolysis under physiological PO₂ values, although the proportion of ATP derived from oxidative phosphorylation would still be predominant. This would also apply after antigenic challenge.

The enhanced activity of the pentose phosphate pathway that occurred after antigenic stimulation indicated that more ribose and NADPH were being generated. Since ribose is required for nucleotide and nucleic acid synthesis, this increased pentose phosphate pathway activity correlated well with the finding that DNA and RNA synthesis in lymphocytes were stimulated during the immune response. NADPH is required for synthetic processes taking place outside mitochondria, such as the synthesis of fatty acids or steroids and the synthesis of amino acids via glutamate dehydrogenase (Harper, 1973;
Conn and Stumpf, 1976). In this respect the stimulation of
the pentose phosphate pathway could also be related to the
increased protein synthesis that occurs in lymphoid cells
stimulated by antigen.

(c) **FUTURE EXPERIMENTS**

The method for monitoring $P_{O_2}$ in lymph developed and
described in this thesis is applicable to the measurement of
$P_{O_2}$ in lymph in other sites or in fluid in other ducts
(e.g. the oviduct, bile duct, etc.) provided the duct can be
cannulated with an appropriate sized tubing. Since $P_{O_2}$ values
of various body fluids have been measured in vivo to only a
limited extent, a significant contribution could be made to
animal physiology by using the present methodology to
undertake a comprehensive study of the $P_{O_2}$ in different body
fluids in different species. Furthermore, an electrode of this
type could probably be converted to measure $P_{CO_2}$ and/or pH
to give a wider application of this methodology.

Lymphocytes collected from popliteal efferent lymph have
proved to be very suitable cells for metabolic studies.
They have merit in that they can be stimulated in vivo under
physiological conditions and collected in a physiologically
dispersed state. Thus it would be interesting to study other
aspects of metabolism of lymphocytes during the immune response
such as changes in activities of important metabolic enzymes
during cellular transformation, the identification of
regulatory steps in various metabolic pathways, the estimation
of the contribution of different metabolic pathways in substrate
metabolism, and the metabolism of other metabolites such as
fatty acids, glycogen, polyamines etc. It may be that after challenge with antigens such as allogeneic lymphocytes, grafted tissues and viruses different metabolic responses are evoked in lymphocytes and this proposition could be studied by the methods described in this thesis.
(1) The Oxygen Tension of Papillital Tissue Lymph

The PO₂ of papillital afferent lymph measured in vivo before antigenic challenge was in the range 41.64 mm Hg (6.1 - 8.22 mm Hg) with a mean and standard deviation of
46.8 ± 8.3 mm Hg (7.4 ± 0.82 mm Hg). The PO₂ of the lymph changed significantly following antigenic challenge, and was found to vary inversely with the concentration of cells in the lymph. The consumption of O₂ by the cells in the lymph was determined, at least in part, in vivo.

(11) The Rate of Gaseous Exchange in Lymphocytes Collected from Papillital Lymph

The rate of O₂ consumption by normal lymphocytes was 1.254 ± 0.204 mmoles/hr/2 x 10⁶ cells as determined by "Direct" Warburg manometry, 2.676 ± 0.204 mmoles/hr/2 x 10⁶ cells as determined by "Indirect" Warburg manometry and 1.165 ± 0.026 as determined by the Clark oxygen electrode method. The rate of CO₂ production was 0.373 ± 0.145 mmoles/hr/2 x 10⁶ cells as determined by "Direct" Warburg manometry and 0.426 ± 0.046 as determined by "Indirect" Warburg manometry. After antigenic stimulation with Salmonella organisms, the rate of oxygen exchange in lymphocyte populations increased significantly. Maximum values were always recorded when the percentage of blast cells in the cell population reached its peak. This phenomenon was observed with all three methods of monitoring the respiration of lymphocytes and was not related to the antigenic stimulus. Polymorphonuclear neutrophils which appeared in the lymph in large numbers on day 1 after antigenic challenge were found to respire at much higher rates than normal lymphocytes.

SECTION B

SUMMARY
(i) The Oxygen Tension of Popliteal Efferent Lymph

The PO₂ of popliteal efferent lymph measured in vivo before antigenic challenge was in the range 41-54 mm Hg (6.3 - 8.2% O₂) with a mean and standard deviation of 46.6 ± 5.3 mm Hg (7.1 ± 0.8% O₂). The PO₂ of the lymph changed significantly following antigenic challenge, and was found to vary inversely with the concentration of cells in the lymph. The consumption of O₂ by the cells in the lymph thus determined, at least in part, its PO₂ in vivo.

(ii) The Rate of Gaseous Exchange in Lymphocytes collected from Popliteal Efferent Lymph

The rate of O₂ consumption by normal lymphocytes was 1.254 ± 0.204 µmoles/hr/2 x 10⁸ cells as determined by "Direct" Warburg manometry, 2.476 ± 0.476 as determined by "Indirect" Warburg manometry and 1.699 ± 0.226 as determined by the Clark oxygen electrode method. The rate of CO₂ production was 0.973 ± 0.145 µmoles/hr/2 x 10⁸ cells as determined by "Direct" Warburg manometry and 2.476 ± 0.476 as determined by "Indirect" Warburg manometry. After antigenic stimulation with Salmonella organisms, the rate of gaseous exchange in lymphocyte populations increased significantly. Maximum values were always recorded when the percentage of blast cells in the cell populations reached its peak. This phenomenon was observed with all three methods of monitoring the respiration of lymphocytes and was not related to the antigenic stimulus. Polymorphonuclear neutrophils which appeared in the lymph in large numbers on day 1 after antigenic challenge were found to respire at much higher rates than normal lymphocytes.
(iii) The Michaelis Constant and the Critical Oxygen Concentration for Lymphocyte Respiration

It was found that the rate of O₂ consumption by lymphocytes given a limited O₂ supply could be related to the P⁰₂ by the Michaelis-Menten equation. The concentration of O₂ at which the respiration rate of the cells began to fall (the critical O₂ concentration, [O₂]crit) was 1.998 ± 0.197 µM (1.477 ± 0.144 mm Hg). No significant differences were observed in [O₂]crit value before or after antigenic stimulation. Since the [O₂]crit value was very low compared to the P⁰₂ of the popliteal efferent lymph, it was concluded that lymphocytes in lymph were respiring at maximal rates all the time. The O₂ concentration at which the rate of O₂ consumption for normal lymphocytes was half maximum (the Michaelis constant, Kₘ) was 0.462 ± 0.108 µM with a range of 0.342 - 0.628 µM. This value was about one-quarter of the [O₂]crit value. After antigenic challenge, the Kₘ values changed significantly and reached a maximum when the cellular response was at its peak. It was found that a linear correlation existed between the Kₘ and the Vₘₐₓ (maximum rate of O₂ consumption). The increased Kₘ values observed when large numbers of blast cells were present in the lymph was thought to be due to the large cytoplasmic volume of these cells representing an increased diffusion distance for O₂ from the suspending medium to the mitochondria.

(iv) Glucose Uptake and Lactate Production by Lymphocytes responding to Salmonella muenchen Organisms

In normal sheep, the level of glucose in lymph was found to vary from 3.52 - 4.70 mM with a mean and standard deviation of 4.01 ± 0.33 mM. Following the injection of antigen, no
significant change occurred in the glucose level of the lymph except for the first few hours after antigen administration when an obvious fall in glucose concentration was always observed. This phenomenon was thought to be due to the high rate of glucose uptake by the large numbers of neutrophils which appeared in the lymph at this time. Normal lymphocytes had a rate of glucose uptake of $0.970 \pm 0.290 \, \mu\text{moles/hr/}2 \times 10^8 \, \text{cells}$ and a rate of lactate production of $1.306 \pm 0.368 \, \mu\text{moles/hr/}2 \times 10^8 \, \text{cells}$. These rates changed significantly following antigenic challenge over a similar time course to that observed for gaseous exchanges. The maximum values occurred when the cellular response was at its peak in both primary and secondary responses. Polymorphonuclear neutrophils were found to have high rates of glycolysis.

(v) The Metabolism of C-1- and C-6-labelled Glucose by Lymphocytes

It was observed in radiorespirometric studies that sheep lymphocytes produced $^{14}\text{C}O_2$ from C-1-labelled glucose at a much higher initial rate than from C-6-labelled glucose. This suggested that the pentose phosphate pathway, as well as the glycolytic pathway plus Krebs cycle were operating actively in these cells. After antigenic challenge the rates of $^{14}\text{C}O_2$ production from C-1- and C-6-labelled glucose increased and reached maximum values when the number of blast cells in the lymph reached its peak. Polymorphonuclear neutrophils produced $^{14}\text{C}O_2$ from C-1-labelled glucose at much higher rates than did lymphocytes. These observations indicated that the pentose phosphate pathway, glycolysis and the Krebs cycle were stimulated in sheep lymphocytes following in vivo antigenic challenge.
(vi) The Effect of Oxygen Tension on the Energy Metabolism of Lymphocytes

It was found that the rates of energy metabolism of sheep lymphocytes were affected by the \( P_{O_2} \) of the gas phase under which the cells were metabolizing. With decreasing \( P_{O_2} \) from 21% to 0% \( O_2 \) the rates of glucose uptake and lactate production increased while the rates of \(^{14}CO_2 \) production from C-1- and C-6-labelled glucose decreased. These phenomena were explicable in terms of the effect of availability of \( O_2 \) on the formation of ATP. The \( O_2 \) supply affected the relative activity of glycolysis versus Krebs cycle and oxidative phosphorylation. The biosynthesis of ribose through the oxidative route of the pentose phosphate pathway compared with that produced through the nonoxidative route of the pathway was also affected by the \( P_{O_2} \). This finding indicated that most of the results on energy metabolism studies carried out by other workers under air were not applicable to the physiological activities of lymphocytes in vivo.

(vii) The Incorporation of \(^3\)H-thymidine, \(^3\)H-uridine and \(^3\)H-leucine by Lymphocytes

Rates of incorporation of \(^3\)H-thymidine, \(^3\)H-uridine and \(^3\)H-leucine by lymphocytes into DNA, RNA and protein respectively were found to be greatly enhanced in the blast cells which appeared in the lymph during an immune response to Salmonella organisms. This meant that the blast cells were actively synthesizing nucleic acids and proteins. Polymorphonuclear leucocytes did not show any significant incorporation of these isotopes. Autoradiographic studies confirmed that blast cells incorporated \(^3\)H-uridine and \(^3\)H-leucine at much higher rates than
small and medium lymphocytes. $^3$H-thymidine was found to be incorporated into nuclear DNA only by the transforming blast cells. Few small or medium cells were found to be synthesizing DNA. The $^3$H-thymidine was incorporated only into the nucleus of the cells, the majority of the $^3$H-uridine was also found in the nucleus but some was present in the cytoplasm while the $^3$H-leucine was incorporated throughout the cytoplasm.

Energy Requirements of Lymphocytes

Normal small and medium lymphocytes have relatively low energy requirements for processes such as protein and nucleic acid synthesis. Their major energy requirements would appear to be related to their migratory habits. While only 5-10% of normal lymphocytes exhibited active movement in coverslip preparations in vitro, the fact that most of these cells had arrived in the lymph following migration from the blood stream through the tissues indicated that the entire small and medium lymphocyte population is, at some stage, actively motile. Although no significant increase occurred in the number of motile small and medium lymphocytes in the in vitro assay after antigenic challenge, the tremendous increase in cell output during the "recruitment" phase of the response (the period in between "shutdown" and the peak of blast cell response) indicates the high degree of motility of these cells.

The blast cells which appeared in the lymph in large numbers were highly motile and as many as half of them could be identified moving rapidly in the coverslip assay. These cells which were migrants from the lymph node would have a significant requirement for metabolic energy for their movements and migrations. In addition to this, the large
blast cells required energy for the high rates of protein, RNA and DNA synthesis associated with their enlargement and proliferation in response to antigenic challenge. A further requirement for energy would relate to the synthesis and secretion of specific antibodies by these cells, which is their primary responsibility during the immune response.
REFERENCES

Protein synthesis and ribosome-ribinucleic acid during the early
stages of phytohemagglutinin lymphocyte stimulation
Exp. Cell Res. 32, 519-525.

Differences in vivo in incorporation of tritiated
deyoxythymidine and tritiated thymidine into human lymphocytes.
Experimenta 26, 677-678.

ALTHAN, P. L. and BITTER, D. S. (1971)
Stimulation of Glucuronidase (Biological) Nucleotidase

Optimal oxygen tension for human lymphocytes in culture.
J. Cell Physiol. 72, 149-166.

ATTAWI, G. and ALBERTI, G. (1970)
Structure and synthesis of ribosomal RNA.

AXELROD, B. (1969)
Other pathways of carbon-14 metabolism.
In Metabolism Pathways (H. Greenberg, ed.), vol. 2, pp. 272-554.
Academic Press.

BAUSCH, A., MILLER, O.J. and UNGER, D. (1968)
Observations on chromosome duplication in cultured human
lymphocytes.
Exp. Cell Res. 41, 400-412.

Arch. Exp. Path. Pharmak. 274, 327-331.
Quoted by Leonard (1957).

BAILEY, N. R. O. (1963)

Tritiated-thymidine uptake in acid lymphocyte cultures; effect of specific activity and exposure time.

BAIK, H., YAS, M. K. and LONIESTEIN, L. (1964)
The development of large immature mononuclear cells in mixed
lymphocyte cultures.
Blood 21, 105-116.

BARLOW, A. D. (1970)
Deoxycytidin transport and pyrimidine deoxynucleotide
metabolism in phytohemagglutinin-stimulated pig lymphocytes.
AHERN, T. and KAY, J.E. (1975)
Protein synthesis and ribosome activation during the early
stages of phytohemagglutinin lymphocyte stimulation.

AKASAKA, T.L., OLDS-ARROYO, L. and MILLER III, J.J. (1979)
Differences in in vitro incorporation of tritiated
deoxyctydine and tritiated thymidine into human lymphocytes.
Experientia 35, 673-674.

ALTMAN, P.L. and DITTMER, D.S. (1971)
'Respiration and Circulation (Biological Handbooks)'.

ANDERSON, V., HELUNG-LARSEN, P. and SORENSEN, S.F. (1968)
Optimal oxygen tension for human lymphocytes in culture.
J. Cell Physiol. 72, 149-152.

ATTARDI, G. and AMALDI, F. (1970)
Structure and synthesis of ribosomal RNA.

AXELROD, B. (1967)
Other pathways of carbohydrate metabolism.
In "Metabolic Pathways" (D.M. Greenberg, ed), vol.1, pp.272-308.

BADER, S., MILLER, O.J. and MUKHERJEE, B.B. (1963)
Observations on chromosome duplication in cultured human
leukocytes.
Exp. Cell Res. 31, 100-112.

BAI, B., VAS, M.R. and LOWENSTEIN, L. (1964)
The development of large immature mononuclear cells in mixed
leukocyte cultures.

BARLOW, S.D. (1976)
Deoxycytidine transport and pyrimidine deoxynucleotide
metabolism in phytohaemagglutinin-stimulated pig lymphocytes.
BARLOW, S.D. and ORD, M.G. (1975)
Thymidine transport in phytohaemagglutinin-stimulated pig lymphocytes.

BARRON, E.S.G. and HARROP, G.A. JR. (1929)
Studies on blood cell metabolism. V. The metabolism of leukocytes.
J. Biol. Chem. 83, 89-100.

BECK, W.S. (1958)
Occurrence and control of the phosphogluconate oxidation pathway in normal and leukemic leukocytes.

BECK, W.S. and VALENTINE, W.N. (1953)
The carbohydrate metabolism of leukocytes: a review.
Cancer Res. 13, 309-317.

BEECHEY, R.B. and RIBBONS, D.W. (1972)
Oxygen electrode measurements.
In "Methods in Microbiology" (J.R. Norris and D.W. Ribbons, eds), vol. 6B, pp. 31-51.


D-glucose: determination with glucose oxidase and peroxidase.

BERGOFSKY, E.H. et al. (1964)
Quoted by Altman and Dittmer (1974).

The use of lymph for the measurement of gas tensions in interstitial fluid and tissues.

BOYLE, W. (1968)
An extension of the $^{51}$Cr-release assay for estimate of mouse cytotoxins.
Transplantation 6, 761-764.

BÖYUM, A. (1968)
Isolation of mononuclear cells and granulocytes from human blood.
BÖYUM, A. (1976)
Isolation of lymphocytes, granulocytes and macrophages.

Separation of lymphocytes from peripheral blood by means of
glass wool column. A method for in vitro culture of
lymphocytes.

Aeration in the submerged culture of micro-organisms.
In "Methods in Microbiology" (J.R. Norris and D.W. Ribbons,

BROWN, E.G., LIU, C.C., MCDONNELL, F.E., NEUMAN, M.R. and SWEET,
A.Y. (1973)
An unique electrode catheter for continuous monitoring of
arterial blood oxygen tension in human infants.

BUTTON, D.K. (1964)
"Continuous culture of Torulopsis utilis with oxygen the
limiting nutrient."
Quoted by Button and Garver (1966).

Continuous culture of Torulopsis utilis: a kinetic study of
oxygen limited growth.

The effects of antigen on the migration of recirculating
lymphocytes through single lymph nodes.
J. Exp. Med. 143, 870-888.

Changes in lymphocyte circulation after administration of
antigen.
Haematologia 8, 321-334.

CARLSTEN, A. and SÖDERHOLM, B. (1961)
Carbon dioxide tension and pH of lymph and arterial blood in
anaesthetized dogs.

CARSTAIRS, K. (1961)
Transformation of the small lymphocyte in culture.
Lancet 2, 984.

CARSTAIRS, K. (1962)
The human small lymphocyte: its possible pluripotential
quality.
Lancet 1, 829-832.
CHAPPELL, J.B. (1964)  
The oxidation of citrate, isocitrate and cis-aconitate by isolated mitochondria.  

CHEN, H.W., HEINIGER, H-J. and KANDUTSCH, A.A. (1975)  
Relationship between sterol synthesis and DNA synthesis in phytohemagglutinin-stimulated mouse lymphocytes.  

CLARK, L.C. JR. (1956)  
Monitor and control of blood and tissue oxygen tensions.  

Continuous recording of blood oxygen tensions by polarography.  
J. Appl. Physiol. 6, 189-193.

COCKETT, A.T.K. (1967)  
Invest. Urol. 5, 260.  
Quoted by Altman and Dittmer (1974).

Incorporation of tritium of 3H-5-uridine into DNA.  

CONN, E.E. and STUMPF, P.K. (1976)  
'Outlines of Biochemistry', 4th ed.  

CONWAY, M., DURBIN, G.M., INGRAM, D., McINTOSH, N., PARKER, D.,  
Continuous monitoring of arterial oxygen tension using a catheter-tip polarographic electrode in infants.  
Pediatrics 57, 244-250.

COOPER, E.H. (1961)  
The uptake of [3H]leucine into human lymphocytes in vitro.  

COOPER, E.H. and AMIEL, J.L. (1965)  
La prolifération des lymphocytes humains "in vitro".  
Quoted by Ling and Kay (1975).

COOPER, E.H., BARKHAN, P. and HALE, A.J. (1963)  
Observations on the proliferation of human leukocytes cultured with phytohaemagglutinin.  
Brit. J. Haemat. 9, 101-111.

COOPER, E.H. and MILTON, J.D. (1964)  
The incorporation and degradation of pyrimidine DNA precursors by human leukocytes.  
Brit. J. Cancer 18, 701-713.
COOPER, H.L. (1969)  
Alternations in RNA metabolism in lymphocytes during the shift from resting state to active growth.  
In "Biochemistry of Cell Division" (R. Baserga, ed), pp. 9-112.  
Springfield, Ill.

COOPER, H.L. (1972)  
Studies on RNA metabolism during lymphocyte activation.  
Transplant. Rev. 11, 3-38.

COOPER, H.L. (1973)  
Effect of mitogens on the mitotic cycle: a biochemical evaluation of lymphocyte activation.  
In "Drugs and the Cell Cycle" (A. Zimmerman, G. Padilla and I. Cameron, eds), pp. 138-194.  

COOPER, H.L. and RUBIN, A.D. (1965)  
RNA metabolism in lymphocytes stimulated by phytohemagglutinin: initial responses to PHA.  
Blood 25, 1014-1027.

CROXTON, F.E., COWDEN, D.J. and KLEIN, S. (1969)  
Prentice-Hall, New Delhi.

CULVENOR, J.G. and WEIDEMANN, M.J. (1976)  
Phytohaemagglutinin stimulation of rat thymus lymphocyte glycolysis.  

Antibody formation by single cells from lymph nodes and efferent lymph of sheep.  
J. Exp. Med. 124, 701-714.

DACIE, J.V. and LEWIS, S.M. (1975)  
"Practical Haematology", 5th ed.  
Churchill Livingstone, Great Britain.

DARNELL, J.E. (1968)  
Ribonucleic acids from animal cells.  

Relationship between RNA content and progression of lymphocytes through S phase of cell cycle.  
PHA stimulation of human lymphocytes during amino acid deprivation. Protein, RNA and DNA synthesis. 
J. Cell. Physiol. 91, 357-367.

DAVIES, H.C., KARUSH, F. and RUDD, J.H. (1965)
Effect of amino acids on steady state growth of group A hemolytic streptococcus. 
J. Bact. 89, 421-427.

DAVIS, P.W. and BRINK, F. JR (1942)
Physical instruments for the biologist: microelectrodes for measuring local oxygen tension in animal tissues. 

DAVY, J. (1823)
Observations on air found in the pleura in a case of pneumatothorax: with experiments on the absorption of different kinds of air introduced into the pleura. 
Quoted by Witte, Clauss and Dumont (1967).

DORE, C.F. and BALFOUR, B.M. (1965)
A device for preparing cell spreads. 
Immunology 9, 403-405.

DOUGLAS, S.D. (1972)
Human lymphocyte growth in vitro: morphological, biochemical and immunological significance. 
Int. Rev. Exp. Path. 10, 41-114.

DRINKER, C.K., FIELD, M.E. and WARD, H.K. (1934)
The filtering capacity of lymph nodes. 
J. Exp. Med. 59, 393-405.

DULBECCO, R. and FREEMAN, G. (1959)
Plaque production by the polyoma virus. 
Virology 8, 396-397.

Short-term radiorespirometry of cell suspensions. 
Biochem. J. 144, 487-496.

DUTTON, R.W. (1967)
In vitro studies of immunological responses of lymphoid cells. 

EAGLE, H. (1959)
Amino acid metabolism in mammalian cell cultures. 
Science 130, 432-437.

"Immunoglobulin Synthesis by Lymphoid Cells in the Sheep". 
ENGLISH, L.S., ADAMS, E.P. and MORRIS, B. (1976)
The synthesis and secretion of immunoglobulins by lymphoid cells in the sheep. The primary response to Salmonella lipopolysaccharide.
J. Exp. Med. 144, 586-603.

ESTABROOK, R.W. (1967)
Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios.

The function of lymph nodes in the formation of lymph.
Monogr. Allergy (Karger, Basel) 16, 82-99.

FATT, I. (1964)
Rapid-responding carbon dioxide and oxygen electrodes.
J. Appl. Physiol. 19, 550-553.

Uptake of 3-O-methyl-14C-D-glucose by rat thymic lymphocytes: insensitivity to cortisol and inhibitors of ribonucleic acid and protein synthesis, and sensitivity to cytochalasin B.
J. Reticuloendothel. Soc. 16, 318-326.

The chemical composition of the lipopolysaccharide of Pseudomonas aeruginosa.
Biochem. J. 114, 185-196.

FORD, W.L. and HUNT, S.V. (1978)
The preparation and labelling of lymphocytes.
Blackwell Scientific Publications, Great Britain.

FORSDYKE, D.R. (1967)
Quantitative nucleic acid changes during phytohaemagglutinin-induced lymphocyte transformation in vitro.

Enzymatic studies in the different types of normal and leukemic human white cells.
Blood 18, 317-327.

Factors affecting the early stimulation of uridine uptake by lymphocytes incubated with phytohaemagglutinin.
Biochem. Soc. Trans. 6, 1077-1079.
Use of a continuously recording intravascular oxygen electrode in the newborn.

GOMORI, G. (1955)
Preparation of buffers.
Method. Enzymol. 1, 143.

GORER, P.A. and O'GORMAN, P. (1956)
The cytotoxic activity of isoantibodies in mice.

GOWANS, J.L. (1966)
Life-span, recirculation and transformation of lymphocytes.

GOWANS, J.L. and McGRegOR, D.D. (1965)
The immunological activities of lymphocytes.
Progr. Allergy 2, 1-78.

GRANT, C.K. and CAMERON, B. (1975)
Cytotoxic effector mechanisms detected in the central lymph of sheep following immunisation with allogeneic or xenogeneic cell suspensions.

L-(+)-lactate: determination with lactate dehydrogenase and NAD.

HAGIHARA, B. (1980)
Intravascular oxygen monitoring with a polarographic oxygen cathode.
To be published.

HAGIHARA, B., ISHIBASHI, F., SASAKI, K. and KAMIGAWARA, Y. (1978)
Cellulose acetate coatings for the polarographic oxygen electrode.

HALL, J.G. and MORRIS, B. (1962)
The output of cells in lymph from the popliteal node of sheep.
Quart. J. Exp. Physiol. 47, 360-369.

HALL, J.G. and MORRIS, B. (1963)
The lymph-borne cells of the immune response.
Quart. J. Exp. Physiol. 48, 235-247.

The ultrastructure and function of the cells in lymph following antigenic stimulation.
J. Exp. Med. 125, 91-110.
HARDT, N. and PANIJEL, J. (1976)
DNA synthesis in rabbit spleen cell population stimulated by various doses of concanavalin A. I. Autoradiographic analysis. II. Equilibrium density sedimentation analysis. Exp. Cell Res. 101, 78-86(I), 87-96(II).

The mitotic activation of lymphocytes. Biochemical and immunological consequences.
In "The Cell Cycle in Development and Differentiation" (M. Balls and F.S. Billet, eds), pp. 397 Cambridge University Press. Great Britain.
Quoted by Ling and Kay (1975).

HARPER, H.A. (1973)

HARRIS, R. and NUGENT, M. (1973)
Continuous arterial oxygen tension monitoring in the newborn infant.
J. Pediatrics 82, 929-939.

HARRIS, G. and OLSEN, I. (1976)
Cell division and deoxyribonucleic acid (DNA) synthesis in cultures of stimulated lymphocytes.
Immunology 31, 195-204.

HAUGAARD, N. (1968)
Cellular mechanisms of oxygen toxicity.
Physiol. Rev. 48, 311-373.

HAUGAARD, N., HESS, M.E. and ITSKOVITZ, H. (1957)
The toxic action of oxygen on glucose and pyruvate oxidation in heart homogenates.
J. Biol. Chem. 227, 605-616.

On the synthesis of RNA in lymphocytes stimulated by PHA. The activity of deoxyribonucleoprotein-bound and soluble RNA polymerase.
Eur. J. Biochem. 9, 542-549.

HAY, J.B. (1970)
"The Role of Fixed and Migratory Cells in Immunological Reactions".

The kinetics of antigen-reactive cells during lymphocyte recruitment.

HAY, J.B., LACHMANN, P.J. and TRNKA, Z. (1973a)
Kinetic studies on the production of sensitized lymphocytes and soluble lymph node factors.
HAY, J.B., LACHMANN, P.J. and TRNKA, Z. (1973b)
The appearance of migration inhibition factor and a mitogen in lymph draining tuberculin reactions.

HAY, J.B. and MORRIS, B. (1976)
Generation and selection of specific reactive cells by antigens.

HAYHOE, F.G.J. and QUAGLINO, D. (1966)
Autoradiographic investigations of RNA and DNA metabolism of human leukocytes cultured with phytohaemagglutinin; uridine-5-¹H as a specific precursor of RNA.

HEATH, E.H. (1966)
Leukocyte culture for chromosome analysis. I. Review of the literature.
Cornell Vet. 56, 364
Quoted by Ling and Kay (1975).

HEATLEY, N.G. and WEEKS, J.R. (1964)
Fashioning polyethylene tubing for use in physiological experiments.
J. Appl. Physiol. 19, 542-545.

HEDESKOV, C.J. (1968)
Early effects of phytohaemagglutinin on glucose metabolism of normal human lymphocytes.

HEDESKOV, C.J. and ESMANN, V. (1966)
Respiration and glycolysis of normal human lymphocytes.

A PO₂ catheter microelectrode for in vivo intravascular use.

HELMREICH, E. and EISEN, H.M. (1959)
The distribution and utilization of glucose in isolated lymph node cells.

The continuous culture of bacteria; a theoretical and experimental study.

HILL, D.K. (1948)
Oxygen tension and the respiration of resting frog's muscle.
J. Physiol. 107, 479-495.
An acute effect of high oxygen tension on the uptake of
$^3$H-deoxycytidine into thymocyte deoxyribonucleic acid.
Biochem. Pharmacol. 23, 3216-3218.

HOHORST, H.J. (1957)
Enzymatische bestimmung von L(+) -milchsäure.
Biochem. Z. 328, 509-521.

HOSPODKA, J. (1966)
Biotechnol. Bioenging. 8, 117-134.
Quoted by Brown (1970).

HUCH, A., HUCH, R., NEUMAYER, E. and ROOTH, G. (1972)
Continuous intra-arterial PO$_2$ measurements in infants.
Acta Pediatri. Scand. 61, 722-723.

HUGGETT, A.S.T.G. and NIXON, D.A. (1957)
Use of glucose oxidase, peroxidase and O-dianisidine in
determination of blood and urinary glucose.
Lancet 2, 368-370.

The chromosome constitution of a human phenotypic intersex.

HUNT, S.V. (1978)
Separation of lymphocyte sub-populations.
In "Handbook of Experimental Immunology" (D.M. Weir, ed),
Blackwell Scientific Publications, Great Britain.

A flexible catheter-type oxygen sensor.
J. Appl. Physiol. 37, 435-438.

INDYK, L. (1975)
PO$_2$ in the seventies.
Pediatrics 55, 153-156.

Conversion of glucose and galactose to lipids by normal and
phytohemagglutinin-stimulated lymphocytes.
J. Biochem. (Tokyo) 76, 791-799.

Messenger ribonucleic acid content of phytohaemagglutinin-
treated lymphocytes.
Biochem. Soc. Trans. 4, 783-785.

JAKOBSEN, L.K. (1960)
Quantitative determination of blood glucose using glucose
oxidase and peroxidase.
An indwelling PO₂ electrode used to monitor foetal vascular PO₂ in the chronically catheterized foetal sheep.
J. Physiol. 271, 8p-9p.

The pentose cycle, triose phosphate isomerization, and lipogenesis in rat adipose tissue.

KATZ, J. and WOOD, H.G. (1963)
The use of ^14C yields from glucose-1 and -6-C¹⁴ for the evaluation of the pathways of glucose metabolism.
J. Biol. Chem. 238, 517-523.

KAY, J.E. (1966)
RNA and protein synthesis in lymphocytes incubated with PHA.
In "The Biological Effects of PHA" (M.W. Elves, ed), pp. 37.
R. Jones and A. Hunt Orthopaedic Hospital, Oswestry.
Quoted by Ling and Kay (1975).

KAY, J.E. (1968)
Phytohaemagglutinin: an early effect on lymphocyte lipid metabolism.

KAY, J.E., AHERN, T. and ATKINS, M. (1971)
Control of protein synthesis during activation of lymphocytes by PHA.

KAY, J.E., AHERN, T., LINDSAY, V.J. and SAMPSON, J. (1975)
The control of protein synthesis during the stimulation of lymphocytes by phytohaemagglutinin. III. Poly(U) translation and the rate of polypeptide chain elongation.

KEMPNER, W. (1937)
Quoted by Hill (1948).

KEMPNER, W. (1939)
Quoted by Hill (1948).

Monitoring of PO₂ in human blood.

KREBS, H.A. and DE GASQUET, P. (1964)
Inhibition of gluconeogenesis by α-oxo acids.
Biochem. J. 90, 149-154.
Renal glucoioeneration. The effect of diet on the
gluconeogenic capacity of rat-kidney-cortex slices.

KREUZER, F., HARRIS, E.D. JR. and NESSLER, C.G. JR (1960)
A method for continuous recording in vivo of blood oxygen
tension.
J. Appl. Physiol. 15, 77-82.

The flow and composition of lymph from the mammary gland in
merino sheep.
Quart. J. Exp. Physiol. 46, 206-215.

LEHNINGER, A.L. (1975)
"Biochemistry", 2nd ed.

Energy metabolism in thymic lymphocytes of normal and leukemic
AKR mice.
Cancer Res. 38, 1113-1119.

LEPINE, et al. (1909)
"Le diabête sucré", Paris.
Quoted by Levine and Meyer (1912).

Oxygen electrode measurements in biochemical analysis.
In "Methods of Biochemical Analysis" (D. Glick, ed), vol. 17,
pp. 1-30.

LEVINE, P.A. and MEYER, G.M. (1912)
The action of leukocytes on glucose.
J. Biol. Chem. 11, 361-370.

LI, J.G. and OSGOOD, E.E. (1949)
A method for the rapid separation of leukocytes and nucleated
erythrocytes from blood or marrow with a phytohaemagglutinin
from red beans (Phaseolus vulgaris)
Blood, 4, 670-675.

LINDAHL-KIESSLING, K. and BOOK, J.A. (1964)
Effects of phytohaemagglutinin on leukocytes.
Lancet 2, 591.

LINDAHL-KIESSLING, K. and MATTSSON, A. (1971)
Mechanism of phytohemagglutinin (PHA) action. IV. Effects
of some metabolic inhibitors on binding of PHA to lymphocytes
and the stimulatory potential of PHA-pretreated cells.
LINDAHL-KIESSLING, K., WERNER, B. and BOOK, J.A. (1963)  
Short-term cultivation of human thoracic duct lymphocytes  
with Phaseolus vulgaris extract.  
Meeting of the Medical Society of Uppsala.  
Quoted by Ling and Kay (1975).

LING, N.R. and HOLT, P.J.L. (1967)  
The activation and reactivation of peripheral lymphocytes  
in culture.  

LING, N.R. and KAY, J.E. (1975)  
"Lymphocyte Stimulation".  

LINZELL, J.L. (1960)  
The flow and composition of mammary gland lymph.  

LONDON, I.M. (1960-61)  
The metabolism of the erythrocytes.  
Harvey Lect. 56, 151-189.

LONGMUIR, I.S. (1954)  
Respiration rate of bacteria as a function of oxygen concentration.  
Biochem. J. 57, 81-87.

LONGMUIR, I.S. (1957)  
Respiration rate of rat-liver cells at low oxygen concentrations.  

LONGMUIR, I.S., MILESI, J. and BOURKE, A. (1960)  
Diffusion of oxygen through a bacterial cell wall.  

MACHAFFIE, R.A. and WANG, C. (1964)  
Carbohydrate metabolism in lymphocytes.  
Amer. College Health Ass. J. 13, 126-127.

The effect of phytohemagglutinin upon glucose catabolism  
in lymphocytes.  
Blood 29, 640-646.

MACLEAN, H. and WEIR, H.B. (1915)  
The part played by the different blood elements in glycolysis.  
Biochem. J. 9, 412-419.

MAXIMOW, A.A. (1902)  
Quoted by Ling and Kay (1975).

MELLMAN, W.J. (1965)  
"Human Peripheral Blood Leukocytes Cultures".  
Rapid changes in initiation-limited rates of protein
synthesis in rat thymic lymphocytes correlate with
energy charge.

MILTON, J.D., COOPER, E.H. and HALLE-PANNENKO, O. (1965)
Thymidine degradation by altered lymphocytes.
Quoted by Ling and Kay (1975).

MITCHELL, J. (1964)
Autoradiographic studies of nucleic acid and protein
metabolism in lymphoid cells. I. Differences amongst
members of the plasma cell sequence.

Transport of RNA from nucleus to cytoplasm following
mitogenic stimulation of human lymphocytes.

MONOD, J. (1942)
Récherches sur la croissance des cultures bactériennes.
Hermann et Cie, Paris.
Quoted by Button and Garver (1966)

MONOD, J. (1949)
The growth of bacterial cultures.

MONOD, J. (1950)
La technique de culture continue: théorie et applications.
Ann. Inst. Pasteur 79, 890
Quoted by Button and Garver (1966).

MORRIS, B. (1966)
Lymphoid cells - their role in the establishment of systemic
immunity.
Proc. XIth Congress of the International Society of
Haematology.

McCABE, M. and GILBERT, D.A. (1965)
Kinetic parameters for oxygen in reactions involving
purified oxidase and tissue slices.
Nature 208, 450-452.

NAGY, S. et al. (1969)
Acta Physiol. 35, 87.
Quoted by Altman and Dittmer (1974).

NATVIG, J.B., PERLMANN, P. and WIGZELL, H. (1976)
"Lymphocytes. Isolation, Fractionation and Characterization".
(Natvig et al., eds).
NEIMAN, P.E. and MACDONNELL, D.M. (1971)  
Studies on the mechanism of increased protein synthesis in human PHA stimulated lymphocytes.  
Proc. 5th Leucocyte Culture Conf., p.61.  
Quoted by Ling and Kay (1975).  

NOWELL, P.C. (1960)  
Phytohaemagglutinin: an initiator of mitosis in cultures of normal human lymphocytes.  
Cancer Res. 20, 462-466.  

OPPENHEIM, J.J. (1968)  
Relationship of in vitro lymphocyte transformation to delayed hypersensitivity in guinea pigs and man.  

PACHMAN, L.M. (1967)  
The carbohydrate metabolism and respiration of isolated small lymphocytes.  
Blood 30, 691-706.  

PAPPENHEIMER, A.M. (1917)  
Experimental studies upon lymphocytes. I. The reactions of lymphocytes under various experimental conditions.  

PARKES, A.B. and HOWELLS, R.D. (1975)  
Glycolytic enzyme levels in phytohaemagglutinin stimulated porcine peripheral lymphocytes.  
Biochem. Biophys. Res. Commun. 64, 1231-1236.  

PELEIDERER, G. and DOSE, K. (1955)  
Eire enzymatische bestimmung der L(+)-milchsäure mit milchsauredehydrase.  
Biochem. Z. 326, 436-441.  

PETERS, J.H. and HAUSEN, P. (1971)  
Effects of phytohaemagglutinin on lymphocyte membrane transport.  

RNA synthesis and histone acetylation during the course of gene activation in lymphocytes.  

Glycolysis as an energy source for stimulation of lymphocytes by phytohemagglutinin.  

"Studies on the Regulation of the Immune Response".  


RUBIN, A.O. and COOPER, H.L. (1965)
Evolving patterns of RNA metabolism during transition from resting state to active growth in lymphocytes stimulated by phytohemagglutinin.

Method for collection and determination of $^{14}$CO$_2$ for in vitro metabolic studies.
J. Lipid Res. 7, 566-567.

Alterations in hexose monophosphate shunt during lymphoblastic transformation.

SAID, S.I., DAVIS, R.K. and BANERJEE, C.M. (1965)
Pulmonary lymph: demonstration of its high oxygen tension relative to systemic lymph.

Metabolic crowding effect in suspension of cultured lymphocytes.
Blood 50, 337-346.

SEVERINGHAUS, J.W. (1968)
Measurements of blood gases: PO$_2$ and PCO$_2$.

SIGMA TECHNICAL BULLETIN, NO. 510
Glucose: enzymatic colorimetric (425-475 nm) determination.

SIGMA TECHNICAL BULLETIN, NO. 826 UV
Lactic acid: quantitative determination (340 nm).

SLOSSE, A. (1912)
Arch. Internat. Physiol. 11, 154.
Etude sur la glycolyse aseptique dans le sang.
Quoted by Barron and Harrop (1929).

SMITH, J.B. (1967)

The role of the lymphatic system and lymphoid cells in the establishment of immunological memory.

SMITH, J.L., LAWTON, J.W.M. and FORBES, I.J. (1967)
Characteristics of protein synthesis in vitro by lymphocytes from human peripheral blood.
The traffic of cells through tissues: a study of peripheral lymph in sheep.
J. Anat. 107, 87-100.

SMITH, J.B. and MORRIS, B. (1970)
The response of the popliteal lymph node of the sheep to swine influenza virus.

SÖREN, L. and BIBERFELD, P. (1973)
Quantitative studies on RNA accumulation in human PHA-stimulated lymphocytes during blast transformation.

SOSKIN, S. and LEVINE, R. (1952)
Carbohydrate Metabolism. Revised ed.
The University of Chicago Press, Chicago.

A system for monitoring arterial oxygen tension in sick newborn babies.

DNA synthesis in tonsil lymphocytes. I. Changes in cell population during culture.

Oxygen tension of nearly pure pulmonary lymph in unanesthetized sheep.
Lymphology 7, 96-100.

Dissolved oxygen measurement in yeast propagation.

SUTER, D. and WEIDEMANN, M.J. (1975)

SUTER, D. and WEIDEMANN, M.J. (1976)
Regulation of carbohydrate metabolism in lymphoid tissue. Nature of the endogenous substrate and their contribution to the respiratory fuel of the sliced rat spleen in vitro.

Adenosine metabolism in phytohaemagglutinin-stimulated human lymphocytes.


WANG, C.H. (1972)
Radiorespirometric methods.
In "Methods in Microbiology" (J.R. Norris and D.W. Ribbons, eds.), vol. 6B, pp. 185-230.

Quoted by Duncombe (1974).

The catabolic fate of glucose in Bacillus subtilis.
J. Biol. Chem. 237, 3614-3622.

WANG, T., MARQUARDT, C. and FOKER, J. (1976)
Aerobic glycolysis during lymphocyte proliferation.
Nature 261, 702-705.

Comparative study of glucose catabolism by the radiorespirometric method.

WARBURG, O. and KUBOWITZ, F. (1931)
Biochem. Z. 214, 5.
Quoted by Hill (1948).

WEAST, R.C. (1976-1977)
"Handbook of Chemistry and Physics", 57th ed.
Chemical Rubber Publishing Co., Ohio.

Effect of concanavalin A on the oxidative metabolism of rat thymus lymphocytes.

WESTPHAL, V.O., LÜDERITZ, O. and BISTER, F. (1952)
Über die extraktion von bakterien mit phenol-wasser.

WHITE, A., HANDLER, P. and SMITH, E.L. (1973)

WINZLER, R.J. (1941)
Quoted by Hill (1948)

WISE, W.C. (1978)
Amino acid transport in thymic- and spleen-derived lymphocytes.

WITTE, C.L., CLAUSS, R.H. and DUMONT, A.E. (1967)
Respiratory gas tensions of thoracic duct lymph: an index of gas exchange in splanchnic tissues.
WITTE, C.L., COLE, W.R., CLAUSS, R.H. and DUMONT, A.E. (1968)
Splanchnic tissue oxygenation: estimation by thoracic duct
lymph PO$_2$.
Lymphology 1, 109-116.

WITTE, C.L., CONNELL, P.N. and WITTE, M.H. (1976)
Direct on-line monitoring of the hepatic tissue oxygen
tension: a new technique.
J. Surg. Res. 21, 33-44.

Estimation of pathways of carbohydrate metabolism.

Activation of 3-O-methyl-glucose transport in rat thymus
lymphocytes by concanavalin A. Temperature and calcium ion
dependence and sensitivity to puromycin but not to cycloheximide.