THE PATHOPHYSIOLOGY OF
TRYPANOSOMA BRUCEI BRUCEI IN THE
RABBIT CIRCULATION

by

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A thesis submitted for the degree of PhD from the
University of London

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To Mum and Dad

with much love
ABSTRACT

The Pathophysiology of *Trypanosoma brucei brucei* in the rabbit circulation

This study presents data on pathophysiological changes occurring in the microvasculature during the infection. Significant accumulations of labelled albumin occurred in heart, kidney, ear and skeletal muscle and to a lesser extent in skin, lung, aorta and mesentery. These changes, thought to indicate increased vascular permeability to macromolecules, were often correlated with the length of the infection. Evidence is presented for alterations to the interstitial matrix, affecting protein exclusion properties.

Trypanosomiasis in the rabbit was found to be characterized by hypotension and increased plasma volume but no oedema of the tissues studied except in cremaster muscle.

An *in vivo* microscopic study on the mesenteric microvasculature of infected rabbits was undertaken. Infected rabbits showed evidence of inflammation including leucocyte rolling and sticking, red cell sludging and cellular infiltration of the mesenteric tissue. Visible signs of increased permeability to Evans' blue dye were not seen although some signs of increased permeability to $^{125}$I-albumin had already been found.

Anaemia is a well known feature of trypanosomiasis and, in view of this, some estimation of plasma erythropoietin were made. Results were inconclusive though there was some evidence for increased erythropoietin levels as the infection progressed.
The effects of some trypanosome-derived immune complexes on arterial blood pressure were studied. The response to injection of in vitro formed immune complexes was negatively correlated with the length of infection.
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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>1</td>
</tr>
<tr>
<td>Dedication</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>5</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>6</td>
</tr>
<tr>
<td>List of Figures</td>
<td>14</td>
</tr>
<tr>
<td>List of Tables</td>
<td>16</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>17</td>
</tr>
<tr>
<td>1.1 Object of study</td>
<td>17</td>
</tr>
<tr>
<td>1.2 The importance of trypanosomiasis</td>
<td>20</td>
</tr>
<tr>
<td>1.3 The vector-parasite interaction</td>
<td>22</td>
</tr>
<tr>
<td>1.3.1 The parasite life cycle</td>
<td>22</td>
</tr>
<tr>
<td>1.3.2 The tsetse fly</td>
<td>22</td>
</tr>
<tr>
<td>1.3.3 Parasite taxonomy</td>
<td>24</td>
</tr>
<tr>
<td>1.3.4 Laboratory parasites</td>
<td>27</td>
</tr>
<tr>
<td>1.3.5 The model</td>
<td>28</td>
</tr>
<tr>
<td>1.4 Antigenic variation</td>
<td>29</td>
</tr>
<tr>
<td>1.5 General pathology</td>
<td>33</td>
</tr>
<tr>
<td>1.5.1 Clinical features of human trypanosomiasis</td>
<td>33</td>
</tr>
<tr>
<td>1.5.2 Diagnosis</td>
<td>35</td>
</tr>
<tr>
<td>1.5.3 Chemotherapy</td>
<td>36</td>
</tr>
<tr>
<td>1.5.3.1 Prophylaxis</td>
<td>38</td>
</tr>
<tr>
<td>1.5.3.2 Mode of action</td>
<td>38</td>
</tr>
<tr>
<td>1.5.3.3 Outlook for chemotherapy of African trypanosomiasis</td>
<td>39</td>
</tr>
</tbody>
</table>
1.5.4 Trypanosomiasis in domestic animals .......... 39
1.5.5 Gross pathology of T.b. brucei 427 in rabbits .... 41

1.6 Immunopathology .................................. 43
1.6.1 Immune complexes ................................ 43
1.6.1.1 Detection of immune complexes ............... 45
1.6.1.2 Circulating immune complexes .................. 45
1.6.1.3 Immune complex deposition in tissues .......... 47
1.6.2 Immunosuppression ................................ 48
1.6.2.1 B cell system ................................ 49
1.6.2.2 T cell system ................................ 51
1.6.2.3 The mononuclear phagocyte system (MPS) ...... 52
1.6.3 Protective immunity ................................ 55
1.6.4 Autoimmunity .................................... 56
1.6.5 Trypanotolerance .................................. 57

1.7 Microcirculation .................................... 58
1.7.1 Structure ....................................... 59
1.7.1.1 Basement membrane ............................ 60
1.7.1.2 Interstitium .................................. 60
1.7.2 Transport ....................................... 61
1.7.2.1 Quantitative transport ......................... 61
1.7.2.2 Qualitative transport .......................... 67
1.7.3 Microvascular heterogeneities .................... 73
1.7.3.1 Red cell transit times from arteriole to venule .. 73
1.7.3.2 Red cell distributions .......................... 73
1.7.4 Blood rheology .................................. 75
1.7.4.1 Viscosity ..................................... 75
1.7.4.2 Haematocrit .................................. 77
1.7.4.3 Fahraeus effect .............................................78
1.8 The immunology of inflammation ..........................79
  1.8.1 The complement system (C') ..........................79
  1.8.2.1 The coagulation system ............................82
  1.8.2.2 Fibrin(ogen)olysis ................................82
  1.8.2.3 Coagulation and fibrinolysis in trypanosomiasis ...85
  1.8.3.1 The kinin/kallikrein system ........................86
  1.8.4 Inflammation .............................................90

1.9 Detailed pathological considerations ...................95
  1.9.1 Early events ...........................................95
  1.9.2 Lymph nodes ..........................................96
  1.9.3 Spleen .................................................99
  1.9.4 Liver ..................................................99
  1.9.5 Rabbit ears ...........................................100
  1.9.6 Heart ..................................................101
  1.9.7 Kidney .................................................102
  1.9.8 Skeletal muscle ......................................103
  1.9.9 Genitalia ..............................................105
  1.9.10 Blood ................................................105
    1.9.10.1 Anaemia ..........................................105
    1.9.10.2 Thrombocytopenia ...............................105
    1.9.10.3 Red cell abnormalities .........................110

1.10 Experimental determination of microvascular permeability .............................................111
  1.10.1 Single vessels ....................................111
  1.10.2 Indicator diffusion method ........................112
1.10.3 Isogravimetric methods ........................................... 113
1.10.4 Tissue uptake methods ........................................... 115
1.10.5 Transcapillary escape rate ....................................... 116

2 PERMEABILITY STUDIES ............................................... 117
2.1 Introduction ............................................................. 117

2.2 Materials and methods ............................................... 119
2.2.1 Parasites ............................................................. 119
2.2.2 Infection of rabbits ................................................. 120
2.2.3 Preparation of $^{51}$Cr-RBC ....................................... 121
2.2.4 Preparation of $^{125}$I-albumin .................................... 124
2.2.5 Procedure ............................................................. 124
2.2.6 Radioactivity measurements ........................................ 126
2.2.7 Tests of assumptions ............................................... 127
2.2.8 Index of infection and C-reactive protein estimations ......... 128
2.2.9 Calculations ............................................................ 129
2.2.9.1 Whole body data ................................................. 129
2.2.9.2 Tissue-specific data ............................................. 130
2.2.10 Extravascular albumin ............................................. 133
2.2.11 Tissue blood fraction .............................................. 134
2.2.12 $^{125}$I-albumin in plasma ......................................... 134
2.2.13 Water contents and dry weights ................................... 134
2.2.14 Lymph ................................................................. 135
2.2.15 Statistics ............................................................. 135

2.3 Results ................................................................. 137
2.3.1 Whole body data ..................................................... 137
2.3.1.1 Arterial haematocrit ................................................. 137
2.3.1.2 Whole body haematocrit ......................................... 137
2.3.1.3 Blood, plasma and red cell fractions ....................... 137
2.3.1.4 $^{125}$I-albumin in plasma ........................................ 139
2.3.1.5 Cx-reactive protein .................................................. 139
2.3.2 Tissue specific data ..................................................... 142
2.3.2.1 Tissue blood fraction ............................................... 142
2.3.2.2 Tissue water content ............................................... 142
2.3.2.3 Extravascular $^{125}$I-albumin ................................... 147
2.3.3 Lymph ................................................................. 159

2.4 Discussion ................................................................. 161
2.4.1 Whole body parameters ............................................... 161
2.4.1.1 Cx-reactive protein .................................................. 161
2.4.1.2 Transcapillary escape rate (TER) of $^{125}$I-albumin from plasma ........................................... 162
2.4.1.3 Haematological data ............................................... 164
2.4.2 Tissue water content .................................................. 169
2.4.3 Tissue blood fractions ................................................. 171
2.4.4 Tissue containing continuous capillaries ....................... 174
2.4.4.1 Heart ................................................................. 174
2.4.4.2 Abdominal wall muscle ............................................ 175
2.4.4.3 Ear ................................................................. 176
2.4.4.4 Cremaster muscle .................................................. 177
2.4.4.5 Scrotal skin ......................................................... 178
2.4.4.6 Mesentery ......................................................... 179
2.4.4.7 Aorta .............................................................. 180
2.4.4.8 Lung .............................................................. 181
2.4.4.9 Kidney ............................................................ 181
2.4.5 Tissues containing discontinuous capillaries .....188
2.4.5.1 Spleen ........................................ 188
2.4.5.2 Liver ........................................189
2.4.6 Lymph ........................................ 190
2.5 Summary ........................................ 192

3. IN VIVO OBSERVATIONS OF THE MESENTERIC MICROVASCULATURE
3.1 Introduction ..................................194
3.2 Materials and methods .......................... 196
3.2.1 Animals .......................................196
3.2.2 Procedure .....................................196
3.2.3 Precautions ...................................199
3.3 Results .......................................201
3.3.1 General observations ........................... 201
3.3.2 Permeability ..................................204
3.4 Discussion ....................................207

4. EFFECTS OF TRYPANOSOME-DERIVED MATERIAL IN THE SYSTEMIC
CIRCULATION ...................................213
4.1 Introduction ...................................213
4.2 Materials and methods ..........................215
4.2.1 Parasites ..................................... 215
4.2.2 Parasite hosts .................................215
4.2.3 Immunologic procedures ......................215
4.2.3.1 Preparation of trypanosome antiserum .......... 215  
4.2.3.2 Agglutination test ............................ 217  
4.2.3.3 Precipitation test ................................ 217  
4.2.3.4 Preparation of IgG ............................. 218  
4.2.3.5 Preparation of IgM ............................. 218  
4.2.4 Preparation of immune complexes ............... 218  
(a) Crude complexes .................................. 218  
(b) IgG and IgM complexes ........................... 219  
(c) Live complexes ................................... 219  
(d) Nature of the immune complexes .................. 219  
4.2.5 Procedure ...................................... 219  
4.2.6 Statistics ...................................... 221  

4.3 Results ........................................ 222  
4.3.1 Arterial blood pressure .......................... 222  
4.3.2 Reaction of arterial blood pressure to challenges . 222  
4.3.2.1 Disrupted and partially disrupted trypanosomes ... 222  
(a) Control rabbit .................................... 222  
(b) Infected rabbit ................................... 225  
(c) Sensitized rabbit .................................. 225  
4.3.2.2 Bradykinin .................................... 227  
(a) Control rabbits .................................. 227  
(b) Sensitized rabbit .................................. 229  
4.3.2.3 Live trypanosomes .............................. 229  
(a) Infected rabbits ................................. 229  
(b) Sensitized rabbit .................................. 232  
4.3.2.4 Crude complexes ................................ 232  
(a) Control rabbits .................................. 232  
(b) Infected rabbits .................................. 235  
(c) Sensitized rabbit .................................. 238
4.3.2.5 IgG complexes (IgGIC) .................................................. 240
(a) Control rabbits .................................................. 240
(b) Infected rabbits ................................................ 244
4.3.2.6 IgM complexes (IgMIC) ................................................. 244
4.3.2.7 Trasylol .................................................. 247

4.4 Discussion ................................................................. 250
4.4.1 Blood pressure measurements ........................................... 250
4.4.2 The involvement of the kinin/kallikrein system ... 254
4.4.3 The formation and nature of complexes .................. 257

5. Erythropoietin .............................................................. 261
5.1 Introduction ............................................................. 261
5.2 Erythropoietin assay ...................................................... 261
5.3 Animals ................................................................. 262
5.4 Results ................................................................. 263
5.5 Discussion .............................................................. 263

6. Concluding remarks .......................................................... 265

Appendix 1 Buffers and solution ........................................... 270
Appendix 2 ............................................................. 274

References ................................................................. 282
**LIST OF FIGURES**

1.1 Parasite life cycle ..................................23
1.2 Capillary transport pathways .........................62
1.3 Pathways across the membrane .......................68
1.4 Transport of dextrans in dog leg ....................69
1.5 Complement activation ................................80
1.6 Coagulation system ..................................83
1.7 The interaction of the Hageman factor-dependent pathways ..................................84
1.8 Acute phase proteins ................................87
1.9 Trypanosome pathology ..............................97

2.1 Plasma levels of $^{125}$I albumin .....................140
2.2 Cx reactive protein titres ...........................141
2.3 Tissue blood fraction ...............................143
2.4 Tissue blood fraction (corrected for tissue hct) .144
2.5 Tissue water contents ...............................145
2.6 Tissue water:dry weight ratios .......................146
2.7 E/P ratios for heart ...............................148
2.8 E/P ratios for abdominal wall muscle ...............149
2.9 E/P ratios for ear ..................................150
2.10 E/P ratios for scrotal skin ..........................151
2.11 E/P ratios for cremaster muscle ....................152
2.12 E/P ratios for mesentery ............................153
2.13 E/P ratios for aorta ................................154
2.14 E/P ratios for lung ................................155
2.15 E/P ratios for kidney ...............................156
2.16 E/P ratios for liver ................................157
1.1 Parasite taxonomy ..................................... 25
1.2 Capillary permeability to various solutes .......... 66
1.3 Red cell transit times .................................. 74
1.4 Biochemical changes ................................... 98

2.1 Clinical presentation of infected rabbits .......... 122
2.2 Distribution of rabbits in experimental groups ... 123
2.3 Whole body haematological data ..................... 138
2.4 Calculation of colloidal osmotic pressure .......... 167
2.5 Tissue haematocrits .................................. 172
2.6 Tissue blood fractions ................................ 173
2.7 Extravascular albumin in the present study and values from the literature ......................... 186

4.1 Clinical presentation of infected rabbits .......... 216
4.2 Initial mean arterial blood pressure in 3 groups of rabbits .............................................. 223
4.3 Effects of disrupted and partially disrupted trypanosomes and bradykinin .......................... 230
4.4 Effects of live trypanosomes ......................... 234
4.5 Effects of crude immune complexes .................. 239
4.6 Effects of IgG - immune complexes .................. 245
4.7 Effects of IgM - immune complexes .................. 248
4.8 Trasylol ................................................. 249
4.9 Arterial blood pressure compared with literature values ...................................................... 251
4.10 Other cardiovascular parameters in trypanosome infections .............................................. 252
2.17 E/P ratios for spleen ........................................ 158
2.18 Lymph .................................................. 160

3.1 Experimental set up for in vivo microscopy .......... 197
3.2 Mesenteric blood vessels in a control rabbit ...... 202
3.3 Mesenteric blood vessels in a rabbit infected for 32 days ........................................ 203
3.4 Mesenteric blood vessels in another rabbit infected for 32 days .................................. 205
3.5 Mesenteric blood vessels in a rabbit infected for 19 days ........................................... 206

Arterial blood pressure traces in rabbits under the following conditions:

4.1 Partially disrupted trypanosome in C1 ........... 224
4.2 Disrupted trypanosomes in I1 .................... 224
4.3 Disrupted trypanosomes in S2 .................... 226
4.4 Bradykinin in C2 .................................... 228
4.5 Bradykinin in S2 .................................... 228
4.6 Live trypanosomes in I4 ......................... 231
4.7 Live trypanosomes in S1 ......................... 233
4.8 Crude complexes in C4 ........................... 233
4.9 Live crude complexes in C5 ....................... 236
4.10 Crude complexes in I8 ........................... 237
4.11 Crude complexes in S3 ........................... 237
4.12 IgG complexes in C7 ............................... 241
4.13 Live trypanosome-IgG complexes in C10 ........ 243
4.14 IgG complexes in I9 ............................... 243
4.15 Soluble IgG complexes in I3 ..................... 246
4.16 IgM complexes in C11 ............................. 246
4.17 Cardiovascular parameters during trypanosome infection ............................................ 255

5.1 Erythropoietin titres .................................. 264
1. INTRODUCTION

1.1 Object of Study

To elucidate some of the fundamental pathophysiological changes occurring in the cardiovascular system of mammals infected with African trypanosomiasis (sleeping sickness).

Trypanosomiasis is an important parasitic disease of many species, in particular, humans and domestic animals. Infection of rabbits with \textit{Trypanosoma brucei brucei} was chosen as the laboratory model for this disease because rabbits are relatively large and the disease is fairly well characterized in this species.

In this study, particular emphasis has been placed on the microvasculature as it is a prime target for damage during the disease. Alterations in physiological parameters of the microcirculation during trypanosomiasis are largely inferred from histological studies (e.g. Edeghere, 1980; Goodwin, 1970; 1971; Goodwin and Hook, 1968; Murray and Morrison, 1980) and from the production of inflammatory mediators known to play a role in microvascular damage (see Richards, 1965; Goodwin and Boreham, 1966; Boreham, 1966; 1979; Tabel, 1982; Basson et al, 1977; Tizard et al, 1980; Nielsen et al, 1979; Facer, 1974.)

Direct physiological studies are not common, possibly due to emphasis on the immunological features of the disease and the limitations of working with humans and large domestic animals.

Goodwin and Hook, (1968) and Goodwin, (1971) carried out pathophysiological and histopathological studies on the ears and cremaster
muscles of rabbits infected with T. b. brucei, and suggested that the major lesion was a chronic angiitis.

Seed, (1969) showed that intradermal injection of trypanosomes caused increased macromolecular permeability in the skin.

Dargie, (1979b) measured transcapillary escape rate (TER) of albumin from the blood in bovine trypanosomiasis.

Other workers measured red cell kinetics (Dargie, 1980; McCrorie et al, 1980; Jenkins and Facer, 1985) and blood, red cell and plasma volumes (Dargie, 1979a; 1980; Amole et al, 1982; Maxie and Valli, 1979).

The present study provides data on pathophysiological changes in the microvascular permeability to macromolecules, complementing histological studies, particularly that of Edeghere, (1980).

The reason for assessing macromolecular transport is that microvessels have a very low but finite permeability to serum proteins, especially serum albumin. If the microvascular wall becomes damaged, large molecules can leak out of the blood vessels and into the tissues. By the use of radiolabelled macromolecules, changes in the permeability of the microvasculature can be assessed quantitatively. Such a technique was adapted for use in rabbits with trypanosomiasis.

Complementary to this part of the study was the observation and photography of living, in situ, mesenteric microvessels. Erythropoietin levels were measured because a major feature of the disease is an anaemia of complex aetiology. Some systemic cardiovascular effects of injected trypanosome-derived material were examined. These minor
sections, however, are mainly qualitative rather than quantitative in nature.

The complexity of the disease and its causative organism necessitates the introduction of a wide variety of topics (microcirculation physiology and pathology, trypanosomes and their vector, host reaction to the disease, diagnosis and treatment) allowing the contributions in this thesis to be viewed in a wider context.
1.2 The Importance of Trypanosomiasis

The African trypanosomiases comprise a group of diseases caused by protozoan parasites, genus *Trypanosoma*, which are transmitted by tsetse flies (genus *Glossina*). Several different species of trypanosome exist, many of which are pathogenic. As many as 45 million people and 80 million cattle, as well as other animals may be at risk (WHO 1976; UNDP/World Bank/WHO 1982; Goodwin, 1985). The disease is endemic in about 7 million Km$^2$ of sub-Saharan Africa (the area of tsetse fly distribution) and is responsible for some of the most serious obstacles in the peacetime social and economic development of the continent, particularly in the case of bovine trypanosomiasis (nagana). Recent wars have exacerbated the problems.

In humans, the disease is called sleeping sickness and may be acute or chronic depending on the strain of the infecting species. It is fatal unless treated, but even then has a high morbidity.

For many years control of trypanosomiasis had been attempted mainly through eradicating the tsetse fly vector. Many workers and aid organizations now recognize that massive applications of toxic insecticides are bringing about environmental deterioration as well as the problems of resistance and tolerance. (Alozie, 1982; Rafatjah, 1982).

As Poltera (1985) points out, further studies of trypanosomiasis in man and experimental animals are needed to clarify the complex pathogenesis of the disease. Such studies may uncover some novel approach to treatment and diagnosis, but may also improve understanding of pathological processes in general.
Trypanosomiasis can be defined as a disease characterized by chronic inflammation (Goodwin, 1970; Ormerod, 1970), a complex and poorly understood phenomenon, applicable to many other disease states.
1.3 The Vector-Parasite Interaction

1.3.1 The Parasite Life Cycle

The parasite vector, the tsetse fly (commonly Glossina palpalis, G. morsitans and G. fuscipes) acquires a lifelong infection after a blood meal from an infected animal. The two mammalian forms of the parasite are known as the slender and stumpy forms. The stumpy forms do not divide, but, on ingestion by the fly, they lose their surface (antigenic) glycoprotein coat and transform into an actively dividing form (the procyclic) in the fly's midgut (see fig. 1.1).

From here they migrate to the salivary glands where they change via the epimastigote stage to the metacyclic form. The metacyclic parasite has an antigenic coat and is now infective to the primary host. When the fly next has a blood meal, it inoculates the metacyclics into its victim. Here the metacyclics transform into the rapidly dividing slender forms. These are common in the ascending phase of the parasitaemic peak. The descending phase has many more stumpy forms. Intermediate forms are also known (see review by Vickerman, 1985).

1.3.2 The Tsetse Fly

These are large Dipterans, genus Glossina similar in appearance to large houseflies or horseflies. They have a rather curious life cycle. The fertilized female adult does not produce eggs but lays a single larva about every 9 days. The larva burrows into the soil and pupates. After about a month, a fully grown adult emerges to continue the cycle. This low rate of reproduction may allow for very specific methods of control such as the sterile male technique (Dame and Schmidt,
Figure 1.1 The life cycle of the African trypanosomes in the mammalian (primary) host and vector (secondary host). A red blood cell (RBC) of diameter approx. 7 m is shown to compare size with the parasites.

* actively dividing
The distribution of trypanosome infections amongst the mammalian host population depends on the feeding preferences of the tsetse fly vector. Work in Liberia (Sachs et al., 1980) showed that humans are non-preferred hosts for many tsetse flies. However, Gouteux et al., 1982a,b) showed that for G. palpalis, the feeding habits vary with the flies' age.

A long term study in Zimbabwe has investigated the use of traps baited with chemical attractants mimicking the preferred host (Vale, 1981) and transparent (to the flies) gauze impregnated with insecticide. This cuts down the insecticide needed to about 1% that used for ground spraying (Vale et al, 1985 (in press) cited in Jordan, 1985).

An integrated approach to tsetse control is very attractive but until some of the alternative methods have been more fully developed, the only realistic method is the use of insecticides (Jordan, 1985) still the major control method (Allsopp, 1984).

1.3.3 Parasite Taxonomy

Hoare (1966, 1972) classified the genus Trypanosoma as shown in table 1.1. It is the Trypanozoon sub-genus that most concerns the present study. The so-called T. brucei complex is made up of 3 noso-demes (clinical races) that are morphologically indistinguishable from each other - T. (T.) brucei brucei, T. (T.) b. rhodesiense, and T. (T.) b. gambiense. T. (T.) b. brucei is thought to be the ancestral species.
Table 1.1 Trypanosome Taxonomy (not complete)

After Hoare, (1972).
Although the W.H.O. (1978) recommended the use of a binomial nomenclature (i.e. T. brucei, T. rhodesiense and T. gambiense) recent evidence has suggested that the former two are much more closely related (see below) and hence they will be referred to as T.b. brucei, T.b. rhodesiense, and T.b. gambiense.

These latter two differ from T.b. brucei only in their ability to infect humans. Human serum has a trypanocidal action on T.b. brucei but not T.b. gambiense nor T.b. rhodesiense. This is the basis of the blood incubation infectivity test (B.I.I.T.) (Rickman and Robson, 1970). Isolated cases are known where inoculation of T.b. brucei in humans presented as an acute infection indistinguishable from T.b. rhodesiense (Robertson et al, 1980; Herbert et al, 1980).

Isoenzyme analysis has shown that T.b. brucei and T.b. rhodesiense are much more closely related than T.b. gambiense (Gibson et al, 1980). Indeed, Rickman et al, (1984) have shown that if T.b. brucei infected tsetse flies (G.m. morsitans) are maintained on human serum, the parasites can become human serum resistant (i.e. infective to humans). This suggests that T.b. rhodesiense may just be a human-adapted form of T.b. brucei. Barry (J.D. - personal communication) considers T.b. rhodesiense to be a variant of T.b. brucei. Hudson et al, (1981) found that some T.b. brucei surface antigens are sensitive to human plasma but others not.

Although T.b. brucei infects domestic animals, it is less pathogenic (Goodwin, 1970; Losos and Ikede, 1972) than T. congolense (mainly East Africa) and T. vivax (mainly West Africa), Losos and Ikede, (1972). These species cause the classic wasting disease of cattle, nagana. The geographical locations overlap somewhat so it is not un-
usual to find animals infected with more than one species of trypanosome (Losos and Ikede, 1972) and even more than one parasite genus. Millott and Cox (1985) have studied the effect in mice of infection with trypanosomes, malaria (genus Plasmodium) and babesiosis (genus Babesia) to try and elucidate some of these complicating features.

African pathogenic trypanosomes can be divided into two groups on the basis of their distribution within the mammalian host and the characteristic lesions they produce during the infection (Losos and Ikede, 1972). The haematic group (T. congolense and T. vivax - the major cattle infections) are mainly plasma parasites and the major clinical symptom is a profound anaemia. The humoral group (T.b. brucei, gambiense and rhodesiense) live largely as connective tissue parasites. However, the division is not absolute. Luckins and Gray (1978) provided light and electron microscopic evidence of T. congolense developing and multiplying outside blood vessels. T. vivax is predominantly vascular but can be found in the tissues (Murray and Morrison, 1980). The T. brucei group of trypanosomes produce varying degrees of parasitaemia. In severe, acute infections in rats and mice (in which death usually occurs within 2-3 days), very large numbers of parasites are seen in the peripheral blood with little or no solid tissue invasion. This is in contrast to naturally occurring and chronic infections where trypanosomes are found throughout the connective tissue.

1.3.4 Laboratory Parasites

Under laboratory conditions outside Africa, it is not generally feasible to infect large animals by their natural vector. Isolates of naturally occurring parasite strains are cryopreserved and usually grown in the laboratory by syringe passage in rats and mice (see 2.2.1).
Methods of growing trypanosomes in culture have been developed, (Brun et al, 1978) but had not reached a sufficiently reliable state when the experiments reported here were started. Later developments in trypanosome culture have proved much more successful (Brun et al, 1981; Brun and Jenni, 1985).

1.3.5 The Model

The model of T.b. brucei in rabbits is similar to T.b. rhodesiense infection in humans but there are lower levels of parasitaemia and cerebral symptoms are apparently absent. The relapse of treated infections in humans and animals may be due to parasites in the central nervous system (CNS) (Jennings and Gray, 1983) which makes this rabbit model less than ideal. This feature is not so important in the present study because curative measures were not undertaken.

Obviously no disease model can exactly correspond to that in another species but the generalized tissue lesions and the biochemical and immunological changes are thought to resemble those in humans and other animals.
1.4 Antigenic Variation

The relative lack of success in combating the trypanosomiases is in large part due to the ability of the parasites to change their antigenicity by altering their surface glycoprotein coat. This ability is called antigenic variation.

Metacyclic and blood forms of trypanosomes possess a 12-15 nm thick coat (Vickerman, 1984) which is shed and replaced by another antigenically dissimilar glycoprotein. In chronic infections, this gives rise to a series of trypanosome populations causing fluctuations in parasitaemia as the variant specific glycoproteins (VSG) are produced (Vickerman, 1978), each population in turn being subject to immunological attack. Van Meirvenne et al (1975) showed that clones of trypanosomes produce a distinct repertoire of VSG's, some of which may be common to different clones.

It appears that there are two mechanisms involved in the emergence of the parasitaemic peaks, the change in the glycoprotein coat of a trypanosome arising spontaneously (genetically controlled) and selection pressure provided by the hosts' immune response (Diggs, 1982).

The molecular biology and genetic control of antigenic variation have been extensively studied (Cross, 1975; Agabian et al, 1980; Hoeijmakers et al, 1980; Pays et al, 1981; Williams et al, 1979 and reviewed by Turner, 1983; 1985; Steinert and Pays, 1985; Donelson and Turner, 1985).

Each VSG is a matrix of $10^7$ identical glycoprotein molecules, mol. wt. 67,000 daltons, which makes up 10% of the entire cell protein
and 30% of the soluble protein, and contains 7-17% carbohydrate, (Cross, 1975).

The host produces antibodies to the coat antigens which may kill, for example, 99% of the trypanosome population. One percent of the parasites escape (because of their different VSG) which divide and multiply to give a new population. The host again mounts an immunological attack and again a small proportion escapes to form the basis of a new population. This usually recurs until death. A further complication is the heterogeneity of the inoculated trypanosomes thus producing many variants simultaneously.

Each VSG is thought to be the result of expression of a single gene. Many VSG's have been characterized but there may be several hundred in the repertoire indicating that 5-10% of the total genetic complement may be involved in antigenic variation. (Donelson and Turner, 1985; Steinert and Pays, 1985).

Briefly, the gene product is a 500 amino acid (AA) protein. The first 20-30 AA is a signal peptide allowing the VSG to spread across the surface of the trypanosome and then the peptide is cleaved off leaving 470-480 AA. The next 360 AA comprise the variable region and are VSG specific. The C-terminal 120 AA belong to two different homology groups. The last 20 AA are not present in mature VSG and the anchoring function is done by an oligosaccharide attached to a phosphoglyceride and two fatty acid chains. These two chains penetrate the cell membrane and hold the VSG in place. The anchoring mechanism is the same for all VSG and provides a rapid and universal mechanism for stripping off one coat and substituting another. The non-variable regions could stimulate the production of antibodies common to different variants but are usually
covered up by the variable regions (Donelson and Turner, 1985).

The process of antigenic variation has been studied by the use of well-defined clones. In natural infections a given population of blood metacyclic parasites is heterogenous in terms of antigenic coat (Esser et al, 1981; Barry et al, 1979). The metacyclic population in a tsetse fly may be much more limited than in a mammalian infection (Hadjuk et al, 1981). Jenni, (1977) and Hudson et al, (1980) suggested that the metacyclics of a clone lineage express a single "basic antigenic type". Le Ray et al (1977; 1978), Barry et al, (1979) and Hadjuk and Vickerman, (1981) presented evidence that the metacyclics of one particular clone (AnTaRl serodeme of T.b. brucei) are heterogenous with respect to the VSG.

The cyclical relapsing nature of trypanosome infections, during which the host's immune system repeatedly destroys many parasites, allows the release of many potentially antigenic materials. These are the common antigens, immunogenic molecules making up the parasite organism, such as nucleoproteins, enzymes, structural proteins, etc. These tend to remain common to the same trypanosome species throughout its development (Lumsden, 1972).

At each peak of parasitaemia, release of common antigens by the host's destruction of the parasites allows the production of trypanosome-specific antibodies. These facilitate the rapid elimination of trypanosome debris. When animals are immunized with disrupted trypanosomes, antibodies are produced primarily against these antigens. Although used in immunodiagnosis, they seem to have little relevance to protective immunity and have received little attention (Terry, 1976).
Parasites also shed their surface antigens (exo-antigens - Weitz, 1960) which provoke antibody production (Herbert and Inglis, 1973; Herbert and Lumsden, 1968) though Black et al, (1982) have suggested that antigen still attached to the parasite surface is more important in this respect.

The outlook for a vaccine seems fairly gloomy. Even the limited antigenic variation in the metacyclic populations (Hadjuk and Vickerman, 1981; Vickerman, 1985) poses a formidable obstacle for vaccination (Goodwin, 1985). Further research into the molecular biology of the parasites may reveal antigen(s), immunity to which confers protection against the disease.
1.5 General Pathology

1.5.1 Clinical Features of Human Trypanosomiasis

*T.b. gambiense* causing the chronic form of human trypanosomiasis, tends to occur largely in western and central areas of Africa, particularly near lakes, rivers and waterholes. The chronicity of this disease (death occurring, when untreated, 2-3 years or more after the initial infection) makes for a prolonged period of cerebral trypanosomiasis from which the common designation, sleeping sickness, arose.

By way of contrast, *T.b. rhodesiense* occurs in central, eastern and southern Africa and causes an acute form of trypanosomiasis with death occurring within 3-9 months of initial infection. Whereas the Gambian form appears to be largely confined to humans, Rhodesian trypanosomiasis can infect a variety of wild animals as well as man.

The bite by an infected tsetse fly can give rise to a lesion at the inoculation site 5-15 days later. This is the first stage of the disease. There is erythema and tenderness and, histologically, the lesion (the trypanosome chancre) shows inflammatory infiltration of leucocytes.

The second stage is where the trypanosomes invade the tissues and bloodstream. It has recently been shown that parasites enter the lymphatic system before the bloodstream and then enter the tissues (Barry and Emery, 1984). A fever may occur when the parasites enter the bloodstream and invasion of the connective tissue commences. In both types of human trypanosomiasis, peaks of parasitaemia can be detected in the peripheral blood but often in *T.b. gambiense* infections,
the parasitaemia is so low as to be undetectable by microscopic examination of a simple wet blood film during much of the infection. *T.b. rhodesiense* infection has a much higher parasitaemia. Irregular pyrexia occurs at intervals coinciding with parasitaemic peaks. Headache and joint pain may occur with episodes of hyperaesthesia. The patient becomes more debilitated with anaemia, thrombocytopenia, splenomegaly and lymph node enlargement. In particular, posterior cervical lymphadenopathy (Winterbottom's sign) is a classic symptom of the more chronic infections.

Other symptoms occur when trypanosomes invade particular organs. Nephritis, myocarditis, fatty degeneration in liver, generalized swelling of the face and extremities, emaciation and, later on, damage to the CNS are all common manifestations of the disease. In *T.b. gambiense* these changes may be spread over a few years and, in fact, may not be really noted by the patient until cerebral damage occurs (Robinson *et al.*, 1980). However, with *T.b. rhodesiense* infections, the patient can rapidly become severely ill. Clinical features of both diseases are well established but do vary from patient to patient (Basson *et al.*, 1977) and intermediate forms of the disease are also known to exist (Apted, 1970).

The third stage of trypanosomiasis is invasion of the central nervous system. There is general chronic inflammation of the brain, meninges and spinal cord (meningoencephalomyelitis). The outward signs are of gradual loss of central control such as unsteady gait, lack of facial expression, languor, pronounced oedema of the face, speech difficulties, progressive tremor of tongue and hands, and later on, convulsions, severe muscular wastage, coma and death. Death may occur from secondary infections. For further information see section 1.9;
Other features of the disease include macroglobulinaemia, mainly fibrinogen and IgM (causing increased plasma viscosity and red cell rouleaux), anaemia, generalized immune stimulation and paradoxical immune suppression.

1.5.2 Diagnosis

Early diagnosis in natural infections is important because the T. brucei sub-group trypanosomes frequently invade the CNS in the later stages of the diseases. Acute trypanosomiasis in humans caused by T.b. rhodesiense can have a high parasitaemia, even in the early stages. Usually a thick blood film will demonstrate parasites (Baker, 1970). However, since the parasitaemia varies, a rapid, reliable method of concentrating blood (or lymph node aspirate) is often necessary to detect the parasites (Van Meirvenne and Le Ray, 1985). This is particularly true for suspected T.b. gambiense infections where parasites are usually undetectable in peripheral blood preparations. Lanham, (1968) developed an anion exchange method for separating trypanosomes from rodent blood. This was adapted and miniaturized for clinical use in the field by Lumsden et al, (1979). A method based on immunodiffusion, the microcounterimmunoelctrophoresis method (Taylor and Smith, 1983) may prove useful in the field.

To exclude cerebral involvement, the CSF must be examined (Van Meirvenne and Le Ray, 1985). If parasites are not detected, raised IgM in particular, protein and leucocytes (greater than 5/mm$^3$) suggest trypanosomiasis (Manson-Bahr and Apted, 1982).
Differential leucocyte count can also indicate the stage of damage. The presence of plasma cells indicates long standing damage whereas raised lymphocyte counts indicate recent changes (Manson-Bahr and Apted, 1982). IgM can be measured fairly easily and correlates well with the state of the infection and remains elevated in treated but relapsing infections (Whittle et al, 1977).

1.5.3 Chemotherapy

Once diagnosis has been established and the stage of the disease confirmed, chemotherapy can commence (Manson-Bahr and Apted, 1982).

In early stage T.b. rhodesiense and T.b. gambiense infections, suramin is the usual choice though pentamidine (in the isethionate and methanesulphonate forms) is the preferred drug for early chronic gambiense trypanosomiasis.

Both these drugs have to be given by injection and are only effective in the early stages since they do not penetrate the CNS. Pentamidine is administered intramuscularly at a daily dose of 3-4 mg of base/Kg body weight for about 7-10 days. It can cause immediate hypotension and faintness (due to release of histamine) but this can be overcome by slow infusion of adrenaline. Side effects include irritation and pain at the injection site, hypoglycaemia and renal damage.

Suramin is extremely irritant when injected intramuscularly and the usual route is by careful intravenous injection. The side effects can be severe. In 0.02 to 0.05% of cases, injection is followed immediately by vomiting, shock and collapse, which is occasionally fatal
(Gutteridge, 1985). Other more common side effects include vomiting, rashes, photosensitivity, peripheral neuropathy and renal damage. For this reason, treatment starts after a test dose of 100-200 mg. Thereafter a dose of 1g is given at 7-10 day intervals for about 5 weeks.

Berenil (diaminazene) is more commonly used to cure infections in animals. It is effective in early human infections and may be given orally, but is not fully tested for human toxicity.

Once cerebral involvement has occurred the only drugs available are the organic arsenicals because pentamidine and suramin will not penetrate the blood brain barrier and hence the CNS. These drugs are melarsoprol - Mel B (a compound of a trivalent arsenical, melarsen oxide and dimercaprol (British Anti-Lewisite -BAL)), melarsonyl potassium - Mel W (an analogue of melarsoprol) and tryparsamide (a pentavalent arsenical metabolised to the active trivalent form in vivo). These drugs are particularly toxic - the most serious side effect being a reactive encephalopathy which occurs in more than 10% of patients (with a mortality rate of 5-10%). Melarsoprol is dissolved in propylene glycol and administered strictly intravenously because the diluent is extremely irritant. Mel W is water soluble and can be given intramuscularly or sub-cutaneously. However, its side effects are similar or worse than those of melarsoprol. Both melarsoprol and Mel W can rapidly cure all stages of trypanosomiasis. Tryparsamide is only effective against the Gambian form of the disease, its worst effect being optic nerve damage which is permanent unless treatment is stopped (Gutteridge, 1985).
If a relapse occurs after treatment with suramin or pentamidine, melarsoprol is given.

Furacin (nitrofurazone) may be a useful drug but has not been fully evaluated yet. It can be given orally but is still very toxic. If a relapse after melarsoprol occurs, furacin is the only alternative.

1.5.3.1 Prophylaxis

Intramuscular pentamidine can give protection against T.b. gambiense for about 3-6 months but there is no guarantee of protection against T.b. rhodesiense.

1.5.3.2 Mode of Action

Trypanosomes contain a pentamidine transport system (Damper and Patton, 1976) producing high concentrations of the drug in the parasites interrupting DNA synthesis (particularly kinetoplast DNA) (Gutteridge, 1969; 1985).

Suramin inhibits a number of enzymes, particularly L-a-glycero-phosphate oxidase (unique to trypanosomes). As this is in itself not a lethal event, other routes for ATP synthesis being possible, (Fairlamb and Bowman, 1980), suramin's mechanism of action is unclear (Gutteridge, 1985).

The arsenicals appear to act by inhibiting pyruvate kinase (Flynn and Bowman, 1974) and thereby block the glycolytic pathway for energy production.
1.5.3.3 Outlook for Chemotherapy of African Trypanosomiasis

In a recent review, Gutteridge (1985) provided an essentially pessimistic view of drug treatment for both human African trypanosomiasis. The problems are essentially that, as outlined above, the available drugs are very toxic and must be given by multiple injection. Jennings et al. (1983) had some success in treating the CNS phase of T.b. brucei infection in mice using a combination of suramin followed by doses of 2-substituted 5-nitroimidazoles. Sjoerdsma et al. (1984) have reported a successful clinical trial of α-difluoro-methylornithane (an ornithine decarboxylase inhibitor) in the treatment of trypanosomiasis.

Trypanosomes have peculiar biochemical and molecular biological features (Oppendoes, 1985) which may be exploitable, for example, by using novel drug delivery systems for existing drugs. The difficulty with new drug development is the cost together with the lack of adequate returns for commercial companies (Vane, 1979).

1.5.4 Trypanosomiasis in Domestic Animals

Economically, bovine trypanosomiasis due to infections of T. congolense and T. vivax, is probably the most serious of infections in domestic animals.

The initial course of the infections is similar to that in humans. The actual effects of the parasites in general appear to depend on the strain of the parasite and the genetic susceptibility of the host (Dargie, 1980; Clayton, 1978; Levine and Mansfield, 1981; Whitelaw et al., 1980), though lack of stress may be a factor in trypanotolerance (Tizard, 1982). There are many similarities in the course of the disease
but variations in severity. In natural infections (as opposed to laboratory infections) more than one species of trypanosome may be present (see 1.3.3).

Like the human infections, the initial stages in sheep, goats and cattle after being bitten by an infected tsetse fly are the appearance of the trypanosome chancre, detectable parasites in the blood a few days later, accompanied by a fever, and some lymph node swelling (reviewed by Murray and Morrison, 1980). Lymph node swelling becomes generalized due to proliferation of the lymphoid response. This may continue until the death of the host or resolution of the disease. However, regression can occur where the lymph nodes become small and hard (Masake, 1980; Murray and Morrison, 1980). Splenomegaly also occurs during the lymphoid proliferation stage. In T. vivax infections in cattle, the splenomegaly is sustained but in goats, the spleen returns to normal size by about 5-6 weeks after infection.

The major feature of trypanosomiasis in domestic animals is a profound haemolytic anaemia, thought to be dependent on the presence of parasites (Murray and Morrison, 1980). The severity of the anaemia varies with host and trypanosome species. Masake (1980) found that goats infected with T. vivax had higher parasitaemias and more severe anaemia than cattle infected with T. vivax. The severe anaemia found in T. vivax infections (haematocrit can be as low as 0.09-0.17 - Masake 1980) may be partly due to gastrointestinal bleeding because of severe thrombocytopenia (as low as 4,000/mm$^3$ compared to pre-infection levels of 400,000/mm$^3$) (Wellde et al, 1983). Aggregation of platelets may be due to a trypanosome-derived factor (Davis et al, 1974) or by antigen-antibody complexes (Maxie et al, 1979). Microthrombi have been found in the lymph nodes, lung, liver and kidney (Masake, 1980; Van den Ingh...
et al, 1976; Murray, 1979) and provide evidence for disseminated intravascular coagulation. However, like anaemia, the severity of thrombocytopenia varies with host and parasite species (Wellde et al, 1983; Van den Ingh et al, 1976; Maxie et al, 1979). Jenkins and Facer (1984) extensively review the haematology of African trypanosomiasis.

The heart is particularly damaged in domestic animals infected with T. congolense, T. vivax and T.b.brucei. The damage is similar in T. vivax and T.b. brucei - there is massive cellular infiltration (lymphocytes, plasma cells and macrophages) of the heart tissue including the conducting fibres. The heart microvasculature becomes dilated with leucocyte accumulation and there is perivascular accumulation of fluid (Murray and Morrison, 1980). In natural conditions, infected cattle die of congestive heart failure due to anaemia, microvascular damage and myocarditis (Murray and Morrison, 1980). Other organs may be affected in similar ways, particularly bone marrow and kidney (Masake, 1980).

The wasted appearance of animals infected with trypanosomiasis is a classical observation. Dargie (1980) suggested that this was due to reduced food intake (voluntary) and diversion of amino acids and energy to immunoglobulin and cellular production rather than building up muscle. Goodwin and Guy (1973) found evidence for muscle breakdown in T.b. brucei infection in rabbits.

1.5.5 Gross Pathology of T.b. brucei 427 in Rabbits

The rabbits are usually infected subcutaneously with about $10^7$-$10^9$ parasites isolated from rodents. The first outward signs of the disease occur about 10-14 days after infection. There is progressive
swelling of the ears, face, nares, paws and genitals and the fur becomes course and yellow. Breathing may become laboured and rhinitis and ocular inflammation are often evident. As the infection progresses, the eyes may close completely and necrotic lesions and scabs appear on the face, scrotum and, occasionally, at the inoculation site. When the ear vasculature is illuminated with a spotlight, the veins and arteries are seen to be tortuous and constricted. Venesection becomes difficult and patches of erythema and scabs are often present. Any of these external signs may occur but vary in their severity between animals. Although the animals usually continue to eat, they become thin and wasted. Parasitaemia is low but there are detectable peaks approximately every 10-14 days until death (M.J. Parry - personal communication).

The present study used T.b. brucei 427 from which the rabbits died about 5-7 weeks after infection. Death is thought to result from secondary infections, kidney failure or circulatory collapse.
1.6 Immuncpathology

1.6.1 Immune Complexes


Immune complex damage is also implicated in other parasitic diseases such as malaria (Allison et al, 1969; de Graves and Cox, 1983; Weiss et al, 1983; Soni and Cox, 1975; Musoke et al, 1977; June et al, 1979; Shear, 1984; Contreras et al, 1980); leishmaniasis (de Brito et al, 1975; Pearson et al, 1983; Kharazmi et al, 1982); schistosomiasis (D'Amelio et al, 1981); amoebiasis (Nuti et al, 1981) and babesiosis (Annable and Ward, 1974).

The formation of immune complexes in vivo is a defense mechanism occurring when antibody binds to a suitable substrate (antigen). The resultant complex can have a variety of actions depending on its size. Factors affecting size, and hence complex solubility, include the class of antibody, commonly immunoglobulin G (2 antigen binding sites) and IgM (5 or 10 antigen binding sites), the antigen valencies and relative proportions of antibody (Ab) and antigen (Ag).

The effects of immune complexes (IC) in vivo also depend on their location, concentration and persistence.

In a condition of great antigen excess, the complexes tend to be small, soluble and may be of the form $Ag_1Ab_1$ or $Ag_2Ab_2$ (of size 7-11S).

As the relative antibody to antigen ratio increases, medium sized IC (size approximately 11-19S) are formed. These can strongly activate (fix) complement (see 1.8.1), an early stage in the complex mechanisms of inflammation (see 1.8.4).

Large insoluble IC (greater than 19S in size) are quickly removed from the circulation (Mannik et al, 1971) by selective adherence to, and phagocytosis by mononuclear phagocytes or macrophages (Arend and Mannik, 1972). They can also be precipitated where they are formed, causing local damage.

Complex molecules such as glycoproteins have many antigenic sites (determinants). Trypanosomes, having $10^7$ identical glycoprotein molecules on their surface (Cross, 1975), have a vast number of determinants and a very high valency (number of repeating determinants).

This would be expected to provoke a strong immunological response. The binding of specific antibody to the trypanosome surface kills the parasite, releasing common antigens. Antigens in a range of sizes would be expected, provoking different sized IC. Circulating and deposited IC have been found in mammals with trypanosome infections and are predominantly associated with complement components (see 1.6.1.2, 1.6.1.3).

The composition of circulating and tissue bound immune complexes during trypanosomiasis shows considerable variation between parasite strains (Sacks et al, 1980), host species and immunological responsiveness of the host, in the case of inbred mice and rats.
Evidence suggests that a large proportion of the immunoglobulin produced during the infection is not specific for trypanosome antigens (Houba et al, 1969; Mackenzie and Boreham, 1974; Lambert et al, 1981). This is thought to be due to a generalized mitogenic effect acting via macrophages on B-lymphocytes (Greenwood and Oduloju, 1978; Greenwood, 1974; Houba et al, 1969; Hudson et al, 1976; Clayton et al, 1979b; Sacks et al, 1982; see 1.6.2.1 below).

However, it has been shown that much of the antibody produced is in fact trypanosome-specific (Musoke et al, 1981; Lindsley et al, 1981).

1.6.1.1 Detection of Immune Complexes

The involvement of parasite antigens in the IC in trypanosomiasis is somewhat controversial. The usual failure to detect parasite antigens in the complexes may be due to specific antibodies binding to, and masking antigenic determinants or the absence in the assay of a specific substrate for all the antigens in the complex (Lindsley et al, 1981). In addition, frequently used techniques for detecting IC components are often non-specific for parasite antigen (Houba, 1982) and may also be detecting aggregated immunoglobulin, rheumatoid factor, immunoconglutinins or anti-idiotype antibodies (Houba, 1982; Rose et al, 1982; Rickman et al, 1981; see chapter 1.6.1.2). Antigen-specific methods are much more complex unless the antigens are well-defined (Zubler and Lambert, 1977), particularly difficult in trypanosomiasis.

1.6.1.2 Circulating Immune Complexes

Circulating immune complexes (CIC) have been detected in the serum and cerebrospinal fluid (CSF) of humans and experimental animals
infected with trypanosomes (Lambert et al., 1981; Parry, 1980; Poltera, 1985; Rose et al., 1982; Basson et al., 1977; Whittle et al., 1980 and Lindsley et al., 1981).

The composition of CIC is controversial, possibly, according to Zubler and Lambert, (1977), because of difficulties in identifying the antigens.

In rabbits infected with *T. b. rhodesiense* (Lindsley et al., 1981) and *T. b. brucei* 427 (Parry, 1980) CIC were found to contain IgG, IgM and complement (Lindsley et al., 1981) and some other unidentified material of molecular weight 72,000 (Parry, 1980). Levels of CIC were found in both studies to be correlated with trypanosome-specific IgG but not IgM. Lindsley et al., (1981) occasionally found trypanosome antigen in CIC. Elevated CIC were found by day 10 of the infection reaching a plateau 14-21 days and increasing again by day 28 (Lindsley et al., 1981).

Although the presence of circulating complexes does not necessarily imply a pathological condition (Delire et al., 1978) and may indeed have a role in normal physiological processes (Contreras et al., 1982), immune complex-mediated pathology is well established both in disease and under experimental conditions (reviewed by Haakenstad and Mannik, 1977).

Similarities between the effects of serum sickness (type III hypersensitivity due to IC formation in conditions of antigen excess) and infection with trypanosomes suggest an IC-mediated pathology. These symptoms include pyrexia, lymph node enlargement, generalized urticaria, inflamed painful joints, low serum complement and transient proteinuria.
Anti-idiotype antibodies have been demonstrated in both chronic serum sickness-induced glomerulonephritis in rabbits (Zanetti and Wilson, 1983) and in trypanosomiasis (Rose et al, 1982).

### 1.6.1.3 Immune Complex Deposition in Tissues

Deposits of IC-type material have been found in a variety of tissues in human and animal trypanosome infections.

In renal glomeruli, IgG, IgM and complement deposits have been demonstrated in rabbits infected with *T.b. rhodesiense* (Nagle et al, 1980) and *T.b. brucei* (Facer et al, 1978); IgM and complement in monkeys infected with *T.b. rhodesiense* (Nagle et al, 1974) and mice infected with *T.b. brucei* (Murray et al, 1975).

In cardiac and skeletal muscle, deposits of trypanosome material, IgG and IgM were found in mice infected with *T.b. brucei* (Galvao-Castro et al, 1978). Poltera (1980) and Poltera et al (1980a, b) found deposits of immunoglobulins and complement in heart and choroid plexus of mice infected with *T.b. brucei*. This latter model was devised to reflect pathology of human trypanosomiasis.

The importance of immune complexes in disease states in general stems from their ability to trigger endogenous inflammatory reactions. The mechanisms whereby this occurs are very complex. An outline is given in chapter 1.8.
1.6.2 Immunosuppression


Immunosuppression to parasite antigens is not only an important feature of the disease but there can also be impaired response to vaccination for other diseases (Oyejide et al, 1984). Other workers have had equivocal responses to vaccination during trypanosomiasis (Sharpe et al, 1982).

In natural conditions, it is likely that humans or animals are infected with more than one type of pathogen and if immunosuppression occurs, it may impair the ability of the host to combat other infections (Millott and Cox, 1985).

Since the early seventies much interest has centred on immunosuppression in trypanosomiasis. Many workers have used clones of parasites and specific strains of mice (Dempsey and Mansfield, 1983a; b; Charoenvit et al, 1981; Sacks et al, 1980; 1982; Hudson and Terry, 1979) so that they can look at very fine differences in the immune response. In addition, mice with naturally occurring or induced immune deficiencies such as T lymphocyte deficiencies - athymic nu/nu mice (Grosskinsky et al, 1983; Campbell et al, 1978; Clayton et al, 1979a; Jayawardena and Waksman, 1977); B cell impairment - xid defect in mice (Jones and Hancock, 1983; Finerty et al, 1984) and suppression from birth with anti- μ (Campbell et al, 1977); macrophage and complement deficiencies.
(Jones and Hancock, 1983) have provided useful adjuncts to other work. Some of the drawbacks with the genetic deficiencies is that they may not be fully characterized and, even if an animal can respond immunologically to trypanosomiasis without a particular part of its immune system, it does not not mean that in intact animals this part does not have a role (Diggs, 1982). Diggs (1982) also points out that although much work is being done on immunosuppression, a unifying hypothesis is still elusive.

The three main candidates for mediators of the immunosuppression are the B cell, T cell and macrophage systems. These are, of course, very closely related and interacting, so accessory factors may also be involved.

1.6.2.1 B Cell System

Involvement of the B cell system is indicated by such features of the disease as macroglobulinaemia (Terry, 1976; Facer, 1976); a B cell mitogenic effect of parasite antigens (Assoku et al, 1977; Greenwood, 1974; Mansfield et al, 1976; Corsini et al, 1977; Clayton et al, 1979b), a polyclonal antibody response (Kobayakawa et al, 1979) and expansion of B-cell areas of lymph nodes and spleen (Murray et al, 1974b).

Mansfield and Bagasra (1978) showed that mice infected with T.b. rhodesiense have an enhanced response to T-independent antigens (even when T-dependent responses are nearly completely depressed) compared to uninfected mice. They suggested that the inability to respond to T-dependent antigens is not due to a B cell defect, but may be related to alterations in accessory cell populations. As early as 1973, Terry et al proposed that trypanosome infections break the control link
between T and B cells. Musoke et al., (1981) also favour this hypothesis. They found that IgM and IgG1 in the first three weeks of T. b. brucei infection in cattle were trypanosome-specific. They concluded that polyclonal B cell stimulation may not be responsible for the high levels of these immunoglobulins and suggest that the switch mechanism from IgM to IgG in trypanosome infections is selective for IgG1. This switch can also be impaired by hypo-complementaemia (Nielsen and White, 1974), a common feature in trypanosomiasis (Greenwood and Whittle, 1976b).

Other work on the B cell system by Campbell et al. (1977) using T. b. rhodesiense infection in mice with a B cell defect (suppressed from birth with goat anti-μ) indicated that a functioning B cell system is necessary for immunity. Murray et al., (1974b) also provided evidence for B cell dysfunction in trypanosomiasis.

However, Jones and Hancock (1983) found that mice with the xid defect (apparently a B cell maturation defect) survive T. b. rhodesiense infection longer than do control mice. Finerty et al. (1984) using the same model not only found that these mice survived longer, but that they had less immune complex formation and lower parasitaemias in the second week than mice without the defect. In normal mice, they found that antibody specific for T. b. rhodesiense antigen peaked on day 10 and then declined. In the B-cell deficient mice, IgG was detected on day 4 and IgM (specific for trypanosome antigen) detected on day 16. They suggested that trypanosome infection "switches on" whatever is lacking in B cell maturation. Another interesting feature of these experiments was that the deficient mice had no IgG1 antibody possibly indicating that these mice do not "recognize" trypanosome antigens that trigger an IgG1 response. In contrast, Musoke et al. (1981), using 2
clones of *T.b. brucei* in cattle found that trypanosome-specific antibody was IgM and IgG1 (no IgG2 at all). They proposed that the switch mechanism for IgM to IgG in trypanosome infected animals is uniquely selective for IgG1 heavy chains. These differences could be species-dependent.

1.6.2.2 T Cell System

The role of T cells in the immune response to trypanosome infection is not as well-defined as for B cells. This may be due to the complexity of the T cell system or to variations in the efficiency of methods to produce T cell deficiencies (Cooper *et al*, 1979). Jayawardena and Waksman (1977) postulated that T suppressor cells are stimulated nonspecifically in trypanosome infections which may be a feature of the overall suppression. T suppressor cells may "arm" macrophages which then become suppressive (Eardley and Jayawardena, 1977). Alcino and Fresno (1985) have recently provided evidence as to a mechanism. They suggested that macrophages after interaction with living *T.b. brucei* are able to induce the release of suppressor molecules that act preferentially on immune T cells which in turn inhibit the proliferation of helper T cells by either defective interleukin-2 production and/or inhibition of interleukin-2 action.

Corsini *et al* (1977) suggested that in response to B cell mitogenesis (induced by the parasites), T suppressor cells are activated and lead to apparent loss of B cell potential.

However, there is evidence for suppression of the T cell system. T cell areas of the spleen and lymph nodes are diminished, the size and organization of the thymus is reduced and it becomes infiltrated
with plasma cells and macrophages (Murray et al., 1974b; Mansfield and Bagasra, 1978; Mansfield, 1978).

Infection with T. b. brucei can prevent the formation of experimental allergic neuritis in rabbits, an autoimmune disease mediated by T cells (Allt et al., 1971). Mansfield and Bagasra (1978) showed that in vivo induction of helper T cells and antigen-specific suppressor T cell responses were reduced in mice infected with T. b. rhodesiense.

An interesting feature of T cell impaired mice infected with T. b. brucei is that they fail to develop the usual chronic inflammatory lesions in cardiac and skeletal muscle (Urquhart, 1980, Galvao-Castro et al., 1978).

1.6.2.3 The Mononuclear Phagocyte System (MPS)

The MPS in liver, lymph nodes, spleen and bone marrow is greatly expanded in mice infected with T. b. brucei (Murray et al., 1974a).

Macrophage (Mφ) activity has been shown to be increased and there is little evidence that Mφ function is impaired (Jones and Hancock, 1983; Dempsey and Mansfield, 1983a; Murray et al., 1974a) and is apparently crucial to the survival of mice infected with T. b. rhodesiense (Jones and Hancock, 1983).

Macrophages are profoundly immunosuppressive from very early in the infection and parasite-induced changes in them may be responsible for T and B cell malfunction (Clayton et al., 1979b). Grosskinsky and Askonas (1981) found that Mφ mediate immunosuppression in vivo after uptake of parasites in the presence of antibody, possibly by the
mechanism proposed by Alcino and Fresno (1985), as mentioned above.

Macrophages play an important role in the initiation and regulation of lymphocyte proliferation (reviewed by Unanue, 1981). The two major pathways to macrophage activation in response to protozoan parasites are thought to be:

1) Presentation of the parasite antigen in association with Ia antigens (immune response linked antigens - a source of genetic variability in host response) to induce proliferation of a certain T cell subset which can produce macrophage activating lymphokines.

2) Presentation of inappropriate antigen or inappropriate amount parasite antigen in association with the same or different Ia antigens may generate T suppressor cells which suppress lymphokine production and enhance macrophage activation (Blackwell and Alexander, 1983).

There may also be T-independent mechanisms (Ezekowitz and Gordon, 1982; Ezekowitz et al, 1983; Grosskinsky et al, 1983). Continued macrophage activation apparently needs continued stimulation by trypanosomes (or their products) (Ezekowitz et al, 1983). Rossi et al, 1984 have shown that peritoneal macrophages from mice infected with T.b. brucei are activated. Slight activation was seen in normal macrophages when live T.b. brucei was added with specific mouse anti-T.b. brucei serum. Activation was also seen when lymphocytes from infected mice were added to the normal macrophages and increased further with the addition of live trypanosomes.

Macrophages may be deactivated during the later part of infection, possibly due to generalized immunosuppression (Sacks and Askonas, 1980; Charoenvit et al, 1981; Ezekowitz et al, 1983), an effect of the parasites themselves (or their products) (Ezekowitz et al, 1983) or
that immunosuppression is mediated in part by the macrophage (Grosskinsky and Askonas, 1981; Eardley and Jayawardena, 1977).

The mechanisms of macrophage action are unclear but there is mounting evidence for production of oxygen-derived free radicals by activated macrophages (Morgan et al., 1980; Nathan, 1983; Nathan and Nakagawara, 1982). These may have a role in killing parasites (Blackwell and Alexander, 1983; Nathan, 1983) and also in mediating tissue damage (Frank and Massaro, 1980).

Antibody excess can be immunosuppressive (Feldmann and Diener, 1971). The nature of the response to trypanosome infection might be varied due to fluctuations in parasitaemia over the course of the infection possibly allowing occasions of relative antibody excess. This may go some way to explain some of the apparently contradictory work on immunosuppression.

Gershon (1974) suggested that immune complexes in certain antibody:antigen ratios may act as a feedback signal to stimulate suppressor T cells. If immune complexes persisted on T cells of sensitized animals they could cause suppression of antibody formation (Kontiainen and Mitchison, 1975; Gorczynski et al., 1974). T cells will bind complexes containing IgG but not free IgG whereas B cells will bind both (Yoshida and Andersson, 1972). Aggregated IgG (which may occur in trypanosomiasis) binds to lymphocytes by a C'-independent activation (Lawrence et al., 1975). However, the binding of immune complexes to lymphocytes appears to be modulated by C' (Nuzzenzweig, 1974), only the alternative C' pathway being necessary (Miller et al., 1973). There is evidence that the alternative C' pathway is activated in trypanosomiasis (Tabel, 1982). Factor B (necessary in alternate C' activation) is reduced in T.b.
gambiense sleeping sickness (Greenwood and Whittle, 1976b) and properdin has been found in renal deposits in monkeys infected with T.b. rhodesiense (Nagle et al, 1974). If such a mechanism does occur during trypanosome infections, it is likely to be a minor feature of immunosuppression.

1.6.3 Protective Immunity

There are many different facets of the host immunological response to a trypanosome infection. Immunosuppression as discussed previously complicates the development of protective immunity to the parasite. It is of crucial importance in controlling the parasitaemia. For example, if rabbits infected with T.b. brucei are treated with cyclophosphamide (an immunosuppressive cytotoxic drug) the characteristic parasitaemic peaks are absent, replaced instead by an uncontrolled parasitaemia, and the rabbits die within 3 weeks as opposed to 5-7 weeks in untreated animals (Parry, 1980). The appearance of the rabbits was as if the whole course of the infection was accelerated. It has been suggested that chickens infected with trypanosomes will "self cure" because chickens respond more rapidly to each antigen and thus control each VSG before large numbers are reached (Herbert et al, 1981). Chickens also have barely detectable parasitaemias.

Like mechanisms of immunosuppression, protective immunity is similarly complicated. Protective immunity appears to be B cell dependant (Campbell et al, 1977; 1982; Campbell and Philips, 1976). In T.b. gambiense infection in mice, Mitchell and Pearson (1983) suggested that T-independent antibody to variant specific glycoprotein (IgM) may be the most important antibody controlling parasitaemia. Longstaff and Terry, (1982) using T.b. brucei infection in guinea pigs
proposed that T-independent IgM kills successive variants in the blood but not extravascularly. Barry and Emery (1984) showed that in goats, antigenic variation probably occurs first in the lymph where little IgM is found. If IgM is in fact the major trypanosome-specific protective antibody, the lymph and tissues could effectively provide a reservoir for existing and new variants.

T cell mediated responses seen in vitro (Campbell et al, 1982) and in vivo (Tizard and Soltys, 1971) may be directed towards common antigens rather than specific determinants and may not reflect protective immunity. However, Finerty et al (1978) suggest that such a T cell response is a necessary component of protective immunity possibly involving helper T cells.

Musoke et al (1981) studying experimental *T. b. brucei* infections in cattle showed that IgM antibodies formed in the first parasitaemic peak are more effective at neutralizing trypanosomes than IgG1 antibodies but that the reverse was true for the second peak. They found that all the eluted IgM and IgG1 in the first three weeks of the infection was trypanosome-specific suggesting some T-dependent role.

1.6.4 Autoimmunity

The formation of autoantibodies occurs in trypanosomiasis. These include anti-liver and Wasserman antibody (MacKenzie and Boreham, 1974) anti-FDP, fibrin/fibrogen degradation products (a.k.a. anti-F) (Capbern et al, 1977; Boreham and Facer, 1974b), DNA, red cell and platelet autoantibodies (Kobayakawa et al, 1979), anti-idiotype antibody (Rose et al, 1982), antibody to immunoglobulins (including rheumatoid factor) (Kobayakawa et al, 1979; Houba et al, 1969; Lambert et al, 1981),
immunocconglutinin (Rickman et al., 1981), anti-liposomal phospholipids (Richards et al., 1983) and anti-phospholipase A₁ (Hambrey et al., 1980).

The effects of these autoantibodies are unknown, although it is possible that some may enhance pathological processes (e.g. immunocconglutinin) and some may be beneficial (e.g. anti-F).

1.6.5 Trypanotolerance

Trypanotolerance (genetic resistance to trypanosomes) is an important area currently being investigated (Murray et al., 1984). In mice, where genotypes are well known, variations in susceptibility are thought to be under multigenic control (Greenblatt et al., 1984; Sacks et al., 1983; Mitchell and Pearson, 1983). Interestingly there also appears to be a gender difference; females showing a decreased susceptibility (Herbert et al., 1981; Greenblatt and Rosenstreich, 1984; Greenblatt et al., 1984). Environmental factors such as stress and nutritional status, particularly in cattle and game, are probably also important factors (Tizard, 1982).

In general it would appear that in a mammal infected with a chronic strain of trypanosome, the immune system, under continuous antigenic stimulation, controls the parasite load, but is not effective in eradicating it. As the infection progresses, the body becomes less and less able to control the disease due to direct and indirect effects on the immune apparatus, and blood vessel damage (see section 1.7) and hence tissue anoxia due to inflammatory stimuli. The final cause of death varies from species to species, but often includes cardiovascular shock, renal failure and secondary infection (Goodwin, 1974).
1.7 The Microcirculation

This is a complex, 3-dimensional branching system of arterioles, arterial capillaries, capillaries, venous capillaries and venules. Its functions are to regulate the supply of nutrients to, and removal of metabolites from the tissue. It is controlled by a combination of central and local mechanisms both nervous and humoral. The properties and functions of individual microvessels vary from tissue to tissue and within the same tissue, probably reflecting specialist demands and conditions of the local area supplied by the vessels.

For convenience, the terms "microvasculature" and "capillaries" shall be deemed roughly synonymous unless specified otherwise. One of the reasons is that we speak of "capillary permeability", for example, when in fact there is a gradient of permeability from arterial to venous segments (Zweifach and Intaglletta, 1968; Renkin and Curry, 1979) and the greatest permeability is in the post-capillary venules (Fraser et al, 1979).

Components of the microvasculature used to be defined in terms of the vessel calibre, for example, grouping all vessels between 10\(\mu\) and 20\(\mu\). This is no longer acceptable (Cliff, 1976) because of variations in morphology and function.

The microcirculation has the slowest blood flow in the circulation and has a large surface area making it ideal for exchange functions.
1.7.1 Structure

Capillaries can be categorized according to the structure of their endothelial cells and basement membrane (Bennett et al, 1959). These classifications are not absolute but useful in a broad sense.

There are two major groups (L.H. Smaje - personal communication):

1) Continuous capillaries (e.g. brain, skeletal muscle, intestinal smooth muscle, heart, lung and mesentery) have closely interconnected endothelial cells with narrow intercellular clefts and unbroken basement membrane. A major sub-group of continuous capillaries have fenestrae which may be opened or closed. These can be seen in electron micrographs as discrete attenuations in the endothelial cell wall. Tissues with fenestrated capillaries include glomerular and tubular capillaries of kidney, choroid plexus, synovium, intestinal mucosa and glandular tissue.

2) Discontinuous capillaries (liver, spleen and adrenal glands are the main tissues with these capillaries) have completely open intercellular clefts between adjacent cells (sinusoids) and no apparent basement membrane.

These divisions are based on morphological rather than functional data.

The endothelial cells are flattened, thin and have numerous vesicles as well as mitochondria, Golgi bodies and other inclusions common to most cells. These endothelial cells line the entire vascular tree but only in the smallest capillaries do they form the actual vessel. The cells appear to have a glycoprotein coat (the glycocalyx) which may have protein adsorbed (Mason et al, 1977).
The glycocalyx can be demonstrated by the use of ruthenium red on endothelial cells from both small vessels and large arteries (Luft, 1966; Baldwin et al, 1978). As ruthenium red is electron-dense, high resolution is possible and the glycocalyx appears to fill intercellular clefts and may cover fenestrations (where present) and line cytoplasmic vesicles (Shirahama and Cohen, 1972; Clough and Smaje, 1984a).

1.7.1.1 Basement Membrane

The basement membrane surrounding many capillaries was generally thought to be just a supporting structure for the capillary but, although the main filtration barrier between blood and tissue is the endothelium (Majno, 1965), it has been proposed that the basement membrane may participate in the filtration process (Copley and Schiental, 1970; Caulfield and Farquhar, 1974) and may have a role in the compliance of the vessel.

1.7.1.2 Interstitium

Whilst not strictly part of the microcirculation, the interstitium plays an important part in the transport properties of microvasculature. It is made up of glycoprotein and glycosaminoglycans in a gel-like matrix enmeshed in a network of collagen fibres. Both the gel (Comper and Laurent, 1978; Laurent, 1970) and collagen (Meyer et al, 1977; Pearce and Laurent, 1977; Wiederhielm and Black, 1976) have the property of excluding protein. In the presence of hyaluronic and collagen gels, a protein solution will only occupy a fraction of the total space available for water. The amount of protein exclusion depends on the tissue hydration and because of this, slight changes in the properties of the matrix could cause large changes in the accumulation and transport
of protein. If protein accumulates in the interstitium, osmotic swelling may occur which can diminish protein exclusion properties (Pearce and Laurent, 1977; Wiederhielm and Black, 1976; Wiederhielm et al, 1976). However, this may depend on the nature of the tissue, (Spencer, 1984).

1.7.2 Transport

A schematic representation of transport of solvent and solute molecules from the plasma to the lymphatic system and thence to the bloodstream is shown in figure 1.2.

There are two approaches to the study of solute and solvent transport across the microvascular endothelium. The first is to measure the fluxes and the forces which produce them, determining membrane coefficients that are characteristic of that membrane. The second approach is to look at the molecular basis and mechanisms of such transport.

1.7.2.1 Quantitative Transport

It has long been known that the capillary endothelial barrier is selective. Starling (1896) proposed that if capillaries were freely permeable to water and ions of plasma and impermeable to plasma colloids, fluid balance (no net fluid movement) would occur, the difference between plasma and tissue colloidal osmotic pressure (COP or \( \pi \)) balanced by the respective hydrostatic pressures. Though this early proposal has been considerably extended and refined, the "Starling hypothesis of forces" has stood up to experimental investigation for 90 years.
FIG. 1.2: Schematic diagram showing direction of solvent and solute flows and some of the factors affecting them.
Early refinements came with Landis (1927) and Pappenheimer and Soto-Rivera (1948) showing that the net rate of transcapillary ultrafiltration \( (J_V) \) was proportional to the difference between opposing forces:

\[
J_V = PS (\Delta P_H - \Delta \pi) \quad (1.1)
\]

where:

- \( P \) = permeability coefficient
- \( S \) = surface area available for transport
- \( \Delta P_H \) = differential hydrostatic pressure
- \( \Delta \pi \) = differential colloid osmotic pressure

\( PS \) is often considered as the "PS product".

In general, three membrane coefficients are held to be sufficient to describe the permeability characteristics of a particular membrane:

- the hydraulic conductivity (filtration coefficient), \( L_p \)
- the solute permeability, \( P \)
- the solute reflection coefficient, \( \sigma \) or \( \sigma_s \)

(Spencer, 1984)

These can be related by the "Kedem-Katchalsky (1958) equations", first derived by Staverman (1951):
and later expressed by Perl (1975)

\[ J_s = PS (C_p - C_i) + \frac{1}{2} (1 - \sigma_s) J_v (C_p + C_i) \]  

(1.4)

where:

\[ J_s = \text{solute flux} \]
\[ \bar{C}_s = \text{mean solute concentration in the membrane} \]
\[ \text{(an unclear quantity)} \]
\[ C_p, C_i = \text{solute concentrations in the plasma and interstitium respectively} \]

Because of the parallel pathway structure of the microvascular endothelium, the solvent drag coefficient (\(\sigma_s\) of equation 1.2) of the whole membrane does not appear to be the same as the osmotic reflection coefficient (\(\sigma_s\) of equation 1.3) though they may be equal for individual membrane pathways (Osager reciprocity). For Osager reciprocity to occur, it is assumed that osmotic volume flow and hydrostatic pressure-driven flow are similar processes (Renkin et al, 1977b).

These equations are linear and, as actual solute flow is thought to be non-linear, they are only approximations (Thomas and Mikulecky, 1978) and also only apply in equilibrium or near equilibrium conditions (Katchalsky and Curren, 1965).
Equation 1.4 can be conveniently divided into two terms:

\[ J_s^d = PS (C_p - C_i) \]  \hspace{1cm} (1.5)

describing the proportion of \( J_s \) due to diffusive transport and:

\[ J_s^c = \frac{1}{2} (1 - \sigma_s) J_v (C_p + C_i) \]  \hspace{1cm} (1.6)

describing that due to solvent drag or convective transport.

Generally diffusive transport of macromolecular solutes is considered to be blood flow independent in healthy tissue. As \( J_v \) is partially dependent on hydrostatic pressure (equation 1.2) which is in turn dependent on blood flow and resistance, it follows that the convective component of \( J_s \) can be blood flow dependent. Convective transport is thought to be only a small component of the net total body albumin flux in normal animals, calculated as 15% in rabbits (Paaske, 1983) and 14% in humans (Perl, 1975). It follows that if \( J_v \) increases then convective transport will make a larger contribution to total \( J_s \). Renkin et al (1977a) found convective transport of albumin to be dependent on lymph flow, such that as lymph flow increased, convection formed a larger part of total albumin flux. However, Spencer, (1984) points out that in order to obtain such a large \( J_v \) (and hence high lymph flow) it was necessary to raise the venous pressure and that permeability changes could occur at venous pressures over 45mmHg. With \( PS \) being of the same order as \( (1 - \sigma_s) J_v \) the actual measurements of \( J_v \), \( PS \) and \( \sigma_s \) are crucially important in determining the relative importance of convective and diffusive transport. Table 1.2 gives membrane permeabilities for a variety of solutes in different tissues.
<table>
<thead>
<tr>
<th>Species/tissue</th>
<th>P per Unit S</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl(^-), Na(^+)</td>
<td>34 \times 10^{-6} \text{ cm/sec}</td>
<td>Renkin, 1977</td>
</tr>
<tr>
<td>urea</td>
<td>26 \times 10^{-6} \text{ cm/sec}</td>
<td>Renkin, 1977</td>
</tr>
<tr>
<td>glucose</td>
<td>10.7 \times 10^{-6} \text{ cm/sec}</td>
<td>Renkin, 1977</td>
</tr>
<tr>
<td>myoglobin</td>
<td>0.3 \times 10^{-6} \text{ cm/sec}</td>
<td>Renkin, 1977</td>
</tr>
<tr>
<td>Mwt. 17,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>serum albumin</td>
<td>0.01 \times 10^{-6} \text{ cm/sec}</td>
<td>Renkin, 1977</td>
</tr>
<tr>
<td>serum albumin</td>
<td>0.24 \times 10^{-6} \text{ cm/sec}</td>
<td>Paaske, 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>whole body</td>
</tr>
</tbody>
</table>

Table 1.2  A comparison of membrane permeabilities for various solutes
The solute reflection coefficient \( (\sigma_s) \) of a given solute is never more than 1. One indicates complete membrane impermeability to the particular solute and zero, complete permeability. Renkin et al. (1977a) found that \( \sigma_s \) for albumin as determined from lymph flux data from dog paw was 0.83 with \( PS = 0.47 \times 10^{-4} \text{ml/sec} \). However, their data can be fitted by \( \sigma = 1 \) and \( PS = 0.8 \times 10^{-4} \text{ml/sec} \) (Spencer, 1984). This makes conclusions about any of the membrane coefficients or fluxes somewhat controversial.

1.7.2.2 Qualitative Transport

Figure 1.3 is a schematic diagram showing possible pathways for solute and solvent flux between blood and tissue. There is much debate about the relative importance of each of these pathways although some routes are more well established.

Early work by Starling (1896) and Landis (1927) on filtration (see 1.10.1 on experimental methods) and Pappenheimer et al. (1951) on filtration and diffusion pointed towards intercellular junctions as being the major solute transport pathways (see 3 on figure 1.3). The permeability characteristics were thought to be explained by a system of water-filled pores with an "equivalent pore radius" calculated by Pappenheimer et al. (1951). Grotte (1956) using dextrans of various molecular weights, found that transport was proportional to molecular size up to a molecular weight of about 45,000 (size of small plasma proteins), but above that, transport was independent of size (see figure 1.4). He proposed that this indicated a 2-pore system - a large number of small pores, 3.5-4.5nm in radius and a very few large pores, 15-40nm in radius. Electron microscopic studies, using histamine and serotonin (5HT) which are known to increase microvascular permeability
Figure 1.3 Schematic representation of transport pathways across endothelial cells (based on Renkin, 1977 and Spencer, 1984).

1. directly through endothelial cell membranes (water, non-polar solutes or may be a water exclusive channel)
2. vesicular transport and possibly via transient transendothelial channels (Frøkjaer-Jensen and Reece, 1985)
3. junctional transport - probably the major route for macromolecules
4. restriction by the basement membrane
5. restriction by the interstitium
6, 7 closed and open fenestrae (respectively) - applies to fenestrated capillaries only
Figure 1.4 Curves obtained for dextrans in dog leg lymph (fit for pores 4.2 nm) for molecules up to 3.2 nm radius (albumin radius = 3.6 nm). Grotte (1956) suggested that the divergence of the experimental results from the theoretical curve was due to there being a second type of pore (1 large one for every 30,000 small pores).
showed that such permeability increases were associated with widening of intercellular junctions, "gap formation" (Majno and Palade, 1961; Majno et al, 1961). Karnovsky (1967), in an extensive ultrastructural study proposed that in non-fenestrated continuous capillaries, inter-endothelial junctions represented a small pore system and transport via the numerous vesicles present in endothelial cells represented a large pore system. Perl (1971) proposed that the intercellular slits were 20nm wide with a 4nm restriction extending one fifth of their depth. Other workers have estimated large pore radii from 25 to 100nm (Areskog et al, 1964; Carter et al, 1974; Levick and Michel, 1973).

The large numbers of vesicles present in endothelial cells which become labelled with electron-dense macromolecules suggest a transport function. This was thought to occur by vesicles shuttling back and forth across the cells (Palade, 1960; Palade and Bruns, 1968; Casley-Smith, 1984) quantitatively significant only for macromolecules (Renkin, 1964). Later ultrastructural studies are inconsistent with the view that vesicles act as shuttles (Clough and Michel, 1984). Simonescu et al (1975) proposed that confluence of vesicle chains forming transitory open channels may represent large or small pore pathways depending on size.

A meticulous serial section ultrastructural study in conjunction with rapid freezing of tissue by Frøkjær-Jensen and Reese (1985) showed that apparent cytoplasmic vesicles were in fact connected to either luminal or abluminal vesicles. Out of 310 vesicles followed, only 1 had apparent connections with both luminal and abluminal surfaces. This is consistent with expected frequency (i.e. 1 in approximately 500) to provide a pathway accounting for macromolecular permeability.
Spencer (1984) and Smaje (1984) showed that even though fenestrated capillaries are more permeable to small solutes than are continuous capillaries without fenestrations, they are similar in selectivity for macromolecules.

Restriction by basement membrane, thought to be only a coarse filter by Karnovsky (1967), may be more important (Johansson, 1979) when considering interstitium to blood transport of macromolecules.

Protein exclusion properties of the interstitium and its ability to retard movement of molecules across it led Watson (1980) to propose that the interstitial matrix was a substantial barrier to transport in some capillaries. This may only occur in tissues where interstitial distances are large (e.g. mesentery and dog paw) (Spencer, 1984). Elevated venous pressure resulted in a washout of extravascular albumin in rabbit skin and muscle with no change in albumin exclusion volume (Bell and Mullins, 1982a, b). In dog intestine (Granger et al, 1980) and lung (Parker et al, 1980) increased tissue hydration caused changes in albumin exclusion volume. Possibly this is a species difference, or, as suggested by Bell and Mullins (1982b), a difference in collagen content.

The glycocalyx may play a great part in the selectivity of the endothelial barrier. Removal of protein from a microvascular perfusate greatly enhances permeability (Mason et al, 1977) and it would appear that plasma proteins bind to surfaces of endothelial cells (Schneeberger, 1984; Turner et al, 1983) in a receptor-mediated fashion (Baldwin and Chen, 1984) to restrict transport. This feature is incorporated into the fibre matrix theory (Curry and Michel, 1980) which suggests that a network of glycoprotein fibres covers or fills channels through the
endothelium and the nature, organization and spacing of the proposed
fibres determine the permeability characteristics. Variations in
permeability seen in different vessels may be due to variation in the
number of transcapillary channels, the functional resistance of the
channels remaining constant. This fibre matrix theory allows for the
fine fibres of the mesh to condense into a coarser network when albumin
is removed (Curry and Huxley, 1982) thereby increasing permeability.

Curry and Huxley (1982) argue for the fibre matrix theory
against pore theory but Spencer (1984) points out that Curry and Huxley
assume that water and solute share the same route in crossing the
endothelium. Curry et al (1976) themselves suggested that there may
be exclusive water channels such that the area of the channels available
for water would be greater than the area available for solute. According
to Spencer (1984), Curry and Huxley's data (1982) would then fit pore
theory if the transport area available for water is twice that for
solute.

Theories of microvascular transport processes and the physiological
equations do not take into account electrical charge effects of either
the transport "barriers" or the molecules under study. In kidney,
clearance of cationic molecules (positively charged) is many times
greater than anionic or neutral molecules. This is thought to be due
to electrostatic hindrance of anionic molecules by negatively charged
components of the glomerular capillary wall (Brenner et al, 1982). In
some diseases of the kidney (e.g. nephrotoxic nephritis) loss of
negative charge from the glomerulus is associated with significant
proteinuria (Karnovsky, 1979). This may also be a contributing mechanism
in renal damage observed in rabbits with trypanosomiasis (Facer et al,
In contrast, Perry et al (1983) found that intestinal capillaries behave as a positive barrier, that is, enhanced blood-lymph exchange of anionic molecules and reduced exchange of cations. The microvasculature of lung also appears to have a positively charged barrier (Parker et al, 1981; Weaver et al, 1982). Interestingly, the lung has an embryological origin similar to the intestine.

Thus it would appear that regional variation in capillary permeability may in part be due to these charge differences.

1.7.3 Microvascular Heterogeneities

1.7.3.1 Red Cell Transit Time from Arteriole to Venule

These can vary not only between different tissues, but also between different capillaries within the same vascular bed. Some of the variation may also depend on the assumed or measured capillary length (see Table 1.3). Though the time available for exchange of respiratory gases and other small molecules can be very short, it is usually sufficient. This may not be true under pathological conditions such as impaired blood flow.

1.7.3.2 Red Cell Distribution

The distribution of red cells within a microvascular bed is not homogenous because of variation in physical and geometric factors (Johnson, 1971; Lipowsky et al, 1980; Schmid-Schonbein and Zweifach, 1975). One of these factors is a tendency for red cells at bifurcations to enter the branches that have higher red cell velocities (Fung, 1973; Schmid-Schonbein et al, 1980a). Although this preference of red cells
<table>
<thead>
<tr>
<th>RED CELL TRANSIT TIME (seconds)</th>
<th>TISSUE</th>
<th>REFERENCE</th>
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<tr>
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<tr>
<td>2.21 - 4.29</td>
<td>resting skeletal muscle</td>
<td>Honig et al (1977)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Klitzman &amp; Johnson (1982)</td>
</tr>
<tr>
<td>0.39 - 9.07*</td>
<td>skeletal muscle</td>
<td>Klitzman &amp; Johnson (1982)</td>
</tr>
</tbody>
</table>

Table 1.3

Red cell transit times from arteriole to venule demonstrating considerable heterogeneity between and within tissues.

Values are mean times.

* indicates the range of individual values.
to go through high velocity channels has unknown physiological significance (Klitzman and Johnson, 1982), it may have pathological implications - for example, it may provide a protection mechanism when microthrombi are present (see also 1.7.4 below).

Discrepancies are also apparent in estimations of microvascular blood flows and pressures, even within a single tissue. Gore (1982) suggested that this was due to comparisons between indirect isogravimetric and volumetric estimations of capillary blood pressures and direct servo-null measurements in single capillaries. He found that in intestine, the indirect methods gave values a third of those from direct measurements. This was due to intestine comprising three very different beds in parallel (mesentery, intestinal muscle and intestinal mucosa). Measuring the individual capillary pressures in the 3 types of capillaries, it was found that individual measurements agreed with the literature values and the weighted average was comparable to the isogravimetric results (Gore and Bohlen, 1975; 1977).

1.7.4 Blood Rheology

1.7.4.1 Viscosity

In small vessels, inertial forces are negligible and pressure gradients are balanced by viscous forces (Fitz-Gerald, 1972). Blood flow therefore depends on driving pressure, blood viscosity and vessel calibre.

In the microcirculation, some of the vessels have a smaller diameter than an unstressed red cell (approximately 7 μm). The actual number of red cells (haematocrit) and their deformability are of primary
importance. The deformability of blood cells is an estimation of the force required to push them through a restricted space. In a normal state, red cells are highly deformable and alter their shape when passing through the smallest capillaries.

The haematocrit (hct) and deformability are features of the apparent viscosity (\( \eta \)) which can be calculated from Poiseuilles' equation, given as:

\[
\eta = \frac{\Delta P \ D^2}{0.326 \ \bar{V} \ l} \tag{1.7}
\]

(Lipowsky et al, 1978)

where:

\( \Delta P \) = time averaged values of pressure drop (cmH\(_2\)O)

\( \bar{V} \) = bulk velocity (mm/sec)

\( D \) = measured vessel diameter (\( \mu \)m)

\( l \) = length of vessel segment (\( \mu \)m)

Apparent viscosity is proportional to hct., increasing in polycythaemia and decreasing in anaemia. The calculation of \( \eta \) assumes that the red cells are normally deformable and no leucocytes are present.

Red cells are much less deformable in rheumatoid arthritis and systemic sclerosis (I.B. Kovacs - personal communication). In trypanosomiasis many red cells are crenated and rigid (Goodwin, 1970; Jenkins et al, 1980; and personal observations) indicating reduced deformability. This will decrease flow velocity and increase apparent viscosity.

The frequency of leucocytes in the blood is approximately one in 500 (Green, 1981). During an infection, however, this ratio may
change markedly particularly if there is also anaemia, as in trypanosomiasis (see 1.9.10). The diameter of polymorphonuclear leucocytes (PMNs) is almost the same as red cells (Schmid-Schonbein et al, 1980b). However, PMNs are more spherical, have a larger volume and greater rigidity (Braid et al, 1984) with viscosity coefficients about 2000 times greater than red cells (Schmid-Schonbein et al, 1981). The presence of just one PMN within a capillary can profoundly increase the local apparent viscosity and increase the perfusion pressure necessary for passage through the microvessels.

In larger vessels, the normal blood velocity is parabolic, flow being faster at the core than at the wall. Red cells, having a higher velocity, are concentrated towards the centre of the vessel (axial drift). Leucocytes are slower and tend to congregate nearer the walls, or, at least, off-centre (Nobis and Gaehtgens, 19).

In glass tubes less than about 8μm, leucocytes will deform, but their velocity is slower than that of red cells. Red cells are seen to congregate behind the leucocytes forming "trains". In tubes of 8μm to 15μm diameter, a few red cells will "overtake" the slower leucocyte.

Inhomogeneity in microvascular perfusion in skeletal muscle during haemorrhagic shock (Dahlberg, 1979) may be due to leucocyte "plugging" of capillaries (Braide et al, 1984; Bagge et al, 1980).

1.7.4.2 Haematocrit

Haematocrit is a primary determinant of apparent viscosity in the microvasculature. Alterations in systemic hct. cause approximately proportional changes in microvascular hct (Gaehtgens, 1984) and capillary
red cell flux (Lipowsky et al, 1979). Total body hct is between 70 and 95% of large vessel (systemic) hct (Lawson, 1962) indicating that small vessels have a lower hct. Gaehtgens (1984) calculated small vessel hct to be 0.24 when total body hct = 0.40; large vessel hct = 0.44, and assuming that the small vessel (<300μm) compartment was 20% of total cardiovascular volume (Lawson, 1962).

1.7.4.3 Fahraeus Effect

This effect occurs noticeably in vessels <300μm and is a means of reducing apparent viscosity in the microcirculation. When blood is made to flow through a narrow vessel, the volume concentration of red cells within the vessels is lower than that of the outflowing blood. This is due to differences in travelling speed between red cells and plasma, giving a lower hct. within the narrow vessel (Albrecht et al, 1979; Gaehngens, 1984). When leucocytes are present, the Fahraeus effect is reduced thereby increasing apparent viscosity and reducing flow. This is likely to be of particular importance in trypanosomiasis.
1.8 Immunology of Inflammation

The major pathological feature of the T. brucei group of infections is a widespread chronic inflammation (Goodwin and Hook, 1968; Galvao-Castro et al, 1978; Edeghere, 1980). To discuss inflammation in relation to trypanosomiasis, I propose to first outline the in vivo mechanisms involved in response to infection and injury.

1.8.1 The Complement System (C')

This consists of a group of proteins which react in a sequence to form the primary humoral mediator of inflammation. Complement is fixed by the Fc portion of IgM or IgG antibody making antibody-antigen (immune) complexes. This enhances their phagocytosis (opsonization) or promotes cell lysis (Frank, 1975; Muller-Eberhard, 1975).

The first component, Cl, has three fragments - Clq, Clr andCls. Clq interacts with the immune complex (IC) forming Cl esterase from Cls. The rest of the sequence is outlined in figure 1.5. The crucial stage is the conversion of C3 to C3a and C3b which is accomplished via Clq (the classical pathway) or via properdin (the alternative pathway - figure 1.5). The alternative pathway can be activated by IC containing IgA or directly by bacterial lipopolysaccharides. Aggregated IgG or IgM can also fix C' via the classical pathway (Roitt, 1980).

C3 is an important component and is present in large amounts (1.2mg/ml) in normal serum. It may be considered as an acute phase reactant (see chapter 1.8.2.3) (Shirazi et al, 1980).
Figure 1.5 Complement activation showing the classical and alternative pathways. (after Murano, 1978 and Roitt, 1980).
C3a is an anaphylatoxin (releases histamine from mast cells and basophils) and is chemotactic for PMN's. C3b has a short lived binding site which allows it to attach to any membrane bearing C3b receptors such as macrophages, PMN's and, in some species, platelets and red cells. Antibody to this binding site is immunoglobulin. Cells carrying the complex Ag-AbC1423b (see figure 1.5) are susceptible to phagocytosis by macrophages. Whenever C3b is generated by the classical pathway, it will activate the alternative pathway via a positive feedback loop (Lachmann, 1979).

C5a is also an anaphylatoxin and is the major chemotactic factor for PMN's (Snyderman et al, 1970) and enhances increased microvascular permeability (Clough and Smaje, 1984b; Widgren and Williams, 1981).

C5-C8 may act as a polymerizing agent for C9 which actually makes the lesion in the cell membrane (Podack et al, 1982). C'-mediated lysis of bacteria and protozoal parasites is analogous to antibody dependent cell-mediated cytotoxicity (ADCC) (Lachmann, 1983).

The potential for C' activation via IC formation in trypanosomiasis may be inferred from the large amount of antigen present on and released from the parasites. Serum C' is frequently depleted and C' deposited in tissue lesions in trypanosomiasis (Basson et al, 1977; Greenwood and Whittle, 1976b; Nagle et al, 1974; Assoku et al, 1977; Nielsen et al, 1979), features of immune complex disease (McConnell et al, 1981). However, Shirazi et al (1980) found raised C3 levels in T. brucei infection in mice and no evidence that C' played a role in controlling parasitaemia. The reason for such discrepancies may reflect host and parasite species differences.
Soluble trypanosome fractions can activate C' via both the classical (Musoke and Barbet, 1977) and the alternative pathways (Tabel, 1982).

The C' system is just one of the enzyme cascade systems present in plasma. The other three are very closely related through Hageman factor (HF - coagulation factor XII) and are the coagulation, fibrinolysis and the kinin/kallikrein systems (see figure 1.7).

1.8.2.1 The Coagulation System

This is a very complex system (figure 1.6) involving the generation of a proteolytic enzyme thrombin which generates fibrin from its precursor fibrinogen. A series of complexes (consisting of various enzyme precursors, co-factors, calcium ions and suitable surfaces) interact to form active thrombin (reviewed by Murano, 1978). The extrinsic activation of the coagulation includes plasma dilution, collagen, immune complexes and surfaces such as glass and plastics, mediated by HF. Simultaneous with the initiation of clot formation are the steps leading to clot dissolution (fibrinolysis) and kinin generation (figure 1.7).

1.8.2.2 Fibrin(ogen)olysis

Plasmin is the proteolytic enzyme which lyses the fibrin clot (see figure 1.6). It has a strong affinity for fibrin and fibrinogen, as well as activating other clotting factors including HF (Kaplan et al., 1976), components of the C' system (Ratnoff and Naff, 1967) and liberating kinins from kininogen (Habal et al., 1976). The breakdown products of fibrin(ogen)olysis are FDP (fibrin/fibrinogen degradation products) which have a considerable range of pharmacological actions
Fig 1.6: The major components of the Coagulation system. Subscript 'a' indicates the activated form.
FIG. 1.7 (After Murano, 1978)
The interaction of the Kinin/Kallikrein, Coagulation, Complement, and Fibrinolytic systems

*indicates pharmacological/inflammatory activity
(Triantaphyllopoulos and Triantaphyllopoulos, 1970) including:

1) inhibition of the clotting of fibrinogen by thrombin.
2) increased FDP levels leading to inhibition of prothrombin consumption, and decreased FDP to the facilitation of prothrombin consumption.
3) inhibition of thromboplastin activity.
4) reduction in the level of factor V.
5) inhibition of platelet aggregation.
6) increases resistance of red blood cell to hypotonic stimulus and mechanical trauma.
7) inhibition of fibrinolysis.
8) agglutination of certain strains of staphylococci.
9) increases vascular permeability.

and (Barnhart et al, 1970) also potentiates biogenic amines and peptides.

1.8.2.3 Coagulation and Fibrinolysis in Trypanosomiasis

Since the coagulation and fibrinolytic systems are so inter-related, the net pathological effect in the host depends on which system predominates.

There is experimental evidence in trypanosomiasis for both excessive bleeding and the formation of microthrombi (reviewed by Jenkins and Facer, 1984). The presence of microthrombi in the microvasculature of many tissues of animals infected with trypanosomes (Murray, 1974; Murray and Morrison, 1980; Van den Ingh, 1976; Van den Ingh et al., 1976; Edeghere, 1980) indicate disseminated intravascular coagulation (DIC). DIC can be caused by intravascular haemolysis (for
which there is evidence in trypanosomiasis) and release of factors triggering the clotting system (Bick, 1978). The detection of FDP's probably indicates whether or not intravascular coagulation has occurred (Jenkins and Facer, 1984). Elevated serum FDP's have been found in trypanosomiasis (Boreham and Facer, 1974a; Facer, 1974; Barrett-Connor et al, 1973; Robins-Browne et al, 1975).

Fibrinogen levels can increase three-fold during trypanosomiasis (Facer, 1976) which has implications for blood rheology in that it increases blood viscosity. Fibrinogen can be considered an acute phase protein (a group of reactants whose presence in the blood is proportional to the degree of acute tissue damage). Fibrinogen is released from the liver into the blood slower than the release of C-reactive protein (see chapter 2). Stuart (1984) suggests that interleukin-1 (a.k.a. hepatocyte stimulating factor; leucocyte endogenous mediator; molecular weight 13,000; $t_r=9$ minutes) is released by the reticuloendothelial system (RES) cells after stimulation by two early FDP's - fragments D and E (see figure 1.8).

Renal damage, a major feature of trypanosomiasis (Facer et al, 1978; Murray et al, 1975; Lindsley et al, 1980) can reduce elimination of FDP's and thus raise plasma FDP's (Lane et al, 1982; 1984). This may, in part, account for variations in FDP levels seen in trypanosomiasis.

1.8.3.1 The Kinin/kallikrein System

The role of HF in inflammation and pain is via the enzyme kallikrein (figure 1.7). This splits high molecular weight (HMW) kininogen into active fragments called kinins (Sicuteri et al, 1976; Pisano and Austen, 1974). The major plasma kinin is bradykinin.
Figure 1.8 The Role of Acute Phase Reactants in Trypanosome Infections as extrapolated from Stuart (1984).

Abbreviations used:

- **CRP**: C-reactive protein
- **FDP**: fibrin/fibrinogen degradation products
- **D&G**: FDP fragments
- **PG's**: prostaglandins
- **RES**: reticuloendothelial system
The actions of the kinin/kallikrein system in vivo are complex and recent evidence suggests that it is also necessary to differentiate between the plasma and tissue (glandular) systems (Dietze, 1983). Generally, however, it is thought that the plasma system mediates the vasodilatation and increased permeability of the microvasculature during inflammation.

Bradykinin is usually a potent vasodilator although certain vascular beds have receptors which mediate vasoconstriction, such as the rabbit ear (Barabé et al, 1979). It is always present in plasma but in very small concentrations (0.07 to 5.0 ng/ml, Spragg, 1976). This suggests that the activation of kallikrein by HF, and HF by kallikrein is a continuous process.

Physiological roles for the kinin/kallikrein system include regulation of blood flow in rabbit uterus, placenta (Seino et al, 1982) rat salivary glands (Ørstavik et al, 1982), and regulation of salt and water balance (Carretaro and Scicli, 1981).

Bradykinin promotes increased microvascular permeability (Northover and Northover, 1970; Hulstrom and Svensjo, 1977; Saldeen et al, 1983; Graham et al, 1965), contraction of smooth muscle (Elliott et al, 1960) and may have an involvement in leucocyte chemotaxis (Graham et al, 1965) and pain (Cliff, 1976).

The main pharmacological actions of kallikrein are due to the release of kinins, but it also has kinin-independent actions. The vasodilator effects of kallikrein may be mediated by prostacyclin (PGI₂) (Morita et al, 1984). Chao et al, (1981) have shown the kallikrein-induced uterine contraction is independent of kinin formation. Kallikrein
can also release substance P which is vasodilatory (Pals and Micalazzi, 1981).

The actions of increased vascular permeability and local vasodilatation can be separated (Zweifach, 1968; Anderson et al, 1984).

The involvement of kinins in inflammation in general and parasitic diseases in particular has long been known. Mice infected with Babesia rhodhaini (Goodwin and Richards, 1960) and T.b. brucei (Richards, 1965) excrete increased amounts of peptides (including kinins) in their urine. This increase in kinin activity in urine may be due to renal damage (Goodwin and Boreham, 1966). Richards also showed that during the infection, the level of kinins in plasma and especially tissue extracts (particularly ears, skin and feet) increased steadily. Goodwin and Boreham (1966) have suggested that the release of peptides may be a general phenomenon that accompanies pathogenic infections and may play an important role in the pathogenesis of T.b. brucei infections (Boreham, 1968a, b). See also chapter 4.

Because of the extremely short half-life in plasma (less than one complete circulation time - Ferreira and Vane, 1967), measurement of kinins is difficult. An alternative is to measure the levels of the enzyme kallikrein (which also has pharmacological actions).

Parry (1980) showed that high levels of circulating IgG immune complexes in T.b. brucei infection in rabbits were associated with increased levels of kallikrein.
1.8.4 Inflammation

Inflammation is the response by the host to injury (burn and mechanical), invasion by foreign substances (bacteria, viruses, parasites etc.) and certain immunosensitivity reactions. The reactions to such stimuli are very similar. One is increased permeability of the microcirculation which, in the presence of adequate blood flow, can lead to oedema, as in the characteristic weal of the "triple response" (Lewis, 1927). Rapid loss of fluid from the vasculature to the interstitial spaces can lead to vascular stasis. The kinin/kallikrein system, in particular, has been implicated in the increase in vascular permeability, vasodilatation, the accumulation of leucocytes and the production of pain (Lewis, 1964).

Mild injury can be mimicked by the topical application or injection of bradykinin, histamine or other "inflammatory mediators". The reaction of the microcirculation appears to have two phases: an initial transient phase where there is a rapid leakage of plasma proteins into the interstitium and the formation of gaps between adjacent endothelial cells of small venules (Majno et al, 1967); and the second phase where the gaps appear to diminish but macromolecules still leave the blood (Joyner et al, 1974). The initial phase appears to last about 10-20 minutes even when the stimulation is continued (Svensjo et al, 1979; Svensjo and Joyner, 1984). After continuous infusion or superfusion with histamine or bradykinin where the number of leaky sites has declined, immediate application of a different stimulus produces a maximal response, i.e. the microvessels are not refractory to another stimulus (Svensjo and Joyner, 1984). Workers obtaining sustained leakage with histamine or bradykinin usually used whole organs (Grega et al, 1979; Svensjo, 1978; Joyner et al, 1974; McNamee and Grodins, 1975) and Svensjo and Joyner
(1984) point out that this is probably due to the sequential formation and release of many different inflammatory mediators (including histamine, prostaglandins, thromboxanes, leukotrienes, kinins etc.) and possibly explains sustained damage in chronic inflammation.

These mediators often interact, potentiating the action of, or causing the release of others (Wedmore and Williams, 1981). Most of these substances have a plasma half-life of the order of a few seconds or minutes. A candidate for prolonging actions of various substances is calcitonin gene related peptide (CGRP). This is a potent vasodilator potentiating the action of bradykinin, histamine, PAF, C5a and LTB4, though it does not appear to act via prostaglandin release. Injected intravenously, it causes hypotension with an intense peripheral vasodilatation. It is stable in the blood or plasma, long-acting (e.g. 10pM in human skin can cause vasodilatation lasting up to 4 hours) and may turn out to be an important mediator of inflammation (Brain, 1985). However, so far it has mainly been studied in nervous and skin tissue making general conclusions premature.

During the acute inflammatory response, PMN's, predominantly neutrophils can accumulate locally. These apparently interact with vascular endothelial cells to regulate permeability (Wedmore and Williams, 1981). The migration of leucocytes to the periphery of venules during an inflammatory response is known as margination and their slow movement (relative to blood flow) along venular walls is known as rolling. When they stick to the endothelium, the small venules have a "blobby" appearance call leucocyte pavementing. Lloyd et al, (1984) found that patients with Adult Respiratory Distress Syndrome (ARDS) had increased leakage of albumin from the blood to the interstitium and alveolar space associated with the appearance of PMN's in the lymph. In a sheep model
(Staub et al, 1975) this albumin leakage only occurred when PMN and macrophages were detected in the lymph (Lloyd et al, 1984; Flick et al, 1981). Clough and Smaje (1984b) studying individual mesenteric venules, found that fluorescent dye leakage ("hot spots") was associated with the presence of PMN's and independent of histamine and prostaglandins. However, with the addition of C5a, large numbers of PMN's crossed the venular wall, the number of "hot spots" increased and transient leaks were occasionally seen. Malik et al (1982) suggested that FDP's also contributed to vascular injury. Prostaglandins, histamine, 5-HT (serotonin) and bradykinin did not seem to be direct mediators.

Increased vascular permeability induced by PAF acether (platelet activating factor) has two pathways, one PMN-dependent and one independent (Bjork et al, 1983b). The PMN independent pathway is that used by bradykinin, histamine or leukotriene C4 to increase permeability (Wedmore and Williams, 1981; Bjork et al, 1982, 1983a) whereas that due to chemoattractants such as zymosan, C5a, leukotriene B4 or generation of oxygen free radicals is PMN dependent (Wedmore and Williams, 1981; Bjork and Arfors, 1982; Bjork et al, 1982; Smaje and Williams, 1983). Rabbits are particularly sensitive to the effects of PAF.

Williams and Peck (1977) proposed a two-mediator hypothesis for inflammatory plasma extravasation. The first mediator (e.g. C5a) increases vascular permeability by interacting with endothelial cells, probably requiring the presence of PMN's (Wedmore and Williams, 1981). The second mediator potentiates plasma exudation by dilating local arterioles thereby increasing blood flow. Prostaglandin E2 and prostacyclin (PGI2) are candidates for this second mediator. Conversely, Anderson et al (1984) found that histamine produced a concentration-dependent vasodilatation and leakage of labelled albumin into the interstitium of rat cremaster
muscle. Addition of prostaglandin synthetase inhibitors (indomethacin and mefenamic acid) diminished protein leakage induced by histamine but did not alter the arteriole dilatation, indicating the role of prostaglandins in the former, but not the latter. These variations may be due to species and tissue differences. For example, Wedmore and Williams (1981) are of the opinion that bradykinin, 5HT and histamine do not appear to be very important in mediating inflammatory oedema in human skin. Yet human endothelial cells in culture release prostaglandins when stimulated by histamine or bradykinin (Alhend-Gelas et al, 1982).

Bjork and Smedegard (1984) followed the microvascular reactions to acute in vivo immune complex (IC) formation in the hamster cheek pouch. They infused ovalbumin (OA) into sensitized hamsters and found a dose-dependent arteriolar constriction. With the highest dose of OA (100\mu g/ml), the vasoconstriction lasted 4-7 minutes with blood flow ceasing completely. Parry, (1980) and Parry and Whittle (unpublished) found cessation of blood flow in rabbit ears when IC were injected intravenously. Bjork and Smedegard (1984) found that platelet aggregation was also an early event occurring at venular endothelium, often at distinct sites. Platelet aggregates were found to mix with PMN built up on the venular wall (PMN accumulation also dose-dependent) associated with increased macromolecular permeability and occasional haemorrhages. When labelled OA was used, antigen deposited mainly in the post-capillary venule wall and in the PMN's (suggesting ingestion of IC). Immune complex deposition is known to activate C' (resulting in formation of C' split products such as C5a (Hugli, 1981) and PMN accumulation (Clough and Smaje, 1984b)) and to stimulate platelets to release histamine via a C'-dependent (Barbaro, 1961) and C'-independent (Movat et al, 1965) mechanism. Interactions between platelets and neutrophils enhance release of neutrophil-derived leukotrienes and other hydroxy acids (Marcus et al,
Leukotriene C₄, D₄ and E₄ and PAF-acether induce vasoconstriction and increase vascular permeability (Bjork and Smedegard, 1983; Bjork et al, 1983a, b).

The variation in response of the microvasculature to challenge with antigen is probably due to different antibody-antigen ratios resulting in IC of different sizes (Haakenstad and Mannik, 1977; Bjork and Smedegard, 1984).
1.9 Detailed Pathological Considerations

1.9.1 Early Events

In the initial phase after the bite of an infected tsetse fly, parasites enter the lymphatic system before the blood system and there may be a lag before parasites appear in the tissue fluid (Barry and Emery, 1984).

The early events occurring after inoculation are characteristic of localized acute inflammation. Local efferent lymph flow increases (J.D. Barry - personal communication) and leucocytes accumulate. Presumably PMN's accumulate initially later followed by lymphocytes and macrophages. Intradermal injection of *T. b. gambesiense* and *T. lewisi* (a non-pathogenic rat trypanosome) provoked a local skin reaction (Seed, 1969). This was characterized by marked extravasation of Evans' blue dye (bound to albumin in the blood) and a large increase in water content indicating vascular permeability. The *T. b. gambiense* lesion enlarged but the *T. lewisi* lesion healed after 4-5 days (Seed, 1969) suggesting an initial non-immunological reaction (mediated by PMN's) followed by, in the case of *T. b. gambiense* organisms, an immunological response (mediated initially by lymphoid cells and plasmablasts, Murray and Morrison, 1980).

The parasites rapidly divide with a doubling time of 6 hours (Seed, 1978). The populations of parasites in the blood and lymph may be different, with antigenic variation apparently occurring first in the lymphatics (Barry and Emery, 1984). Bijovsky *et al* (1984) suggested that lymphatics are the main route of *T. cruzi* dissemination from the site of inoculation.
The infection spreads via the lymphatics to the lymph nodes and to the bloodstream via the thoracic duct or directly to the skin blood vessels. The localized reaction can thus spread elsewhere and the complex immunopathology of the host's reaction to trypanosome invasion comes into play (see 1.6).

The disease in humans and animals is a progressive degenerative type though certain organs seem more susceptible to trypanosome-derived damage. Histological studies indicate that tissues become infiltrated with inflammatory cells, and, as the parasites multiply and are destroyed by the host's immune defences, trypanosome debris and live trypanosomes (Akol and Murray, 1982; Poltera, 1985; Morrison et al, 1979; Murray and Morrison, 1980; Rudin et al, 1984).

Figure 1.9 and table 1.4 summarize the pathological and biochemical changes seen in rabbits infected with T. b. brucei. The biochemical changes (table 1.4) are indicative of widespread immune system stimulation (globulin release), abnormal lipid metabolism (lipid, cholesterol, FFA and phospholipase A increase), kidney damage (proteinuria, albumin decrease, variable creatinine increase, urea increase), cell breakdown, particularly muscle (increases in aspartate transaminase, creatine phosphokinase) and coagulation abnormalities (fibrinogen and fibrin/fibrinogen degradation products - FDP's increase) and blood rheological disturbances (globulin and fibrinogen increases).

1.9.2 Lymph Nodes

The involvement of lymph nodes in trypanosomiasis has been known since at least the 19th century (Winterbottom's sign - posterior cervical lymph-adenopathy). The lymphatic vessels are involved in the
Fig 1.9  Schematic diagram of the pathology due to infection with trypanosomes

Abbreviations :  Ag-antigen  
Ab-antibody  
C'-complement  
DIC - disseminated intravascular coagulation  
HF - Hageman factor (HFa denotes activated HF)  
FDP - fibrin/fibrinogen degradation products
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<td>fibrinogen (variable)</td>
<td>Facer (1974)</td>
</tr>
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</tr>
<tr>
<td>lipid</td>
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<td>phosphate</td>
<td>Goodwin &amp; Guy (1973)</td>
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Table 1.4. Biochemical changes in trypanosomiasis (blood and serum levels).
earliest stage of trypanosomiasis (Barry and Emery, 1984). Lymph node enlargement occurs in which the lymphoid cells proliferate, medullary cords become thickened and tortuous with large numbers of plasma cells (indicative of B cell stimulation) whereas paracortical areas, T cell dependent, regress (Murray et al., 1974b; Van den Ingh, 1976; Nagle et al., 1980).

1.9.3 Spleen

McCrorie et al., (1980) showed that the spleen has a major causative role in haemolytic anaemia in T. b. brucei infections of rabbits. Splenectomy prior to infection prevented the reduced red cell survival time and delayed the onset of anaemia. The immunological role of the spleen was also evident because splenectomy was associated with significantly raised parasitaemias (control of parasitaemia is thought to be a B cell function - Mitchell and Pearson, 1983). Similar to the lymph node response to infection, spleen B cell dependent areas proliferate and T cell areas regress (Murray et al., 1974a, b; Mansfield and Wallace, 1974; Mansfield and Bagasra, 1978; Diggs, 1982; Mansfield, 1978). The gross splenomegaly seen in trypanosomiasis is probably due to the immunological proliferation and associated with the increased red cell trapping function.

1.9.4 Liver

During T. b. brucei infections in rabbits, the liver appears to have progressively dilated sinusoids, fatty degeneration, inflammatory cell infiltration (Edeghere, 1980), phagocytosis of trypanosomes by neutrophils, macrophages and Kupffer cells, large numbers of parasites within and without the sinusoids and extravasation of red cells (Rudin
et al, 1984). Staining for IgM, IgG and C3, Nagle et al (1980) found IgM and IgG fluorescence along the sinusoidal linings indicating immune complex deposition, IgM in cell cytoplasm in portal areas and C3 in Kupffer cells suggesting phagocytosis of immune complexes. Disruption of sinusoidal endothelium and electron-dense material was found within and without the vascular lumen (Nagle et al, 1980). In mice, the liver apparently has a major role in phagocytosis of trypanosomes, both blood-borne and ones present in liver tissue (Holmes et al, 1982).

There is no evidence for increased protein catabolism during the infection (Dargie, 1980).

1.9.5 Rabbit Ears

During T.b. brucei infection, rabbit ears become pale and heavy with a tortuous vasculature. Many parasites are present in the extra-vascular skin tissue (Goodwin, 1971; Nagle et al, 1980) together with an inflammatory infiltrate, with some cells staining for C3, deposition of IgM at the dermal-epidermal junction (possibly due to autoantibodies against skin antigens or immune complex adsorption to the collagen of this region (Nagle et al, 1980)).

Blood vessel damage in the ears is well documented (Goodwin and Hook, 1968; Goodwin, 1971; Edeghere, 1980). The changes during the infection include a progressive narrowing of the central ear artery and its anastomosing branches and reduced blood flow (Goodwin and Hook, 1968). Histologically, leucocytes (mononuclears rather than PMN's) were found to stick to the vascular endothelium, particularly at venular junctions and sometimes migrated into the tissues (Goodwin, 1971). Platelet aggregates built up where leucocytes were attached to the
endothelium and occasionally the vessel became entirely plugged, (Goodwin, 1971). Electron microscopic studies indicate venular endothelial cell swelling and enlargement of the sub-endothelial space (Goodwin, 1971, Edeghere, 1980). Perivascular cuffing was a major feature. The interstitial hypercellularity was predominantly due to mononuclear cells. The fibroblasts degenerated and collagen appeared fragmented (Goodwin, 1971).

1.9.6 Heart

All parts of the heart show severe inflammatory changes in human and animal trypanosomiasis (Poltera et al, 1976; Poltera, 1980). There is inflammatory infiltrate consisting of lymphoid cells, plasma cells and macrophages (Murray and Morrison, 1980; Murray et al, 1979; Poltera et al, 1976; Poltera, 1980; 1985) as well as trypanosomes in heart connective tissue (Rudin et al, 1984). Poltera and Cox (1977) found muscle breakdown in human trypanosomiasis. In mice, parasites accumulate in the interstitium and draining lymphatics of the heart (Poltera et al, 1980b). Deposits of immunoglobulin and C3 were found (Poltera et al, 1980b) suggesting an immune complex mediated aetiology of cardiac pan-carditis. Cardiac involvement is a prominent feature of T.b. rhodesiense infections and death may be due to heart failure or arrythmias, suggesting damage to the conducting system (Greenwood and Whittle, 1980).

Microvessels are progressively dilated with leucocytes and have swollen endothelial cells (Murray and Morrison, 1980). In advanced infections local lymphatics are occasionally occluded with thrombi consisting of fibrin and leucocytes (Morrison et al, 1979).
Renal damage is a major feature of many trypanosome infections (Murray et al., 1975; Van den Ingh, 1976; Edghe, 1980; Facer et al., 1978; Morrison et al., 1979; Nagle et al., 1974; 1980; Boreham and Kimber, 1970). It is characterized by proteinuria, increased serum creatinine and urea and serum electrolyte imbalance (Goodwin and Guy, 1973; Van den Ingh, 1976).

Histologically there is basement membrane thickening (Van den Ingh, 1976; Facer et al., 1978), deposition of IgG and IgM in capillaries and mesangium (Nagle et al., 1980; Van den Ingh, 1976), immune complex deposition in capillary walls (Nagle et al., 1980) and mesangium (Murray et al., 1975). C3 is often detected in glomerular deposits (Nagle et al., 1974; 1980) and in circulating IC (Lindsley et al., 1981). Nagle et al. (1974) detected properdin in glomerular deposits indicating activation of alternative complement pathway.

Facer et al. (1978) demonstrated that renal damage during trypanosomiasis was two phase, the first being immune complex-mediated glomerulonephritis followed by tubular ischaemic damage.

Haakenstad and Mannik (1977) reviewed the patterns of immune complex (IC) deposition in rabbit renal glomeruli. Briefly, there are 3 deposition patterns; sub-endothelial, mesangial and sub-epithelial. In the former, there are granular deposits along the capillary loops and in the subendothelial space. Some mesangial deposits may be present together with endothelial swelling, decrease in capillary lumen and necrosis. Mesangial deposition is often associated with normal histology but can have increased mesangial cellularity and increased extracellular
mesangial material. In subepithelial deposition, there is increased immunoglobulin deposition in peripheral glomerular loops, patent capillary lumens, thickened basement membrane and sometimes, fibrotic Bowman's capsules. The site of immune complex localization depends on IC size. Small complexes are associated with deposits in glomerular capillary loops, large complexes being associated with deposits in the mesangium. As IC size depends on, amongst other features, the Ab:Ag ratio, the fluctuating parasitaemia could provide several sizes of trypanosome-derived IC.

Whilst deposits of IgM, IgG and C3 are often detected, problems in detecting antigens present in complexes (Lambert and Houba, 1974; Zubler and Lambert, 1977) make it difficult to demonstrate with complete confidence that trypanosome-derived immune complexes deposit in the blood vessels and tissues to cause pathological changes. However, the indirect evidence for such a mechanism is good (e.g. Parry, 1980; Lindsley et al, 1980; Nagle et al, 1980; Galvao-Castro et al, 1978) and new techniques should overcome problems of detecting trypanosome antigens. The similarities between some of the pathogenic lesions in trypanosomiasis and those of well documented immune complex diseases point to trypanosomiasis being such a disease.

1.9.8 Skeletal Muscle

Muscle wastage is a feature of most trypanosomal infections (Goodwin, 1970; Van den Ingh; 1976; Goodwin and Guy, 1973; Dargie, 1980). This may be due to voluntary reduced food intake and transfer of anabolism to immunoglobulins and erythropoiesis rather than structural proteins (Dargie, 1980). However, Galvao-Castro et al (1978) demonstrated in mice that the host's immune response is necessary to develop skeletal and
cardiac muscle lesions. This was characterized by local deposition of IgM and IgG, associated with an inflammatory infiltrate of lymphocytes, macrophages, a few PMN's, trypanosomes and trypanosome antigens, indirectly pointing to an immune complex pathogenesis.

In a light microscopic study of the cremaster muscle in rabbits infected with *T. b. brucei*, Goodwin and Hook (1968) and Edeghere (1980) demonstrated dilated and tortuous venules and veins. Injected colloidal carbon (India ink) located in phagocytes, lined capillaries and venules, indicating increased vascular permeability (Marchesi, 1962). Inflammatory infiltrates of mononuclear cells in granulomas were seen in other skeletal muscles as well as cremaster muscle and ears (Goodwin and Hook, 1968). Edeghere (1980) in an electron microscopic study of rabbit cremaster muscle found that as the *T. b. brucei* infection progressed, more and more vessels became damaged. This damage consisted of inter-endothelial gap formation in venules and venular capillaries, extravasation of leucocytes and red cells, rouleaux formation, swelling of endothelial cells into the lumen which together with leucocyte sticking and microthrombi often completely blocked the microvascular lumen. He too never observed parasites within the vasculature as Goodwin (1971) reported, though they were detected in the interstitium. The basement membrane was always intact even when the microvessels had virtually disintegrated (Edeghere, 1980). This together with observations that colloidal carbon often localizes in the subendothelial space points to the barrier function of the basement membrane. Its role as a coarse filter between the blood and tissues may be important as the endothelial barrier is diminished, as appears to be the case in trypanosomiasis.
1.9.9 Genitalia

In male rabbits infected with *T. b. brucei* one of the major gross manifestations is swelling of the scrotum which develops scabby necrotic lesions as the disease progresses. The scrotal skin has a very intense inflammatory infiltrate of plasma cells, macrophages, lymphocytes with some hyper and parakeratosis (Van den Ingh, 1976). Damage to cremaster muscle is outlined above. Severe changes are evident in the testicles caused by a granulomatous orchitis and periorchitis (Van den Ingh; 1976; Ikede and Akpavie, 1982). As the infection progresses, testicular degeneration is evident with no spermatogenesis. Reproductive disorders have been documented in human trypanosomiasis (Apted, 1970; Greenwood and Whittle, 1980) and in bovines (Losos and Ikede, 1972) and sheep (Ikede, 1979), though *T. congoense* and *T. vivax* infections cause more degenerative than inflammatory changes (Ikede and Akpavie, 1982). An important facet of such disorders is that male infertility does not resolve as soon as a cure in effected, and it may take many months (Ige and Amodu, 1975; Ikede and Akpavie, 1982).

1.9.10 Blood

The major features in trypanosomiasis are reduced haematocrit (anaemia), thrombocytopenia, leucocytosis, increased red cell rigidity, rouleaux formation, platelet aggregation and microthrombi formation, increased plasma viscosity (mainly due to increases in immunoglobulins and fibrinogen) and elevated levels of circulating immune complexes.

1.9.10.1 Anaemia

In *T. b. brucei* infections of many host species, anaemia is usually moderate and not a major clinical feature though severe anaemia can occur
in T. b. rhodesiense infections in humans (Barrett-Connor et al., 1973) and in the more acute laboratory strains of T. b. brucei in rabbits such as S42 (Jenkins et al., 1980). In contrast anaemia is the chief feature of T. congolense and T. vivax infections of domestic animals and a major cause of death (Namo and Holmes, 1975).

There are many reports of decreased red cell survival time in T. congolense and T. vivax infections of domestic animals (Valli et al., 1979; Anosa and Isoun, 1980; Dargie, 1980) and the T. brucei group of infections (Jenkins et al., 1980; Jennings et al., 1974; McCrorie et al., 1980; Woodruff et al., 1973) indicating a haemolytic mechanism, probably involving the spleen (McCrorie et al., 1980 and see 1.8.2 above). Labelled red cells accumulate in the spleen during the infection (see chapter 2 and McCrorie et al., 1980) but it is unknown whether this is because homologous red cells are damaged and thus are more easily destroyed and/or there is a non-specific destruction of red cells by the enlarged spleen, as suggested by McCrorie et al., (1980).

An immune-mediated mechanism of haemolytic anaemia is also indicated. Red cell membranes have been studied during trypanosome infections and the following substances have been noted attached to the red cell surfaces: complement during T. b. rhodesiense infections in man (Woodruff et al., 1973) and T. vivax in calves (Facer et al., 1982); T. b. brucei antigens in mice (Herbert and Inglis, 1973) and rabbits (Woo and Kobayashi, 1975); trypanosome-derived IC in mice infected with T. b. brucei (Amole et al., 1982) and T. evansi (Assoku, 1975); IgG and IgM during T. vivax in calves (Facer et al., 1982). De Graves and Cox (1983) showed that immunoglobulin (anti fixed C3) and IC contribute to the concurrent anaemia and thrombocytopenia found in malaria and suggest that a similar mechanism may operate in Trypanosomiasis, as
indicated by Rickman et al (1981). Blood cells of rats infected with T.b. rhodesiense become coated with fibrinogen (not surprisingly since blood fibrinogen levels are increased during the infection (Facer, 1976) and given the affinity of red cells for fibrinogen) or its products, possibly in the form of fibrin/fibrinogen and anti-F complexes (Rickman et al, 1981; Rickman and Cox, 1979).

Levels of circulating IC are greatly increased during trypanosomiasis (Parry, 1980; Lindsley et al, 1981; Lambert et al, 1981; Rose et al, 1982) and red cells might act to remove soluble IC (Fruit et al, 1977; Lindsley et al, 1980).

There is some evidence for a trypanosome-derived haemolysin (Huan et al, 1975), possibly phospholipase A (Tizard et al, 1978), large amounts of which have been found in the tissue fluid of rabbits infected with T.b. brucei (Hambrey et al, 1980). Phospholipase A catalyses the release of free fatty acids, one of which, linoleic acid, has a haemolytic action (Tizard et al, 1979) but more importantly, linoleic acid is a precursor of prostaglandin formation. The effects of the kinin/kallikrein system are modulated by prostaglandins (McGiff, 1979) particularly the E series and prostacyclin (PGI₂) (Seino et al, 1982). Bradykinin can also release prostaglandins (McGiff et al, 1976) by activating phospholipase A (Hong and Levine, 1976) and kallikrein can stimulate the production of PGI₂ (Morita et al, 1974). The diverse roles prostaglandins have (particularly prostacyclin and thromboxanes) in inflammation (see section 1.8.4) and local control of the cardiovascular system (McGiff, 1979) could easily be involved in red cell abnormalities during trypanosomiasis.
Another mechanism for haemolysis may be secondary to microvascular damage, microangiopathic haemolytic anaemia (Brain et al, 1962) characterized by microthrombi, DIC, angiitis, rouleaux formation, red cell crenation and reticulocytosis, all features of trypanosomiasis (Jenkins et al, 1974).

Haemodilution has been proposed as a contributing factor to trypanosome anaemia in T.b.brucel infected mice (Amole et al, 1982), T. congolense infected cattle (Naylor, 1971) and T. vivax infected sheep (Anosa and Isoun, 1976). However, as Maxie and Valli (1979) point out, unless simultaneous measurements are made of red cell space and plasma space and corrected for haematocrit, accurate assessment of blood volume cannot be made. Thus haemodilution has only been shown to occur in T. vivax infected sheep and goats (Anosa and Isoun, 1976) and rabbits infected with T. congolense and T.b. brucel (Holmes and Jennings, 1975). Even if independent measurements are taken, however, if labelled albumin or transferrin are used but not sampled within at least the first 30 minutes in control animals (and probably much less in infected animals), the apparent plasma space is overestimated because the microvasculature is not completely impermeable to such proteins (see chapter 2 and 1.7). Amole et al (1982) sampled venous blood between 1 and 3 hours after injecting labelled serum proteins. This may have allowed significant extravasation of the tracer and thus no firm conclusions can be made for mice infected with T.b. brucel. Dargie (1979) found no change in blood volume in cattle infected with T.b. brucel or T. congolense. Simultaneous measurements of red cell and plasma space were carried out in rabbits infected with T.b. brucel in the present study (see chapter 2.4. 1.3).
The other main feature of anaemia is dyshaemopoiesis. The brisk reticulocytosis seen early in the infection is presumably a response to accelerated haemolysis. This is borne out by the early bone marrow hyperplasia (Colvin et al as cited by Jenkins and Facer, 1985; Saror, 1980; Van den Ingh, 1976) but there is some evidence that the bone marrow response is less efficient in chronic conditions such as megaloblastic anaemia (Jenkins and Facer, 1985) which may be a factor in the progressive anaemia of trypanosome infections. However, measurements of red cell iron utilization indicate diminished or ineffective erythropoiesis (Dargie et al, 1979; Dargie, 1980) in bovine trypanosomiasis. Jenkins and Facer (1985) note that Noyes et al (1982) measured erythropoietin levels in malaria and that it would be helpful to make such estimations in trypanosome infections. To this end, a pilot study was undertaken. (see chapter 5).

1.9.10.2 Thrombocytopenia

Though this occurs in most trypanosome infections, there are variations in severity. The most extreme thrombocytopenia appear in domestic animals infected with T. vivax and T. congolense (Wellde et al, 1983). The thrombocytopenia is mild in T. b. gambiense infections (Greenwood and Whittle, 1976a) but much more severe in T. b. rhodesiense infections (Greenwood and Whittle 1980) and T. b. brucei S42 in rabbits (Jenkins et al, 1980). Like anaemia, the causes of thrombocytopenia are varied. It may occur as a result of reduced production, particularly later in the infection (see "anaemia" above). Early in the infection, though, it may be the result of increased platelet aggregation and reduced plasma half-life, as suggested by evidence of DIC (reviewed by Jenkins and Facer, 1985) and platelet aggregates in the microcirculation (Edeghere, 1980). Slots et al (1977) showed that T. vivax- derived immune
complexes released serotonin in vivo and in vitro from platelets (which release their contents when aggregated).

1.9.10.3 Red Cell Abnormalities

These include red cell crenation, anisocytosis and spherocytes (Jenkins, 1980; Boreham and Goodwin, 1967; present study). These features have important implications for haemorheology in trypanosomiasis in that they indicate reduced red cell deformability.

In the microcirculation, anaemia will tend to reduce the apparent viscosity whilst the reduced red cell deformability will tend to increase it (see 1.7.4). In addition, platelet aggregation, microthrombi, red cell rouleaux formation and macroglobulinaemia will all contribute to increased apparent viscosity and slowing flow in the microcirculation. The presence of leucocytes in the microcirculation with their high viscosity coefficient reduces the Fahraeus effect, which also increases apparent viscosity and reduces blood flow (see 1.7.4)

It would appear that the overall pathological effects are to reduce blood flow in the microcirculation. Changing microvascular parameters may, in fact, be responsible for some of the hypotension seen in some rabbits infected with T.b. brucei (Boreham and Wright, 1976; Yates, 1978) as indicated by Gaehtgens (1984).

Damage to microvessels during T.b. brucei infections appears to be localized within a tissue with more vessels becoming damaged as the infection progresses (Edeghere, 1980; Goodwin and Hook, 1968) rather than diffuse changes affecting the whole tissue equally. This effect was seen in the mesenteric microcirculation (see chapter 3).
1.10 Experimental Determinations of Microvascular Permeability

1.10.1 Single Vessels

Landis (1927) devised a method for measuring the net movement of water across single capillaries. If a capillary was occluded with a microneedle, red cells sometimes flowed towards the occlusion or sometimes away from it. He argued that movement towards the occlusion was the result of water being filtered out of the plasma between the red cells and the point of occlusion. Reabsorption of fluid would cause the cells to move away from the occlusion. These linear movements of red cells can be converted into fluid flow and Landis found good experimental agreement with the Starling concept (1896) (see equation 1.1).

This technique was refined by Zweifach and Intaglietta (1968) on rabbit omentum. By comparing transcapillary fluid movement before and after transient alterations of colloid osmotic pressure (with intravenous albumin) it was possible to calculate filtration coefficients (hydraulic conductivity) and the net driving pressure. Smaje et al (1970) made further modifications, applying the technique to rat cremaster muscle. Since then, many studies have been carried out in a variety of tissues and species, though mesentery (see chapter 3), omentum, hamster cheek pouch and rat cremaster muscle remain the most accessible. Some of these studies have been undertaken to compare different methodologies (Gore, 1982) and to develop models of capillary permeability (Curry and Michel, 1980).

To look at solute permeability in single vessels, Levick and Michel (1973) used a micropipette system (based on Landis, 1926 and Michel et al, 1969) where Evans blue dye (Tl824) alone and complexed with
albumin were used to perfuse single frog mesenteric capillaries. Albumin is frequently used as a marker of macromolecular permeability. It may be labelled with visible dyes, fluorescent dyes (e.g. FITC) or radioactive markers (e.g. \( ^{131}\text{I} \) and \( ^{125}\text{I} \)).

Often single vessel experiments are performed in conjunction with histological methods. Ferritin can be used as a macromolecular permeability marker and being electron-dense, its exact location can be determined electron microscopically (Turner et al., 1983). Ferritin can also have its net charge altered so that charge-dependent effects can be studied (e.g. Clough and Smaje, 1984; Turner et al., 1983; Baldwin and Chen, 1984). Ultrastructural studies, though seemingly straightforward, have given rise to furious debate about the role of vesicles and fundamental aspects of microvascular transport (see also 1.7.2.2).

1.10.2 Indicator Diffusion Method

In the single injection multiple indicator technique (Crone, 1963; Martin de Julian and Yudilevich, 1964) two indicators are injected simultaneously into an artery close to the tissue or vascular bed under study. One of the indicators (reference) is confined to the vasculature whilst the other tracer (test) is known to cross the endothelial cell wall. Samples are drawn in rapid succession from a vein draining the bed. The concentrations of test and reference tracer in any sample of venous blood provide a figure for the fraction of the test tracer which has left the blood (the extraction, \( E \)). Crone (1963) assumed that the test tracer crossed the capillary wall by diffusion and that there was no backflux of the test solute. The permeability of the capillary area could be expressed as:
\[ P = \frac{Q}{S} \log_e \frac{1}{1 - E} \]  \hspace{1cm} (1.8)

where the extraction, \( E \) is given by:

\[ E = 1 - \frac{C_{\text{TEST}}}{C_{\text{REF}}} \]  \hspace{1cm} (1.9)

where:

\[ \begin{align*}
\dot{Q} & = \text{blood flow} \\
S & = \text{surface area} \\
C & = \text{concentration of tracer}
\end{align*} \]

The PS product is determined if \( S \) is unknown.

Disadvantages of this technique are that capillary heterogeneities of flow and lengths may lead to underestimations of PS for rapidly diffusing solutes (Levitt, 1970). If high flow rates are used to minimise the heterogeneity, solvent drag of the solute increases (Spencer, 1984) leading to overestimations of PS, particularly for larger molecules.

1.10.3 Isogravimetric Methods

The effect of inflammatory mediators such as histamine and bradykinin in producing solute and fluid efflux from the blood means that small changes in fluid balance can be detected using isogravimetric methods (Grega et al, 1972a; b; Renkin et al, 1977a; Diana et al, 1972; Kline et al, 1973; Marciniak et al, 1977). However, if input and output blood pressure and flow are controlled and lymph flow and protein concentrations are measured, much more information about inflammatory mediators (or other substances) on microvascular transport may be gathered
Renkin (1964) devised an analysis of lymph whereby PS of a solute could be determined as:

$$PS = \frac{LR}{1 - R}$$

(1.10)

where

- $R =$ the lymph: plasma ratio for the solute
- $L =$ lymph flow

This assumes that transport is entirely diffusive (and thus PS is an overestimate) and that $L$ is the same as capillary $J_v$.

Renkin's group carried out many studies on dogs' paws over the years using lymph analysis to look at a variety of transport problems and mechanisms occurring during inflammation (for example, Joyner et al., 1974; Garlick and Renkin, 1970; Carter et al., 1974). Later work (Renkin et al., 1977a, b) manipulating lymph flows allowed them to analyse convective as well as diffusive components of transport across microvascular endothelium (though Spencer, 1984 has reservations about their conclusions). The disadvantage of working on lymph is that it has an exceedingly low flow rate and to obtain sufficient lymph for analysis either long time intervals must be used or the production of lymph increased. Renkin and co-workers used the latter method, flexing dog paws 100 times per minute and collecting lymph at 30 minute intervals. There are also technical difficulties associated with the maintenance of patent lymphatic cannulae.
If a solute is added to the arterial blood, its permeability in a given tissue can be assessed by sampling its concentration in the tissue at various times after addition. Regional permeabilities can also be assessed in whole body experiments. This method is particularly suitable for relatively impermeable solutes as they can be followed for as much as several hours or days. However, unless an external monitoring system is used (e.g., a gamma camera for solutes labelled with a $\gamma$-emitting source), multiple sampling is not possible. This makes the method animal intensive and subject to greater experimental errors.

Iodinated serum proteins have long been used to assess macromolecular exchange between intra- and extravascular compartments (e.g., Sterling, 1951; Rothschild et al., 1955). If a multiple tracer technique is used, volumes of distribution can be determined. The method is similar to the indicator diffusion method in that a reference tracer confined to the vasculature is used in conjunction with a diffusible test tracer. Commonly used pairs of tracers are $^{59}\text{Fe}-\text{RBC}$ and $^{131}\text{I}\text{-albumin}$ or other serum proteins (Studer and Potchen, 1971) and $^{51}\text{Cr}-\text{RBC}$ and $^{125}\text{I}\text{-albumin}$ (Owen and Triffitt, 1976). In the present study, $^{51}\text{Cr}\text{-RBC}$ and $^{125}\text{I}\text{-albumin}$ were used. The labelled red cells are distributed within the vasculature so that by injecting a known concentration, total blood or red cell volume can be determined. The diffusible tracer volume can be corrected by the intravascular component. The assumptions made in this method are discussed in chapter 2.

The use of this method for determining macromolecular permeability is not nearly as common as some of the other methods. One of the reasons may be that it is not suitable for looking at finest details of capillary
permeability. It is of more use when comparing control, normal animals/organisms with an "altered" or pathological condition (as in the present case). An increasing number of workers are now using lymph analysis methods because, once the technical difficulties are surmounted, a great deal of information may be obtained. Chronic studies are also possible, such as the sheep lung lymph fistula (Staub et al, 1981).

1.10.5 Transcapillary Escape Rate (TER)

This is measured in a whole body where an invasive method is not preferred (e.g. in a clinical situation). A known amount of tracer (e.g. $^{125}$I-albumin which is expected to behave as a native albumin) is injected intravenously. Blood samples are taken at intervals so that the time course of the tracer in the blood or plasma can be monitored. The slope of the decay curve (log scale) gives the TER. In an analysis of such data based on Perl (1975) together with information from the literature, Paaske (1983) concluded that the TER "undoubtedly reflects the disappearance of albumin from the bloodstream but it has nothing to do with the capillary permeability". This will be discussed further in chapter 2 where similar data was obtained for control and T.b. brucei infected rabbits, not to measure TER as such, but to monitor the blood levels of labelled albumin and red cells.
2. Permeability Studies

2.1 Introduction

Histological studies of animals infected with *T. b. brucei* indicated that substantial damage occurred in the cardiovascular system, particularly in the smallest blood vessels (Boreham and Goodwin, 1967; Goodwin and Hook, 1968 & Goodwin, 1971). These changes resembled those resulting from inflammatory reactions. The most damaged region appeared to be the capillary-venular segments where large interendothelial cell gaps were seen (Edeghere, 1980). Although there are a variety of pathways postulated for exchange of substances across the microvascular wall (Renkin, 1977; Spencer, 1984; chapter 1.7.2.2), gap formation is thought to indicate increased transport, particularly of macromolecules (Majno and Palade, 1961).

The present study was undertaken to assess physiological changes in macromolecular permeability that may occur during trypanosomiasis in rabbits.

The reference tracer, which is confined to the vasculature, was \(^{51}\text{Cr}\) bound to homologous red cells (\(^{51}\text{Cr-RBC}\)). Permeability changes were assessed with \(^{125}\text{I}\)-labelled serum albumin (\(^{125}\text{I-alb}\)). Commonly used pairs of tracers are \(^{59}\text{Fe-RBC}\) and \(^{131}\text{I-alb}\), and other serum proteins (Studer and Potchen, 1971) and \(^{51}\text{Cr-RBC}\) and \(^{125}\text{I-alb}\). (Owen and Triffitt, 1976). The method of the latter authors was adapted for our use. \(^{51}\text{Cr}\) was preferred to \(^{59}\text{Fe}\) since in order to label erythrocytes, the latter must be injected in a donor animal seven days prior to an experiment, and in view of the anaemia in trypanosomiasis, the iron balance may be affected (Jenkins and Facer, 1984). Homologous red cells were also preferred to
minimise any errors due to host reaction.

Several assumptions have to be made in the use of tracer techniques:

1) following injection, there is rapid and complete mixing in the blood.

2) the tracers are not metabolised or excreted during the experiment.

3) the radio-labelling does not change the behaviour of the material being labelled.

4) the label is strongly and irreversibly bound to the material.

5) the intravascular tracer is confined exclusively to the blood.

As described below, precautions were taken to ensure the validity of these assumptions and tests were made of some of them.
2.2 Materials and Methods

2.2.1 Parasites

_**Trypanosoma brucei brucei**_ 427 was obtained from the London School of Hygiene and Tropical Medicine, Winches Farm, St. Albans. Though this strain is thought to be non-infectious to humans, there is evidence that it is very closely related to _T. b. rhodesiense_ (Hudson _et al._, 1981). _T. b. rhodesiense_ may in fact be a human-adapted variant of _T. b. brucei_ (J.D. Barry - personal communication).

The parasites were grown in the blood of mice in _vivo_. _Trypanosoma b. brucei_ 427 produces a very high parasitaemia with little or no solid tissue involvement, death occurring 2-3 days after inoculation.

The parasitaemia of an infected mouse is ascertained by a wet blood film made from tail venous blood. If the parasitaemia is sufficiently high (of the order or higher than numbers of red cells), the mouse is quickly killed (usually by cervical dislocation) and blood taken from the right side of the heart with a 2ml syringe and 25 gauge needle. The plastic syringe container was filled with sterile saline and a few drops of the mouse blood was added (a dilution of approximately 1/10 - 1/20, depending on parasitaemia).

Diluted blood (0.1ml) was injected intraperitoneally into each of 2 clean mice (male or female T.O., Biochemistry Animal Unit, Imperial College, London) and the process repeated every 2-3 days. With experience, it was easy to manipulate dilutions and injections to obtain the desired parasitaemia at a given time.
During periods when parasites were not needed, stabilates were prepared using a technique demonstrated by C.D. Kimber (Winches Farm) which is based on the method of Cunningham et al (1963). Heavily parasitized blood from mice was mixed with glycerol to give a concentration of 7.5% glycerol. Blood/glycerol was drawn into fine capillary tubes, sealed and gradually cooled to -70°C over 8 hours. The tubes were immediately transferred to liquid nitrogen where they remained viable as long as the temperature remained constant.

2.2.2 Infection of Rabbits

To obtain sufficient parasites to infect 4 rabbits, rats were used as passage animals instead of mice. Outbred albino S.D. rats of either sex (Biochemistry Animal Unit, Imperial College) were inoculated with high parasitaemic mouse blood. After about 2-3 days, the parasitaemia was closely monitored so that blood was obtained having the highest possible parasitaemia. The rats were heavily anaesthetised with chloroform and bled from the right side of the heart into anticoagulant (lithium heparin) tubes and centrifuged in a small bench centrifuge at 3,500 rpm for 10 minutes. The trypanosomes were separated from the plasma using the anion-exchange method of Lanham (1968) using a phosphate-glucose-saline (PGS) buffer eluant (Appendix 1). This method gives trypanosomes free of blood contaminants. Eluted trypanosomes were centrifuged at 3,000 rpm for 5 minutes and washed in PGS three times to remove any remaining contaminants. Resuspension of the parasites in PGS gave approximately $5 \times 10^8 - 7 \times 10^9$ parasites per ml (checked by counting on an Improved Neubauer hemocytometer). Rabbits were infected by subcutaneous inoculation of about $2 \times 10^9$ freshly prepared trypanosomes.
Male New Zealand White rabbits (1.9 to 3.5 Kg) were used in these experiments, with the exception of 2 females (exp. 18 and 121, both infected). Twelve uninfected rabbits acted as controls (Cl-C12). Twenty-one rabbits (11-121) were infected as described above and were killed 12-31 days later, a mean infection length of 21 days.

Table 2.1 gives the details of infected rabbits, namely infection duration, clinical presentation (+ indicates particular severity of external symptoms, absence of +'s indicates the usual appearance for the infection duration (see also chapter 1.5.5 and 1.9)), duration of the experiment (30, 60, 90 or 120 minutes).

For these experiments the severity of the infection was judged primarily by the state of the genitals because it served no purpose to cause the rabbits more distress than was necessary. The allocation of rabbits into experimental sub-groups is shown in Table 2.2.

2.2.3 Preparation of $^{51}$Cr-RBC

Approximately 4-5ml of blood were drawn from the carotid artery of an anaesthetised rabbit into a 10ml heparinised syringe. Approximately 100 µCi Na$^{51}$CrO$_4$ (Amersham International), specific activity 250-500µCi/µg Cr, was added slowly to the blood with continuous gentle mixing. After 30 minutes at room temperature, 50mg of crushed ascorbic acid B.P. was added to reduce unbound $^{51}$Cr to the trivalent state. Saline was then added to give a volume of 10ml, and the erythrocytes were washed 3 times by repeated centrifugation (3000 rpm for 2 minutes) and resuspended. The final resuspension was to the original blood volume. All the supernatants were retained for γcounting for estimation of washing efficiency. A small aliquot of the $^{51}$Cr-labelled RBC was reserved for γcounting and since a
<table>
<thead>
<tr>
<th>RABBIT</th>
<th>DURATION (days)</th>
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<th>GENITALS</th>
<th>SKIN</th>
<th>SPLEEN</th>
<th>EXPER. TIME</th>
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Table 2.1 The presentation of the infected rabbits.

- indicates normal appearance for the length of the infection.
* particular severity of a symptom than is usual for the length of infection; graded * to ** *.
<table>
<thead>
<tr>
<th>Experiment Duration</th>
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<th>Infected Rabbits</th>
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<td></td>
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</table>

**Table 2.2** The allocation of rabbits into experimental sub-groups. The column 'L' is the length of the infection in days. Experiment duration is in minutes.
known weight of $^{51}$Cr-RBC was injected into the rabbit, the actual quantity of radioactivity injected could be calculated.

We used five times the quantity of Na$^{51}$CrO$_4$ (i.e. about 100 $\mu$Ci) recommended by Owen and Triffitt (1976) since the lower quantity gave tissue $^{51}$Cr counts only marginally above background.

2.2.4 Preparation of $^{125}$I-albumin

Bovine serum albumin (BSA) was labelled with $^{125}$I by the chloramine-T method (0.1mg chloramine-T was used to oxidize 1 mCi Na$^{125}$I in the presence of 1mg albumin). Immediately prior to injection into the rabbit, the $^{125}$I-alb was ultrafiltered at least 3 times with saline through a filter with a cut off at 50,000 daltons to remove low molecular weight impurities including free iodide ($^{125}$I$^-$). The final volume was about 2ml. A small aliquot was counted for $^{125}$I to determine the activity of $^{125}$I-albumin and a known weight injected into the rabbit.

2.2.5 Procedure

A drop of blood was taken from the ear of an infected rabbit prior to experimentation to estimate parasitaemia and degree of red cell crenation. If there were more than 2-3 parasites per field (using a x40 objective) and/or a significant degree of crenation the rabbit was not used for at least 3-4 days. Experience showed that such rabbits were difficult to anaesthetise and tended to die suddenly during the experiment. The reason for this is unclear but may be due to large scale release of parasite metabolites and proteolytic enzymes during parasite destruction.
The rabbit selected was anaesthetised with 30mg/Kg sodium pentobarbitone (Sagittal, May and Baker), diluted 1 in 2 with sterile saline, and injected via a marginal ear vein. The carotid artery was cannulated for the injection of tracers and removal of blood samples. This minimised surgical trauma and cut down on experimental time. Infected rabbits are very difficult to operate on, particularly their veins, which become "like wet blotting paper" (Goodwin, 1970).

All injections were washed in with heparinized saline (50IU/ml). Great care was taken sampling the blood so that there was no dilution or contamination of samples.

10mls of blood were removed immediately after surgery, 4-5ml for $^{51}$Cr labelled red cells (RBC) and the remainder used to prepare serum or plasma which was stored at -15°C for C-reactive protein (CRP) determination (see 2.2.8). The rabbit was heparinized (600IU/Kg) and the $^{125}$I-albumin slowly injected (via the carotid artery) over about 1-1½ minutes. The start of the injection was considered to be zero time.

At 2, 5, 10 minutes after the start of injection and at intervals thereafter, depending on the duration of the experiment, blood samples (0.7ml) were removed for measurement of total blood and plasma radioactivity and for duplicate hematocrit determinations. All samples for analysis were placed in numbered and pre-weighed vials with closely fitting labelled caps and weighed immediately after the end of the experiment. $^{51}$Cr-RBC were injected 12-20 minutes prior to termination of the experiment. A blood mixing time of 5 minutes for $^{51}$Cr-RBC has been found to be adequate in control rats (Studer and Potchen, 1971) but in infected animals, longer mixing times could be expected (because of accelerated ESR, increased viscosity and rouleaux formation - Facer, 1976).
After the $^{51}$Cr-RBC injection, 2 or 3 blood samples were taken at intervals. After a final blood sample (3ml) had been taken, sufficient pentobarbitone was given intravenously for very deep anaesthesia and tissues were removed as rapidly as possible in the following order:

testis skin (2-4 samples)
cremaster muscle (2-4)
abdominal wall muscle (3-5)
kidneys (4) - samples removed after clamping renal artery, vein and ureter
mesentery (2-4) - after clamping major arteries and veins
liver (3-4)
spleen (1-3)
lung (3-5)
heart (3-4) - large arteries and veins were clamped if death had not already occurred
aorta (1-3)
ear (3) - sometimes removed earlier

Thoracic lymph was sampled just above the diaphragm immediately after opening the chest. Any blood contaminated samples were discarded. After weighing and estimation of radioactivity, the vials were unstoppered and tissues were dried to constant weight.

2.2.6 Radioactivity Measurements

$^{125}$I and $^{51}$Cr are both $\gamma$ sources which have markedly different disintegration characteristics. Each sample was counted for both isotopes simultaneously in a 2-channel $\gamma$ counter (Hewlett-Packard 5130) for 5 minutes. The raw counts were corrected for background radioactivity, crossover between the 2 isotope channels and converted to corrected
counts per second (cps) per gram of tissue for each isotope by means of a computer program (written by O.R. Tutty, Department of Applied Maths and Theoretical Physics, University of Cambridge).

2.2.7 Tests of Assumptions

When the experiments commenced, only a single channel counter was available and discrimination between $^{131}\text{I}$ and $^{51}\text{Cr}$ was very difficult, $^{125}\text{I}$ was much more suitable. Although the $^{125}\text{I}$-alb was purified immediately before use, some breakdown of the protein may occur to yield free iodide in vivo (Winlove et al, 1978). $^{125}\text{I}^-$ is transported between blood and tissue more rapidly than protein (see Fig. 2.1) and so its concentration was assessed in most experiments by gel filtration chromatography. One ml. of plasma from the final blood sample was passed through a Sepharose 4B or 6B column (Pharmacia Ltd.). The distribution of free $^{125}\text{I}^-$ and labelled protein in the eluted fractions was measured by $\gamma$ counting and the total protein distribution was determined by absorption spectroscopy at 280nm wavelength. Free $^{125}\text{I}^-$ was eluted later than that bound to albumin and the radioactivity of each fraction was determined. Total free $^{125}\text{I}^-$ was expressed as a percentage of total protein bound $^{125}\text{I}$. In 28 out of 34 experiments the free $^{125}\text{I}^-$ to bound $^{125}\text{I}$ values were less than 1%. No differences in tissue uptake could be discovered in the 6 experiments when the ratio was marginally greater than 1%.

The experiments were comparatively short, with the maximum time being 2 hours. This was the longest time for labelled albumin to be in vivo and to be certain of minimal free $^{125}\text{I}^-$ formation (Winlove et al, 1978), as outlined above. The other advantage of a short experimental duration is to minimize metabolism and excretion of the tracer.
$^{51}$Cr levels in the blood reached a constant value 2-5 minutes after injection and remained constant until the end of the experiment. It was therefore assumed that there was no leakage out of the vasculature and it behaved as a reference tracer.

2.2.8 Index of Infection and C-reactive Protein Estimations

Although T. b. brucei 427 infection in rabbits is progressive, the severity of the disease can vary from rabbit to rabbit. Monitoring the parasitaemia indicates when the parasitaemic peaks occur but does not necessarily indicate the extent of other pathological changes (M.J. Parry - personal communication). Cook (1979) suggested that severity of the disease in rabbits could be assessed by estimation of plasma C-reactive protein (CRP). This is an acute phase reactant which appears early in infection. It is associated with the inflammatory reaction and persists only during the active phase. It is non-specific and present in a variety of disease conditions (Hedlund, 1947). CxRP is an analogous protein found in rabbits during infection. In T. congolense infections, increased CxRP concentrations in the early stage rapidly reach a maximum and then slowly decline as tissue necrosis becomes more severe (Thomasson et al, 1973). A similar pattern is seen in rabbits infected with T. b. brucei with no correlation between CxRP levels and parasitaemia or trypanosome agglutinating antibodies (Cook, 1979).

After all the serum samples had been collected from the experimental animals, batch CxRP estimations were performed using a Hyland CR-test kit (Travenol, Ltd.). Although this test kit is designed for estimating human CRP, it detects rabbit CxRP equally well (M.J. Parry - personal communication). Agglutination titres were determined by observing the macroscopic reaction of serially-diluted serum/plasma samples with anti-CRP bound to latex particles.
Previously frozen samples were rapidly thawed at 37°C and then inactivated at 56°C for 30 minutes. Dilutions were made with the glycine-saline buffer supplied with the kit.

2.2.9 Calculations

The available data consisted of:

1) total counts per second (cps) of $^{51}$Cr and $^{125}$I injected
2) $^{125}$I cps per gram of tissue, blood and plasma (denoted as $I_T$, $I_B$ & $I_P$)
3) $^{51}$Cr cps per gram of tissue and blood (denoted as $Cr_T$ and $Cr_B$).
4) arterial haematocrits (hct) - measured in duplicate
5) body weight (bw) in Kg
6) multiple blood and plasma samples at various times during the experiment
7) $^{125}$I cps per gram of lymph where collected ($I_L$)
8) wet (W) and dry (D) weights for each tissue sample

These were used to calculate a variety of useful parameters.

2.2.9.1 Whole Body Data

By the use of two tracers, red cell space (RBC space) and plasma space (PV) can be determined independently.

$$\text{RBC space/Kg} = \frac{\text{injected Cr}}{10 \text{ min. } Cr_B} \times \frac{\text{hct}}{\text{bw}}$$  \hspace{1cm} (2.1)

$$\text{PV/Kg} = \frac{\text{injected I}}{10 \text{ min. } I_P} \times \frac{1}{\text{bw}}$$  \hspace{1cm} (2.2)
From these, the total body blood fraction/Kg can be determined as:

\[
\frac{PV + \text{RBC space}}{bw}
\]  

Blood fraction can also be obtained by simple dilution of isotope:

\[
\text{blood fraction/Kg using Cr only} = \frac{\text{injected Cr}}{10\text{min.Cr}_B \times bw}
\]  

\[
\text{blood fraction/Kg using I only} = \frac{\text{injected I}}{10\text{min.I}_B \times bw}
\]

Total body hct can be calculated from:

\[
\frac{\text{RBC space}}{PV + \text{RBC space}}
\]  

and also expressed as a percentage of arterial hct.

All blood samples taken provided sufficient blood to do duplicate hct. determinations. Fresh blood was drawn into capillary microhaematocrit tubes, sealed and centrifuged. The hct was expressed as a fraction of the total blood volume.

2.2.9.2 Tissue-specific Data

Two methods of calculating extravascular accumulation of \(^{125}\text{I}\)-albumin were used. The first one assumes that the haematocrit of the tissue blood is the same as the arterial hct, and that changes in arterial hct during the infection are reflected to a similar degree in the tissues (Gaehtgens, 1984). Comparisons between the 2 groups of rabbits (control and infected) are possible but not determination of absolute values for the various parameters.
The second method uses data from control rabbits in the 30 minute experiments to calculate tissue hct and thus to determine more accurately the intravascular space and hence extravascular $^{125}$I-albumin. Though this may reflect the actual situation at the microvascular level, certain major assumptions must be made:

a) as above, that there is a corresponding change in tissue hct when arterial hct is changed in the infected rabbits, as is true for control rabbits (Gaehtgens, 1984).

b) that there has been no appreciable leakage of labelled albumin out of the vasculature within the first 30 minutes of the experiment. This is obviously untrue for tissues with discontinuous (sinusoidal) capillaries such as liver and spleen where equilibration of macromolecular tracers can occur within 5 minutes (Studer and Potchen, 1971) and certainly in less than 30 minutes (see 2.2.14). Spencer (1984) found negligible leakage of $^{125}$I-albumin in a tissue with fenestrated capillaries (rabbit salivary gland). Bell et al (1978) found that rabbit kidney (fenestrated capillaries) had an albumin reflection coefficient ($\sigma_s$) of 0.998. This implies that very little, if any, albumin is transported out of the vasculature in healthy rabbits. The long equilibration time of many tissues with continuous capillaries to labelled macromolecules (Mullins and Bell, 1982; Bell and Mullins, 1982a; b; Studer and Potchen, 1971; Owen and Triffitt, 1976) and comparatively high $\sigma_s$ to albumin (Spencer, 1984) suggests that calculation of tissue hct based on the 30 minute controls is valid.

c) tissue hct is susceptible to alterations due to variation in local blood flow and arterial hct and such a correction factor determined and then applied to other animals would be expected to have a large margin of error.
In the following equations, these abbreviations are used:

\[ \text{TBF} = \text{tissue blood fraction} \]
\[ \text{Cr, I} = \text{the activities (cps per gram) of } ^{51}\text{Cr and } ^{125}\text{I respectively}} \]
\[ \text{H} = \text{haematocrit} \]
\[ \text{IVV} = \text{intravascular } ^{125}\text{I-alb. fraction} \]
\[ \text{E} = \text{extravascular } ^{125}\text{I-alb. fraction} \]
\[ \text{E/P} = \text{extravascular } ^{125}\text{I-alb. in plasma equivalents} \]

Subscripts
\[ B = \text{blood} \]
\[ T = \text{tissue} \]
\[ P = \text{plasma} \]

Final sample blood and plasma Cr and I are used unless stated otherwise.

**First Method**

\[ \text{TBF} = \frac{\text{Cr}_T}{\text{Cr}_B} \quad (2.7) \]

\[ \text{IVV} = \text{TBF} \times \text{I}_B \quad (2.8) \]

\[ \text{E/P} = \frac{\text{I}_T - \text{IVV}}{\text{I}_P} \quad (2.9) \]
Second Method (corrected for tissue hct)

\[ H_T = \frac{\frac{Cr_T}{I_T} \times H_B}{\frac{Cr_B \times (1-H_B)}{I_B} + \frac{Cr_T \times H_B}{I_T}} \]  

(2.10)

(from Spencer, 1984)

\[ TBF' = \frac{Cr_T}{Cr_B} \times \frac{H_B}{H_T} \]  

(2.11)

The \( \frac{H_B}{H_T} \) ratio is the correction factor calculated from the 30 minute control experiments. It is tissue specific and applied to both infected and control animals.

\[ IVW' = TBF' \times I_B \times \frac{(1 - H_T)}{(1 - H_B)} \]  

(2.12)

\[ E/P = \frac{I_T - IVW'}{I_P} \]  

(similar to equation 2.9)

2.2.10 Extravascular Albumin

This was expressed as "plasma equivalents" - the E/P ratios (equation 2.9). For both methods of calculation E/P's for each tissue were divided into control and infected groups and subdivided into experimental durations (i.e. 30, 60, 90 and 120 minutes), giving 8 groups in all. Statistical analysis was carried out as detailed in chapter 2.2.15.
2.2.11 Tissue Blood Fraction

TBF and TBF' were calculated for each tissue as outlined above (equations 2.7 and 2.11). The mean of the infected groups was compared to that of the control group.

2.2.12 \(^{125}\)I-albumin in Plasma

The rate of loss of \(^{125}\)I-alb. from the circulation was determined by expressing plasma concentrations of \(^{125}\)I-alb. in each sample as a percentage of the concentration in the 10 minute sample. Exponential regression analysis was carried out.

2.2.13 Water Content and Dry Weights

After gamma counting, tissue samples were dried to constant weight and then re-weighed. Two approaches to the results were used:

1) Water contents expressed as a percentage of tissue wet weight \((W)\) using:

\[
\frac{W - D}{W} \% \quad (2.13)
\]

\(D\) being the dry weight of the tissue.

2) Water to dry tissue mass using the formula:

\[
\frac{W - D}{D} \% \quad (2.14)
\]

For both methods the results for each tissue were divided into control and infected groups, the means being compared using the students t-test.
2.2.14 Lymph

Lymph was collected from a large thoracic vessel at the end of the experiment. The composition of such lymph is generally considered to be visceral but about 50% is thought to be liver lymph (Johnson and Levitt, 1975). Attempts were made to cannulate an abdominal lymphatic vessel. Difficulties encountered were partially due to the low flow of lymph and high resistance in the butterfly cannula. In addition, disadvantages due to surgical disturbance of the abdominal contents were felt to outweigh the limited advantages of determining the lymph $^{125}$I-albumin concentrations. The cannulations were not felt to be of sufficient reliability to make any pronouncements about changes in lymph flow.

Throughout these experiments, surgical and mechanical trauma was kept to a minimum.

2.2.15 Statistics

Two tailed T-tests were carried out to test for the significance of differences between the infected and control groups for TBF, water contents, water:dry tissue mass and whole body haematological data.

The null hypothesis for the E/P ratios and $^{125}$I-albumin distributions is that there is no increase due to the infection. One-tailed T-tests were carried out to test for increases compared to controls.

Where there was a significant difference in the standard deviations (SD) of the given sets of data (as determined using an F-test) a modified T-test was used (Wetherill, 1967).
Regression analysis was done to determine correlations, if any, between the parameter studied and the length of the infection. Exponential regression analysis was carried out between plasma $^{125}$I-albumin and length of infection.

Degrees of probability were determined, with $p > 95\%$ ($p < 0.05$) considered to be significant.
2.3 Results

2.3.1 Whole Body Data

2.3.1.1 Arterial Haematocrit

This was significantly reduced in the infection, as shown in Table 2.3, that is, a significant degree of anaemia. The change in hct. did not correlate with the length of infection.

2.3.1.2 Whole Body Haematocrit

These were expressed as actual hct. and as a percentage of arterial hct. For each rabbit (whole body ± arterial) hct was determined and then averaged for each of the 2 groups (Table 2.3). No significant difference between control and infected groups was found in either of these 2 parameters. During the infection, the decrease in arterial hct was similar to the reduction in whole body hct (13.6 and 14.5% respectively).

2.3.1.3 Blood, Plasma and Red Cell Fractions

These are shown in Table 2.3. Total blood fractions determined using both isotopes (equation 2.3) showed that there was a significant increase during the infection. There was no correlation with the length of the infection.

Total blood fractions measured using either $^{125}$I-alb. or $^{51}$Cr-RBC (equation 2.4 or 2.5) were not significantly different during the infection when compared with controls.
<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CONTROL</th>
<th>INFECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n) $\bar{x} \pm$ SEM</td>
<td>(n) $\bar{x} \pm$ SEM</td>
</tr>
<tr>
<td>ARTERIAL HAEMATOCRIT</td>
<td>(8) 0.349 ± 0.014</td>
<td>(18) 0.307 ± 0.0134*</td>
</tr>
<tr>
<td>(measured)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHOLE BODY HAEMATOCRIT</td>
<td>(8) 0.291 ± 0.022</td>
<td>(18) 0.253 ± 0.018</td>
</tr>
<tr>
<td>RBC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC+PV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL BODY HAEMATOCRIT</td>
<td>(8) 80.60 ± 4.31</td>
<td>(18) 82.55 ± 4.49</td>
</tr>
<tr>
<td>$\frac{\text{ARTERIAL HAEMATOCRIT}}{\text{TOTAL BODY HAEMATOCRIT}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL BODY BLOOD FRACTION</td>
<td>(8) 52.31 ± 2.20</td>
<td>(14) 60.86 ± 2.75*</td>
</tr>
<tr>
<td>PV + RBC (g/Kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL BODY BLOOD FRACTION</td>
<td>(8) 42.96 ± 2.69</td>
<td>(15) 48.91 ± 2.64</td>
</tr>
<tr>
<td>(injected $^{51}\text{Cr}$) (g/Kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(blood $^{51}\text{Cr}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL BODY BLOOD FRACTION</td>
<td>(12) 57.59 ± 3.79</td>
<td>(14) 64.50 ± 4.02</td>
</tr>
<tr>
<td>(injected $^{125}\text{I}$) (g/Kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 min blood $^{125}\text{I}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLASMA FRACTION (g/Kg)</td>
<td>(12) 37.04 ± 2.23</td>
<td>(14) 45.86 ± 2.86*</td>
</tr>
<tr>
<td>RBC FRACTION (g/Kg)</td>
<td>(8) 15.19 ± 1.17</td>
<td>(15) 14.75 ± 1.06</td>
</tr>
</tbody>
</table>

* denotes infected parameter significantly different (p 0.95)
PV = plasma fraction
RBC = red blood cell fraction

Table 2.3 WHOLE BODY HAEMATOLOGICAL DATA

The decrease in measured arterial hct. in the infected rabbits (14.48%) is of a similar magnitude to the decrease in whole body hct. (13.06%) though the latter is a non-significant decrease.
The plasma fraction was significantly increased in the infected rabbits. However, in spite of finding a significant degree of anaemia in infected rabbits, the isotopically determined $^{51}$Cr-RBC red cell fraction (per Kg) was not significantly decreased when compared to controls.

2.3.1.4 $^{125}$I-albumin in Plasma

Exponential regression analysis of $^{125}$I-albumin plasma levels and length of experiment were carried out using the formula: $y = A.e^{B.x}$ and the plots are shown in figure 2.1. The plots are of the control group, 4 infected rabbits (exp. II, I2, I3 & I4) the main group of rabbits (excluding exp. II, I2, I3 & I4) and of 2 additional control rabbits injected with Na$^{125}$I alone. The calculated $t_B$ of $^{125}$I-alb. in the plasma are shown on the figure. The raw data is given in Appendix 2, table A.

2.3.1.5 Cx-reactive Protein

The titres of CxRP are expressed as the reciprocal of the highest dilution giving macroscopic agglutination. The results are shown in figure 2.2, where the titres are plotted logarithmically against duration of infection. There is no correlation between CxRP and length of infection or CxRP and E/P ratios.
Figure 2.1: The time course of $^{125}$I-albumin in plasma using the plasma $^{125}$I activity 10 minutes after injection as 100%. Using exponential regression analysis, the $^{125}$I activity at zero time and half-life in the plasma ($t_{1/2}$) were calculated.

A) Infected rabbits I5 - I21. $t_{1/2} = 370$ minutes
B) Control rabbits C1 - C12. $t_{1/2} = 263$ minutes
C) Infected rabbits II - I4. $t_{1/2} = 122$ minutes
D) Two additional control rabbits injected with Na $^{125}$I only. $t_{1/2} = 54$ minutes (measured, not calculated)
Figure 2.2 Cx-reactive protein titres plotted logarithmically against length of infection.
2.3.2 Tissue Specific Data

2.3.2.1 Tissue Blood Fraction (TBF)

These were calculated using the two methods as outlined in 2.2.9.2, equations 2.7 and 2.11. For each tissue, the mean TBF was determined for the control and infected groups. The results are plotted in figure 2.3 (no tissue hct. correction) and figure 2.4 (corrected for tissue hct.). The data is given in Appendix 2, tables B and C. Calculated tissue hcts. for the 30 minute controls (C8, C9 and C10) are given in Appendix 2, table D.

In both calculation methods, there is a significant increase in TBF in abdominal wall (skeletal) muscle, spleen and lung of infected rabbits.

2.3.2.2 Tissue Water Content

Water content expressed as a percentage of tissue wet weight and as a ratio of water to dry tissue mass are shown in figures 2.5 and 2.6 respectively.

Significant enhancement (p<0.05) in tissue water content in infected animals was found only in cremaster muscle. Spleen had a significantly reduced (p<0.05) water content in infected animals.

Water to dry tissue ratio was significantly enhanced in cremaster muscle (p<0.01) of infected animals but diminished in abdominal wall muscle (p<0.01).
Figure 2.3. Tissue blood fraction (mean ± SEM) assuming $H_i = H_b$.

For each tissue, control is on the left and infected on the right.
Figure 2.4. Tissue blood fraction (mean ± SEM) corrected for Ht.

For each tissue, control is on the left and infected on the right.
For each pair of values, Control is on the left
Infected is on the right.

**FIG. 25**
Tissue water contents %
± SEM to 95% C.L.
For each pair of values, control is on the left, infected is on the right.

**Fig. 2.6** Water: dry tissue ratios ± SEM to 95% confidence limits (c.l.)
2.3.2.3 Extravascular $^{125}\text{I}$-albumin

The mean E/P for each tissue was expressed graphically as a function of the length of the experiment (figs. 2.7-2.17). Each figure is in 2 parts, A and B. A is the result of assuming similar haematocrits in blood and tissue. B shows the E/P ratios when the discrepancy between blood and tissue haematocrit is accounted for (see 2.9.2.2).

Both of these graphs for each tissue are presumed to indicate the rate and extent of tissue uptake of $^{125}\text{I}$-alb. All statistical tests were carried out as described in 2.2.15 and significant increases during the infection compared to control rabbits are shown on the figures. The data with standard deviations are in Appendix 2, Tables E&F. The distribution of the rabbits within each subgroup is shown in Table 2.2.

The tissues containing mainly continuous capillaries are heart (fig. 2.7), abdominal wall muscle (fig. 2.8), ear (fig. 2.9), cremaster muscle (fig. 2.10), scrotal skin (fig. 2.11), mesentery (fig 2.12), aorta (fig. 2.13) and lung (fig. 2.14). Kidney has mainly fenestrated continuous capillaries (fig. 2.15). Spleen and liver have capillaries with a discontinuous or sinusoidal structure (fig. 2.16-2.17). Although the method taking into account the tissue hct in these 2 tissues are shown, the use of 30 minute data to determine tissue hct is not valid for these tissues.

Significant correlations between E/P ratios and length of infection are shown on the figures.
Figure 2.7. Extravascular $^{125}$I-albumin in HEART. $\times$ = control, $o$ = infected.
(a) assuming $H_1 = H_0$, (b) corrected for $H_1$. $+$ denotes slg. at 95% level, $++$ at 99%.
$r$ denotes positive correlation with length of infection.
Figure 2.8. Extravascular $^{125}$I-albumin in ABDOMINAL WALL MUSCLE. $\times$ = control, $o$ = infected.

(a) assuming $H_1 = H_b$, (b) corrected for $H_1$. $+$ denotes sig. at 95% level, $++$ at 99%.

$r$ denotes positive correlation with length of infection.
Figure 2.9. Extravascular $^{125}$I-albumin in EAR. $\times$ = control, $\circ$ = infected.

(a) assuming $H_t = H_b$, (b) corrected for $H_t$. $+$ denotes sig. at 95% level, $++$ at 99%.

$r$ denotes positive correlation with length of infection.
Figure 2.10. Extravascular $^{125}$I-albumin in SCROTAL SKIN. $\times$ = control, $\circ$ = infected.

(a) assuming $H_i = H_b$, (b) corrected for $H_i$. + denotes sig. at 95% level, ++ at 99%.

$r$ denotes positive correlation with length of infection.
Figure 2.11. Extravascular $^{125}$I-albumin in CREMASTER MUSCLE. $\times$ = control, $\circ$ = infected.
(a) assuming $H_1 = H_0$, (b) corrected for $H_1$. $+$ denotes sig. at 95% level, $++$ at 99%.
$r$ denotes positive correlation with length of infection.
Figure 2.12. Extravascular $^{125}$I-albumin in MESENTERY. $\times$ = control, $\circ$ = infected.

(a) assuming $H_1 = H_2$, (b) corrected for $H_1$. + denotes sig. at 95% level, ++ at 99%.

$r$ denotes positive correlation with length of infection.
Figure 2.13. Extravascular $^{125}$I-albumin in AORTA. $\times =$ control, $\circ =$ infected.

(a) assuming $H_1 = H_b$, (b) corrected for $H_b$. + denotes sig. at 95% level, ++ at 99%.

r denotes positive correlation with length of infection.
Figure 2.14. Extravascular $^{125}$I-albumin in LUNG. $x =$ control, $o =$ infected.

(a) assuming $H_t = H_b$, (b) corrected for $H_t$. $+$ denotes sig. at 95% level, $++$ at 99%.

$r$ denotes positive correlation with length of infection.
Figure 2.15. Extravascular $^{125}$I-albumin in KIDNEY. $x =$ control, $o =$ infected.

(a) assuming $H_t = H_b$, (b) corrected for $H_t$. + denotes sig. at 95% level, ++ at 99%.
$r$ denotes positive correlation with length of infection.
Figure 2.16. Extravascular $^{125}\text{I}$-albumin in LIVER. $\times =$ control, $\circ =$ infected.

(a) assuming $H_t = H_b$, (b) corrected for $H_t$. $+$ denotes sig. at 95% level, $++$ at 99%.

$\ast$ denotes positive correlation with length of infection.
Figure 2.17. Extravascular $^{125}$I-albumin in SPLEEN. $\times =$ control, $\circ =$ infected.
(a) assuming $H_i = H_o$, (b) corrected for $H_i$. $+$ denotes sig. at 95% level, $++$ at 99%.
$r$ denotes positive correlation with length of infection.
2.3.3 Lymph

The concentration of $^{125}$I-albumin in the thoracic lymph was normalized by dividing by final plasma $^{125}$I, giving $L/P$ ratios, as shown in figure 2.18.
Figure 2.18. Lymph counts of $^{125}\text{I}$-albumin, expressed as a percentage of the final plasma $^{125}\text{I}$-albumin count. $\times =$ control, $\circ =$ infected. $+$ denotes significant difference at the 95% level, $++$ at 99%.
2.4 Discussion

Whole body data will be discussed first, followed by tissue-specific information.

2.4.1 Whole Body Parameters

2.4.1.1 Cx-reactive Protein

Of the ten samples from control rabbits, 7 were negative whilst 3 were unexpectedly high. The reason for this is unknown but many disease processes can elevate CxRP levels (Hedlund, 1947). The infected rabbits showed large increases in CxRP between 14 and 25 days of infection, with the exception of rabbits 117 (16 days) and 119 (20 days) during this period.

On the basis of CxRP levels, it is not possible to categorize the severity of the infection. There was no correlation between CxRP and other parameters.

Although it was found that CxRP measurements contributed little to the assessment of the severity of the disease, there is some suggestion that the acute phase of the disease is characterized by elevated CxRP. Van den Ingh (1976) classified T.b. brucei infection into 3 stages.

1) initial - up to 2 weeks duration.
2) acute - about 2-4 weeks, characterized by high levels of CxRP, a strong immunological reaction and invasion of the tissues by leucocytes and active phagocytosis.
3) chronic - falling CxRP levels, steady depletion of immunologically
active cells in lymphoid tissue, severe tissue ischaemia, increasing levels of parasites, fat accumulation and tissue breakdown.

In the present (limited) study, Van den Ingh's findings cannot be confirmed though the 2 studies are consistent. But it is not sufficient to divide the infected rabbits into sub-classes on the basis of CxRP levels.

Naik and Voller (1984) monitored CRP in Africans with falciparum malaria. They found that the negative controls still had elevated CRP levels compared to European negative controls, suggesting that CRP assessment for other uses (e.g. post-operatively) is not possible in endemic malarial areas. This may also be applicable in trypanosomiasis areas.

CRP enhances human neutrophil phagocytosis and may be important in the cellular defence against bacteria (Ganrot and Kindmark, 1969) and possibly trypanosomes (Thomasson et al. 1973; Cook 1979).

2.4.1.2 Transcapillary Escape Rate (TER) of $^{125}$I-albumin from Plasma

The plasma $t_\delta$ of $^{125}$I-albumin was calculated as 263 minutes in control rabbits. Nahmias et al. (1981) using $^{125}$I-human serum albumin (HSA) found $t_\delta$ of 198 minutes in rabbits. This discrepancy could be due to differences in rate of loss of HSA compared to bovine serum albumin (BSA) used in the present study. Gamble et al. (1982) showed that there was variation in TER depending on the labelling method and species of labelled albumin.
The rabbit non-liver TER for albumin is about 5.5% per hour (Paaske, 1983), calculated for rabbit whole body in the present study as 11.4% per hour for controls and 8.1% per hour for the main group of infected rabbits. It is possible that either of the 2 components, liver and non-liver may be altered.

Evidence is presented (fig. 2.16 and chapter 2.4.5.2) that the accumulation of labelled albumin is slower in the infected liver than in controls, suggesting that the liver component of the TER is reduced, thereby prolonging the time $^{125}$I-alb. is in the plasma.

Another facet of this problem is change in lymph flow rate. This was not measured but lymph was consistently much easier to collect in infected rabbits (see 2.4.6) and was also of similar $^{125}$I-alb concentration to that in controls (fig. 2.18) from 60-120 minutes, suggesting that tracer recirculation is faster or more efficient in infected rabbits. If there is enhanced filtration of fluid out of the bloodstream but with little or no increase in protein permeability, lymph flow increases but lymph protein concentration decreases (Mullins and Bell, 1982; Parker et al, 1979).

When considered in terms of the increased extravascular $^{125}$I-alb in some of the tissues during the infection, the slower TER presents a paradox. Dargie (1979), finding no significant relative loss of $^{125}$I-alb from the blood in T.b. brucei or T. congolense infections of Zebu and N'dama cattle, concluded that capillary permeability was not changed during these infections. Paaske (1983) is of the opinion (ch. 1.10.5) that TER does not measure capillary permeability nor lymph flow, but he does not offer any other explanation.
The separate group of four infected rabbits which had a much faster TER ($t_{1/2}=122$ minutes), were infected for 27-31 days (Table 2.1, 2.2, fig. 2.1) and had low or negative CxRP levels indicating that these rabbits were probably in the third chronic stage of the disease (Van den Ingh, 1976). By this time it is reasonable to suggest that $^{125}\text{I}-\text{alb.}$ recirculation via the lymph may be impaired. Tissue damage is widespread by this stage and any increases in macromolecular permeability would be expected to be maximal.

2.4.1.3 Haematological Data

Simultaneous measurements of red cell space and plasma space were carried out. In spite of finding a significant reduction in haematocrit during the infection, red cell space was found to be unaltered. The significantly increased blood volume, measured independently with both isotopes, was due to the increase in plasma space, confirming previous observations of haemodilution in a variety of host and trypanosome species (see ch. 1.9.10.1) (Amole et al., 1982; Naylor, 1971; Anosa and Isoun, 1976; and Holmes and Jennings, 1975).

Care must be taken with the use of diffusible tracers in the determination of blood volume because if the tracer leaves the vasculature, the apparent distribution volume is larger than that of the circulation. Because of this, some reports of haemodilution in trypanosomiasis must be scrutinized carefully.

Muscle and skin contain two thirds of total body interstitial fluid (Aukland and Nicolaysen, 1981). Significant decreases were found in water:dry tissue ratios of abdominal wall muscle in infected rabbits,
providing a possible source of fluid for plasma expansion. Abdominal wall muscle also has an increased blood volume (TBF) suggesting preferential accommodation of extra blood in the structural skeletal muscle.

Haemodilution is a misleading term as it may be construed to imply reduced colloid osmotic pressure (COP) as, for example, in experimental saline volume expansion. COP has not been measured during trypanosomiasis to date, but serum albumin concentration (the major determinant of COP) is decreased during T.b. brucei in rabbits (Goodwin and Guy, 1973; Van den Ingh, 1976). Of the 40% decrease in serum albumin concentration (Goodwin and Guy, 1973), nearly half can be accounted for by a 24% increase in plasma volume (see Table 2.3) suggesting that some measured plasma parameters might be altered as a passive result of such volume increase.

Total serum protein concentration during trypanosomiasis is found to be similar or raised (Goodwin and Guy, 1973; Van den Ingh, 1976) mainly due to increased immunoglobulins and fibrinogen. However, there may also be elevated concentrations of FDP's (Facer, 1974), metabolic products from trypanosomes (Tizard et al, 1978), small molecular weight peptides (Boreham, 1977), etc., which may contribute to plasma COP, offsetting decreased albumin concentration. Clarkson (1968) suggested that large increases in IgM could lead to elevated COP and plasma volume.

There is data relating to the protein, albumin and globulin concentrations in serum during T.b. brucei infections in rabbits (Goodwin and Guy, 1973; Facer, 1976). Using the following relationship, it is possible to calculate COP ($\pi$) (Landis and Pappenheimer, 1963).
where c are the respective concentrations (g/100ml) of albumin and globulin. The calculated COP is shown in Table 2.4. The largest drawback with these calculations is that they are of serum rather than plasma and do not take into account any small molecular weight peptides present during the infection which may add to plasma COP.

In addition, Goodwin and Guy (1973) implanted capsules in the subcutis of rabbits infected with *T. b. brucei*. Albumin, protein and immunoglobulins (as well as various enzymes and metabolites) were measured in the capsular fluid. However, such capsular fluid is not the same as interstitial fluid. The capsular fluid:serum protein concentration was found to be 0.63 (Goodwin and Guy, 1973) which is considerably greater than the ratio found in the liquid paraffin cavity method, i.e. 0.32 in rabbits (Haljamae et al, 1974) and 0.34 (Rutili and Arfors, 1977). Though not ideal, Goodwin and Guy's data is also given in Table 2.4.

There appears to be a reduction in serum COP in the initial stage of the infection but then, presumably because of the globulin increases, it stabilizes around control levels. The initial apparent decreases in serum COP may be accompanied by reductions in tissue COP in which case, as fluid flux is dependent on differential COP (see equation 1.1 ch. 1.7.2.1), no major shift of fluid would be expected.

The maintenance of the red cell space during *T. b. brucei* infection in spite of a measured decrease in hct. was interesting because there is evidence for haemolysis and shortened red cell survival times in
Table 2.4. Calculated colloidal osmotic pressure (COP, mmHg) of serum and capsular fluid during *T.b.brucei* infection in rabbits. Also given (in brackets) is the COP as a percentage of the control value, and difference between serum and capsular values (ΔCOP) for Goodwin & Guy's (1973) results. The column headed LI gives the length of infection (days). Note that *T.b.brucei* 427 (also used in the present study) is the more chronic strain.

<table>
<thead>
<tr>
<th>LI</th>
<th>Fluid</th>
<th>Serum (mmHg)</th>
<th>ΔCOP (mmHg)</th>
<th>Capsule fluid (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Serum</td>
<td>21.51 (100.0%)</td>
<td>7.23</td>
<td>10.24 (100.0%)</td>
</tr>
<tr>
<td>7</td>
<td>Capsule fluid</td>
<td>17.47 (100.0%)</td>
<td>15.82 (90.5%)</td>
<td>10.17 (99.3%)</td>
</tr>
<tr>
<td>10</td>
<td>Serum</td>
<td>16.52 (94.5%)</td>
<td>6.80</td>
<td>9.02 (88.1%)</td>
</tr>
<tr>
<td>14</td>
<td>Capsule fluid</td>
<td>18.05 (103.3%)</td>
<td>8.95</td>
<td>9.10 (88.9%)</td>
</tr>
<tr>
<td>17</td>
<td>Serum</td>
<td>21.36 (99.3%)</td>
<td>18.03 (103.2%)</td>
<td>8.03</td>
</tr>
<tr>
<td>21</td>
<td>Capsule fluid</td>
<td>18.02 (103.1%)</td>
<td>9.54</td>
<td>8.48 (82.8%)</td>
</tr>
<tr>
<td>28</td>
<td>Serum</td>
<td>21.65 (100.7%)</td>
<td>16.12 (92.3%)</td>
<td>4.77</td>
</tr>
</tbody>
</table>

*References:* Facer (1976) and Goodwin & Guy (1973)
T. b. brucei infections (Jenkins et al, 1980; Jenkins and Facer, 198; McCrorie et al, 1980; see 1.8.2 and 1.9.10). However, the more acute T. b. brucei strain (S42) in rabbits provokes a more severe anaemia than 427. Splenomegaly occurred in most of the infected rabbits in the present study (see 2.4.5.1) and McCrorie et al, (1980) suggested that there is increased non-specific destruction of red cells by the spleen. To maintain the same red cell space during the infection, increased erythropoiesis is necessary. This has been shown in T. b. brucei infected rats (Jennings et al, 1974). However as the infection progresses through the third stage (chronic) there is anaemia of chronic disorders (Jenkins and Facer, 1985) where iron stored in the bone marrow is not used for erythropoiesis (see also chapter 5).

Similar decreases in arterial hct and whole body hct (14.5 and 13.6% respectively) during the infection suggests that there is no major shift of blood between different segments in the vasculature.

The increase in blood volume during T. b. brucei infection is discussed in relation to concomitant hypotension in chapter 4. The calculation of blood volumes using each of the isotopes and both together shows that using $^{125}$I-albumin only gives a higher blood volume and $^{51}$Cr-RBC only gives a lower blood volume than both isotopes together. The difference is likely to be due to the slightly different distribution of red cells and plasma within the vasculature. For example, the number of red cells in capillaries is very low (e.g. capillary hct. of 0.09 compared to a large vessel hct. of 0.33 in cat mesentery (Johnson, 1971)).
2.4.2 Tissue Water Content

Cremaster muscle appears to accumulate fluid, even though the increase in water content is only about 2-3%. It is unlikely that this increase in water content could account for the observed genital swelling. Indeed, testis skin, which shows the same degree of swelling, shows no such increase in water content. Occasional accumulations of fluid between the testis skin and the cremaster muscle were found in the infected animals which could explain some of the genital swelling. Extensive damage to male genitalia is a prominent feature of trypanosome infections (Van den Ingh, 1976; Ikede and Akpavie, 1982, see also 1.9.9).

The apparent lack of oedema seen in most tissues, particularly the ear was most surprising. The ears of infected rabbits were usually very thick and heavy. One practical upshot of this was the difficulty in giving injections via the marginal ear vein. Some of the tissue samples from the ear were taken from particularly thick parts and none of these showed increased water content. When the infected ears were cut, they did not "weep" as one would expect with fluid accumulation. The ear tissue appeared almost translucent with little bleeding. Abnormal lipid accumulation may be a major factor here. Raised serum lipids have been found (Goodwin and Guy, 1973; Van den Ingh, 1976) as well as marked accumulations of lipid in rabbits's ears (Goodwin, 1971) and liver (Edeghere, 1980). Hambrey et al (1980) found large amounts of phospholipase A¹ in the tissue fluid of rabbits infected with T.b. brucei. Antibodies against liposomal phospholipids have been found (approximately 3 weeks post-infection) (Richards et al, 1983) possibly resulting from antigenic stimuli by trypanosome surface phospholipids. The significance of these findings is unknown though phospholipase is involved in cell membrane breakdown (and consequent formation of prostaglandins). Hambrey
et al (1980) suggested that respiratory distress might be caused by the phospholipase degrading lung surfactant.

In addition, Goodwin (1971) and Goodwin et al (1973) provided evidence that there was considerable connective tissue damage during T.b. brucei infections, a feature of which was collagen and fibroblast degradation. Where damage occurs, there is inflammatory cell infiltration. No estimations of extravascular populations of T.b. brucei have been made, but the presence of large numbers of parasites would be expected to considerably alter the local environment. It is therefore suggested that of the tissues studied and ear in particular, observed swelling is not due to fluid accumulation, but to extravascular infiltration of inflammatory cells, lipid, trypanosomes and their debris. This is supported by histological evidence (Goodwin and Hook, 1968; Goodwin, 1971; Van den Ingh, 1976; Edeghere, 1980; Akol and Murray, 1982; Morrison et al, 1979; Rudin et al, 1984).

The decrease in water:dry tissue mass seen in abdominal wall muscle may provide a source for the plasma expansion (see 2.4.1.2). Because the tissues investigated in this study were sampled rather than looked at in their entirety, discovering a decrease in water:dry tissue ratio may indicate decreased proportion of water or it may indicate increased dry tissue mass. The former seems more likely though not proven.

The significantly decreased water content of spleen could result from the large changes in size and cellular composition (see chapter 1.9.3).
2.4.3 Tissue Blood Fractions (figs 2.3, 2.4)

These were significantly increased during the infection in spleen, lung and abdominal wall muscle, regardless of which calculation method was used.

It is likely that in spleen, such an increase in TBF is due to preferential uptake of $^{51}$Cr-RBC (Studer and Potchen, 1971), a process enhanced during T.b. brucei infection (C.A. Facer - personal communication; McCrorie et al, 1980).

The increased TBF in lung and abdominal wall muscle during the infection is because of the expanded blood volume. As the systemic blood volume increases, so does that of the pulmonary circulation giving elevated TBF per unit weight of lung tissue. Systemically, there is more scope for adjusting blood flow to certain areas, which in this case seems to point to skeletal muscle being used in this capacity.

Tables 2.5 and 2.6 show some of the haematological parameters obtained for control rabbits in the present study compared to values given in the literature.

No special precautions were taken to prevent blood loss from tissues during excision (apart from clamping large arteries and veins where necessary) whereas both Everitt et al (1956) and Studer and Potchen (1971) froze their rats in liquid nitrogen and dissected them while still frozen. Conversely, there may have been some post mortem pooling in the tissues removed towards the end of the experiment. Despite these factors the present results obtained for control rabbits are broadly in agreement with others in the literature particularly for those tissues
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Present study</th>
<th>Everett et al (1956)</th>
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<tbody>
<tr>
<td>Scrotal skin**</td>
<td>82.10 ± 6.43</td>
<td>96.4 - 103.2</td>
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<tr>
<td>Ear</td>
<td>88.25 ± 6.99</td>
<td>-</td>
</tr>
<tr>
<td>Abdominal muscle*</td>
<td>94.97 ± 3.83</td>
<td>88.2 - 88.6</td>
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<tr>
<td>Cremaster muscle*</td>
<td>96.58 ± 4.25</td>
<td>88.2 - 88.6</td>
</tr>
<tr>
<td>Mesentery</td>
<td>87.16 ± 1.74</td>
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<tr>
<td>Kidney</td>
<td>43.53 ± 6.29</td>
<td>53.3 - 61.0</td>
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<tr>
<td>Heart</td>
<td>81.32 ± 9.29</td>
<td>73.8 - 90.6</td>
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<tr>
<td>Liver</td>
<td>73.17 ± 9.15</td>
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<tr>
<td>Aorta</td>
<td>91.99 ± 8.09</td>
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<td>Lung</td>
<td>105.28 ± 12.10</td>
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<tr>
<td>Spleen</td>
<td>79.80 ± 2.76</td>
<td>130.3 - 138.1</td>
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Table 2.5. Comparison of tissue haematocrits with those of Everett et al (1956), expressed as a percentage of whole body haematocrit. Values for the present study are mean ± SEM for the three control rabbits in the 30 minute experiments. The results from Everett et al (1956) are the range they found in normal rats.

* Everett et al used "skeletal muscle".

** Everett et al used "skin".
Table 2.6. Tissue blood fractions (%), compared with others in the literature. Values from the present study are in the form mean (+ SEM) for control rabbits corrected for tissue haematocrit. The other values are from normal rats.

* other workers used "skeletal muscle".
** other workers used "skin".

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<tbody>
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<td>Scrotal skin**</td>
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<tr>
<td>Ear</td>
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<td>-</td>
<td>-</td>
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<td>Abdominal muscle*</td>
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<td>2.13</td>
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<tr>
<td>Mesentery</td>
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<td>-</td>
</tr>
<tr>
<td>Kidney</td>
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<td>7.3</td>
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<tr>
<td>Heart</td>
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<tr>
<td>Liver</td>
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<td>20.9</td>
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<tr>
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<td>-</td>
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<tr>
<td>Lung</td>
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<td>54.6</td>
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<td>Spleen</td>
<td>33.14 ± 2.92</td>
<td>15</td>
<td>21.9</td>
</tr>
</tbody>
</table>
with lower blood fractions. The variation in the data is consistent with variations in species and technique. Dewey (1959) reported much lower tissue blood contents than others, but he used exsanguinated rats.

2.4.4 Tissues containing Continuous Capillaries

2.4.4.1 Heart (fig 2.7)

There were significant elevations of E/P ratios for all durations of experiment and significant positive correlations with infection length, regardless of the calculation method.

Control heart tissue reached a quasi-steady state by 90 minutes (fig. 2.7A) (later in fig. 2.7B) whereas in infected heart tissue, no such plateau has been reached by 120 minutes. This "steady state" reached in most control tissues with continuous capillaries by 120 minutes is not a true steady state because blood levels of $^{125}$I-alb are falling with time (see fig. 2.1). The longest experimental time used was 120 minutes because significant amounts of free iodide ($^{125}$I$^-$) may be present at longer in vivo times (Winlove et al, 1978).

Damage to the heart of humans and animals during T. brucei group infections is a major pathological feature (Poltera, 1985; Morrison et al, 1979; chapter 1.9.6). The present study shows progressive pronounced increases in magnitude and rate of extravascular accumulation of labelled protein. This provides physiological evidence to back up the observed histopathological and clinical changes seen in this tissue in trypanosomiasis (Poltera et al, 1976; Poltera, 1980; 1985; Greenwood and Whittle, 1980; Murray et al, 1979).
2.4.4.2 Abdominal Wall Muscle (fig. 2.8)

E/P ratios (calculated without tissue hct correction) were elevated for all experiment durations (see fig. 2.8A) and positively correlated with infection length.

E/P ratios (with tissue hct correction) were significantly elevated in the 30, 60, 90 minute experiments (fig 2.8B) and positively correlated with infection length in the 30 and 60 minute experiments. The E/P for the 30 minute control is less than 0. This is because in determining tissue hct (equation 2.10) it was necessary to assume that all $^{125}$I-alb (in tissues with continuous capillaries in 30 minute controls) was contained intravascularly. Because the correction factors so derived were the mean of 3 experiments, (30 minute controls) these 3 experiments would be expected to produce E/P ratios approximately 0 (slightly negative or slightly positive).

The significantly elevated E/P ratios in abdominal wall muscle (7 out of 8 possible comparisons with controls for both calculation methods Fig 2.8A and B) when considered with the significantly reduced water to dry tissue ratio may mean that estimations of extravascular accumulation of $^{125}$I-albumin are underestimates.

The host's immune response is necessary for skeletal muscle lesions to develop during trypanosomiasis (Galvao-Castro et al, 1978). Muscle wastage is a common feature of trypanosome infections (see 1.9.8). However, this may be due in part to fluid loss (see 2.4.1.3) (underlining a major homeostatic mechanism?). The driving force for such a fluid shift to the plasma is unknown. The scanty derived data for COP (on the basis of albumin and globulin) is equivocal and appears to fluctuate as the
infection progresses. There may be a tendency for plasma COP to increase during the fourth week of infection. Most of the rabbits used in the present study were at this stage of the infection.

Damage to skeletal muscle during trypanosome infection has been more thoroughly studied in cremaster muscle (Edeghere, 1980; Goodwin and Hook, 1968). However, there seem to be fundamental differences between this tissue and abdominal wall muscle in terms of their reactions to the disease. This may reflect their disparate functions. (see 2.4.4.4).

2.4.4.3 Ear (fig. 2.9)

Ears often demonstrate the earliest outward signs of trypanosome infection in rabbits, with progressive thickening and droopiness.

The E/P ratios obtained for infected rabbits are significantly elevated for all four experiment durations (no tissue hct correction), see figure 2.9A, and positively correlated with the length of infection in the experiments of 60 and 90 minute duration. Apparent steady state may have been reached by 120 minutes in controls but not in the infected ears.

The E/P ratios calculated with the tissue hct correction were significantly elevated in the 30 and 60 minute experiments (fig. 2.9B) whilst only in the 60 minute experiments are E/P ratios correlated with infection length. The change in infected E/P with time parallel those in controls and apparent steady state has not been reached.

The finding of enhanced extravascular $^{125}\text{I-alb}$ accumulation in 6 out of 8 possible comparisons with controls and yet with no apparent
fluid accumulation in spite of visible swelling of the ears is interesting. Some of the increase in volume of the ear could be due to lipid accumulation (see chapter 2.4.2). Infiltration with cells (whether parasite or host derived) would not be expected to alter the water:solid matter ratio.

Ear blood content is slightly increased though not significantly, probably because of the wide variation between samples (see Appendix 2, Tables B and C). Patchy areas of vascularized granulomas are seen (Goodwin and Hook, 1968). This heterogeneity makes interpretation more complicated. However, it is felt that the lack of oedema is a genuine finding as it does not depend on variable TBF within the tissue.

2.4.4.4 Cremaster Muscle (fig. 2.10)

The genitals show obvious signs of progressive pathological changes in T.b. brucei infections in male rabbits. Goodwin and Hook (1968) observed that in cremaster muscle venular microvessels became blackened when injected with colloidal carbon and that the changes resembled those induced by local injection of histamine into rat scrotum (Majno, 1964). Histological section showed "patches of granulomatous tissue, composed predominantly of mononuclear cells and rich in capillaries, surrounded by dilated veins" (Goodwin and Hook, 1968). Edeghere (1980) also found a patchy nature of cremaster muscle pathology with increasing numbers of damaged microvessels as the infection progressed. The damage to the microvessels took the form of swollen endothelium, gap formation, passage of leucocytes and RBC through these gaps, occasional complete blockage of vascular lumens but more frequent leucocyte "sticking", microthrombi, platelet clumps and deposits of amorphous proteinaceous matter. Parasites were found extravascularly and skeletal muscle appeared
fragmented from time to time. Basement membranes appeared intact even when endothelial cells had virtually disintegrated.

The present study shows that there is significantly increased extravascular \(^{125}\text{I}-\text{alb}\) accumulation in cremaster muscle in the 30, 60 and 120 minute experiments (fig. 2.10A and B) (both methods of calculation) which correlated positively with length of infection for these three experiment times. There was no change in tissue blood fractions (both calculation methods). Water content and water:dry tissue ratios were significantly enhanced in infected cremaster muscle indicating that fluid content was increased per unit weight of tissue (oedema) but could also reflect loss of muscle bulk. This is the converse of changes in abdominal wall muscle. Such differences may reflect the temperature regulation function of cremaster muscle, allowing spermatogenesis to proceed at optimum temperature. The degeneration of the testis (Van den Ingh, 1976; Ikede and Akpavie, 1982) may alter the functional integrity of the scrotal sac, but this is unknown.

Cremaster muscle is a little difficult to handle. Once the artery is cut, it bleeds profusely allowing blood of high activity of both isotopes to contaminate the tissue. This may account for the quite large variation in the TBF in control and infected groups (see Appendix 2, Table B and C). However, as in the ear, the damage due to the infection is patchy (Edeghere, 1980; Goodwin and Hook, 1968).

2.4.4.5 Scrotal Skin (fig. 2.11)

This skin became reddened as the genitals enlarged during the infection. Necrotic scabs were often evident later in the infection. When excised, the tissue appeared thickened and stiff though there was
no significant change in water content or water:dry tissue ratio (fig. 2.5 and 2.6).

E/P ratios, calculated without tissue hct correction, were significantly elevated in the 30, 60 and 120 minute experiments (fig. 2.11A) which was only correlated with infection length at 60 minutes. Calculated E/P ratios using tissue hct correction showed significant elevation only in experiments of 30 and 60 minutes duration which were correlated with infection length for both these times. Apparent steady state may have been reached by 90 minutes in controls and possibly in infected (tissue hct correction) but not in infected (no tissue hct correction).

Tissue blood fractions were not significantly different from the controls, though they tended to be slightly higher (see figures 2.3 and 2.4).

2.4.4.6 Mesentery (fig 2.12)

Observations were made on this tissue because an intravital microscopy study was planned at a later date (see chapter 3). The mesentery is essentially a 2 dimensional structure and, apart from fat cells around the larger vessels, virtually transparent making it well suited for such experiments.

The E/P ratios were significantly elevated at 30, 60, 90 and 120 minutes (no tissue hct correction) but not correlated with the length of the infection. Whilst apparent steady state was reached by 90 minutes in controls, E/P ratios were still rising at 120 minutes in the infected mesentery (fig. 2.12A).
Where E/P ratios were corrected for tissue hct, no significant elevation was seen at 30 or 60 minutes, only at 90 and 120 (fig. 2.12B). Again there was no correlation with length of infection. Steady state (apparent) had been reached by 90 minutes in both infected and control rabbits.

The large variation in E/P ratios, particularly in infected rabbits is thought to be due to the variable amount of adipose tissue adhering to the mesentery. Water contents and water:dry tissue ratios are both very low. Drying to constant weight was impossible because the fat just melted, reflecting the large contribution of adipose tissue to the total tissue mass.

2.4.4.7 Aorta (fig. 2.13)

This tissue was studied because of the observations of extreme fragility of large vessels during trypanosome infection.

There is little change with time of control E/P ratios (no tissue hct correction) but a significant elevation of E/P in infected aorta at 30, 90 and 120 minutes (fig. 2.13A). E/P correlated with infection length at 60, 90 and 120 minutes.

Figure 2.13B shows E/P ratios calculated with tissue hct correction. The 2 plots are virtually parallel with only a significant elevation in infected tissue at 120 minutes. No difference was found in TBF (both calculation methods), water content or watercontent:dry tissue between control and infected aorta.

The high calculated TBF (both methods) probably indicates some binding of $^{51}$Cr-RBC and/or $^{125}$I-alb to the luminal surface of the aorta.
2.4.4.8 Lung (fig. 2.14)

Studer and Potchen (1971) found that extravascular labelled albumin in the lungs of normal rats varied widely from animal to animal. This may mask any change in capillary permeability.

Calculating E/P with no tissue hct, there was only a significant elevation at 60 minutes. Correcting for tissue hct, only E/P ratios at 30 and 60 were significantly elevated in the infection. There was a significant correlation with infection length and E/P (both calculations) only at the 60 minute duration.

There is a significant elevation in TBF (both methods) in infected lung but no change in water contents or water:dry tissue ratios. Apart from TBF, there is very little evidence from the present results that indicate physiological alterations in lung tissue during T.b. brucei infections of rabbits.

The lung hct measured in control experiments of 30 minutes agrees with those of Everitt et al (1956) (Table 2.5). Parker et al (1984) quote dog lung hct as 92% of venous hct, similar to but a little higher than the present study's value of 84% of arterial hct. Discrepancies could be due to venous and arterial hct differences and species.

2.4.4.9 Kidney (fig. 2.15)

Significant elevations of E/P ratios (uncorrected for tissue hct) were found in infected rabbits at 60 minutes and 120 minutes which positively correlated with infection length (fig. 2.15A). There is a high degree of variation within the infected groups, particularly at
60 and 90 minutes. When E/P's are corrected for tissue hct (fig. 2.15B) they are significantly elevated at 60, 90 and 120 minutes. Only the 60 minute experiments correlated with length of infection.

No significant change in the other parameters was measured.

The extravascular albumin (plasma equivalents) obtained for controls (no hct correction) agrees very well with those obtained by Owen and Triffitt (1976).

Kidneys contain mainly fenestrated capillaries which are a type of continuous capillary. They are differentiated from other continuous capillaries in that they are very permeable to small molecular weight solutes but very impermeable to large molecules such as albumin (Clough and Smaje, 1984a; Spencer, 1984). Bell et al (1978) found albumin $a_s$ in rabbit kidney to be 0.998 and thus little if any labelled albumin would be expected to be transported from the blood to the interstitium in 120 minutes. The majority of control E/P (with tissue hct correction) are negative which is consistent with capillary endothelium being very impermeable to albumin and hence control values would reflect the decrease in plasma $^{125}$I-alb with time.

Pappenheimer and Kinter (1956) showed that kidney has an excess of plasma, possibly due to enhanced plasma skimming. During the infection there is likely to be a diminution of the Fahraeus effect and plasma skimming, altering local haemodynamics (see 1.7.4.3). This may enhance E/P ratios but is unlikely to fully account for the spectacular increase in renal E/P's seen in some rabbits.
The 4 rabbits which had a much faster TER, namely I1, I2, I3 and I4 (see 2.4.1.2 and fig. 2.1) also had significantly elevated renal E/P ratios compared to the other infected rabbits at the same experimental times (i.e. 60 and 90 minutes, see Table 2.2). Indications from the CxRP measurements and the length of infection (27-31 days) suggest that these 4 rabbits are in the third phase of the disease (Van den Ingh, 1976) which may mean that the renal damage is in the second phase, tubular ischaemia (Facer et al., 1978; see also chapter 1.9.7). In fact such accumulations of $^{125}$I-alb in the kidneys of these rabbits may contribute to the rapid TER.

Considering the nine tissues with continuous capillaries (both fenestrated and non-fenestrated), of the 36 pairs of mean E/P ratios, there was significant elevation in 75% (27 out of 36), assuming tissue hct was equal to blood hct and 61% (22 out of 36) correcting for tissue hct.

There seemed to be marked variation between the tissues. Heart tissue shows the most clear cut increase in extravascular $^{125}$I-albumin accumulation both in rate and magnitude. This cannot be accounted for by the formation of oedema as water content and water:dry tissue ratio are not changed during the infection.

Tissues showing obvious signs of damage, namely ear, cremaster muscle and scrotal skin, do not show such clear cut alterations in E/P ratio. However, it appears that permeability to macromolecules is
significantly increased in these tissues due to infection with *T.b. brucei*. Because of such variation between tissues and the unrelatedness to albumin "TER", Dargie (1979b) could not conclude from his data on TER that no significant increase in microvascular permeability occurred.

The differences in the E/P's resulting from the 2 methods of calculation show how important tissue hct is. Ear, cremaster muscle and testis skin have a temperature regulation function and estimates of local blood fraction, dependent on flow and hct, would be expected to vary considerably between control animals. Superimposition of a progressive infection would further increase the degree of variability in these tissues, accounting for the wide range of data obtained. However, it is felt that there are indications that both macromolecular permeability and time to reach apparent steady state are increased during the infection. In cremaster muscle however, the increase in water content of the tissue could account for the increased time to reach apparent steady state.

In this latter context, it has been shown that changes in hydration of the tissue interstitial gel matrix may affect the partition of plasma proteins between the extra- and intravascular compartments (Aukland and Nicolaysen, 1981; see 1.7.1.2). As the protein exclusion volume depends on the concentration of the interstitial matrix of glycoaminoglycans (GAG) and collagen (Granger and Shepherd, 1979), excess fluid (oedema) would decrease the concentration of these components thus decreasing the protein exclusion volume, allowing more protein to be present in the interstitium (Parker et al., 1979; Mullins and Bell, 1982; Granger, 1979). This property of the interstitium acts as a protection mechanism against oedema by resulting in a greater decrease in interstitial COP (oncotic) than would result from simple dilution of interstitial protein.
Mullins and Bell (1982) showed that a 10% saline volume expansion of body weight resulted in a 41% increase in extravascular albumin mass in muscle but no change in skin (measured at steady state conditions), a 3-fold increase in lymph flow for both tissues and interstitial volume increase of 20% for skin and 230% for muscle.

Table 2.7 compares extravascular albumin (plasma equivalents/100g tissue) for ear, scrotal skin, cremaster muscle and abdominal wall muscle (control and infected, 120 minutes) with control and expanded volume data obtained by Mullins and Bell (1982). Approx. data from Owen and Triffitt (1976) for rabbit skin and muscle is also shown. This is in the form ml plasma/100g tissue and estimated from miniature graphs (hence the approximation) one hour after $^{125}$I-albumin injection.

The present results for scrotal skin and ear (120 minutes) are much less than those obtained by Mullins and Bell (1982), but they measured albumin directly (correcting for intravascular albumin). There is evidence that skin is slow to reach slow steady state (Owen and Triffitt, 1976; Bell and Mullins, 1982a), though the present E/P ratios are still less than those of Owen and Triffitt for one hour. Where the present results are corrected for tissue hct, the 90 minute experiments are used for comparison with Owen and Triffitt's (1976) one hour results because the 30 minute experiments (controls) are considered to be marginally more than zero (time).

Cremaster muscle E/P is comparable to Mullins and Bell's (1982) muscle but more than that of Owen and Triffitt (1976). Abdominal wall is somewhat less.
<table>
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<th>TISSUE</th>
<th>EXTRAVASCULAR $^{125}$I ALBUMIN (100g of tissue) MEAN ± SEM</th>
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<tr>
<td>SKIN</td>
<td>7.67 ± 0.33</td>
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<td>SCROTAL SKIN</td>
<td>(6) 1.71 ± 0.25</td>
<td>present study (120 minutes)</td>
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<td></td>
<td>(6) 1.19 ± 0.23*</td>
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</tr>
<tr>
<td>EAR</td>
<td>(8) 2.34 ± 0.39</td>
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</tr>
<tr>
<td></td>
<td>(8) 1.70 ± 0.30*</td>
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<td>3.03 ± 0.18</td>
<td>Mullins and Bell (1982)</td>
</tr>
<tr>
<td>CREMASTER MUSCLE</td>
<td>(6) 2.71 ± 0.45</td>
<td>present study (120 minutes)</td>
</tr>
<tr>
<td></td>
<td>(6) 2.25 ± 0.45*</td>
<td>&quot;</td>
</tr>
<tr>
<td>ABDOMINAL WALL MUSCLE</td>
<td>(9) 0.65 ± 0.04</td>
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</tr>
<tr>
<td></td>
<td>(9) 0.44 ± 0.07*</td>
<td>&quot;</td>
</tr>
<tr>
<td>SKIN</td>
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<tr>
<td>SCROTAL SKIN</td>
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<td>present study (60 minutes)</td>
</tr>
<tr>
<td></td>
<td>(8) 1.10 ± 0.25*</td>
<td>&quot; (90 minutes)</td>
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<td>0.7 (see text)</td>
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<tr>
<td>CREMASTER</td>
<td>(8) 1.35 ± 0.05</td>
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<tr>
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<td>(8) 3.58 ± 0.81*</td>
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<td>ABDOMINAL WALL MUSCLE</td>
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<tr>
<td></td>
<td>(10) 0.37 ± 0.02*</td>
<td>&quot; (90 minutes)</td>
</tr>
</tbody>
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**Table 2.7:** Extravascular $^{125}$I-albumin per 100g of tissue (mean ± SEM) in the present study compared to data in the literature. * indicates values corrected for tissue hct.
There seems to be two very different mechanisms operating in cremaster muscle (which apparently shows some degree of oedema) and abdominal wall muscle (which shows fluid loss but still with significantly elevated E/P's at most durations of the experiments). The gross pathological changes seen in the genitals during *T. b. brucei* infection in rabbits may indicate parasite preference for such an environment whilst other skeletal muscles respond to the infection in a non-specific fashion, though this is speculation.

The regional variations in extravascular $^{125}\text{I}$-albumin accumulation point to heart, ear, cremaster muscle, abdominal wall muscle as showing the most pronounced elevations of E/P ratios. However, this depends on the relative merits of the two methods of calculations. The disadvantage of not correcting for tissue hct. is the wide disparity between regional and systemic hct. Kidney is particularly affected as the calculated hct is approximately one third of arterial hct., whereas lung hct is about 81%. Despite the pitfalls involved in calculating tissue hct from the 30 minute controls, it is felt to be preferable to not correcting for tissue hct. On this basis, heart shows the most elevated E/P ratios of the infected rabbits, followed by kidney, cremaster muscle and abdominal wall muscle. Correcting for tissue hct also allows more weight to be placed on the values of the various parameters calculated.

The ratio of blood hct:tissue hct used to correct the TBF and hence E/P is assumed to be the same (or at least similar) in the infected rabbits. There is no way of checking this at present though the similarities in tissue TBF between control and infected rabbits and the similarity in decrease of whole body hct with that of arterial hct during the infection suggests that the blood:tissue hct ratio is reasonably consistent.
2.4.5 Tissues containing Discontinuous Capillaries

2.4.5.1 Spleen

Most of the E/P ratios (no tissue hct correction) for both infected and control are largely less than zero (fig. 2.16A), that is, the calculated intravascular $^{125}$I-alb was greater than the total measured $^{125}$I-alb. This is clearly impossible, suggesting that the tissue hct is greater than that in the blood. The measured tissue hct (based on 30 minute control values - see Table 2.5) expressed as a percentage of whole body hct was 79.80% compared to 130.3-138.1% found by Everitt et al, (1956). This means that intravascular $^{125}$I-alb would be greatly over-estimated when tissue hct was assumed to be equal to the arterial hct (as in the first method of calculating E/P ratios). With the increased sequestration of $^{51}$Cr-RBC in the spleen during trypanosomiasis, as mentioned previously, this error is compounded. There is little evidence that extravascular $^{125}$I-albumin is enhanced during the infection (see fig. 2.17A). In fact, the errors probably mask any such alterations.

The calculation of splenic E/P ratios using an erroneously derived tissue hct would further distort the true picture (unknown) (see fig. 2.16B). The reason for the very low (comparatively) tissue hct is that even though based on 30 minute control experiments, tissues with a sinusoidal discontinuous microvascular structure provide little barrier to the passage of macromolecules and "steady state" is rapidly reached. By 30 minutes, any extravascular $^{125}$I-albumin would reflect the decline of plasma $^{125}$I-alb. with time.
In addition, the changes in splenic size (spleen weight was found to increase as much as 10 fold during T. b. brucei infection) and structure (see 1.9.3) render conclusions about macromolecular permeability highly dubious.

### 2.4.5.2 Liver

Liver has a similar microvascular structure to spleen, equilibration of macromolecules occurring within 5 minutes (Studer and Potchen, 1971). The red cell trapping function of liver is not marked and calculation of tissue hct (as a percentage of whole body hct) is not much less than that found by Everett et al (1956) (70.28% compared to 81.4-83.1%).

When not corrected for tissue hct, control E/P's (fig. 2.17A) decline with time whereas those of infected liver increase with time, though only significantly so at 60 and 120 minutes. Because control E/P have been assumed to have reached steady state and are reflecting the decrease of $^{125}$I-alb. in plasma, it appears that infected liver is slower to reach steady state. This is consistent with observations on lymph $^{125}$I-albumin (see fig 2.18 and 2.4.6).

There was a positive correlation between E/P and length of infection at 60 and 120 minutes.

Calculation of E/P using tissue hct is not really valid (fig 2.17B) for liver, as outlined for spleen above.
2.4.6 Lymph

Lymph was consistently easier to collect and in greater quantities in infected rabbits compared to controls.

In control animals, thoracic lymph appeared to reach a steady state rapidly and the fall partially reflected the rate of loss of $^{125}$I-alb. from the bloodstream (fig 2.1). In contrast, the $L_p/P$ ratios in infected animals increased with time to a steady value at about 60 minutes. There was a significant difference in infected rabbits between values at 30 minutes and 60 minutes ($P < 0.05$); 30 minutes and 90 minutes ($P< 0.05$); and 30 minutes and 120 minutes ($P <0.01$). The only significant difference between values from infected and controls was at 30 minutes duration. This suggests that in infected animals, there is a longer time constant for the establishment of a quasi-steady state. Johnson and Levitt (1975) suggested that about 50% of thoracic lymph comes from the liver. It is interesting to note that the variation of E/P ratio with time for liver in control and infected liver (fig. 2.17A) is very similar to that of the $L_p/P$ ratio. Liver lymph is thought to equilibrate in 30 minutes in normal animals (Perl, 1975).

Lymph from infected rabbits was found to be more concentrated than in controls:

control (n=5)  97.29% water (± SD 1.46)
infected (n=14)  94.94% water (± SD 1.62)

This difference was found to be significant ($P <0.05$) using a 2-tailed t-test. These results probably indicate more protein in the lymph of infected animals. Gel chromatography performed on lymph from both infected and control animals showed that in the infected group, the peak
corresponding to the albumin fraction eluted less cleanly possibly  
pointing to the presence of protein fragments or peptides. Parasites  
are also likely to be present.

The lymphatic system may be of greater importance in trypanosomiasis than previously thought. Barry (J.D. - personal communication) demonstrated increased flow in efferent lymphatics in goats when bitten by an infected tsetse fly. Barry and Emery (1984) proposed that in the initial stages of the infection at least, parasites enter the lymphatic system before the blood system and there may be a lag before the parasites appear in the tissue fluid. Bijovsky et al (1984) found that the lymphatics are the main route of T. cruzi dissemination from the site of inoculation. The apparent response of increased lymph flow in the acute infection and in the chronic infection (if it occurs) would serve to enhance the disease process.

The apparent increase in lymph flow (though not proven) does suggest that lymph flow is more efficient during trypanosomiasis. There is occasional blockage of heart lymphatic vessels in the terminal moribund phase of the disease (Morrison et al, 1979; Poltera, 1980). This may become generalized preventing the "washout" of fluid and protein. This may account for the disparity in plasma $^{125}$I-albumin.
2.5 Summary

As already mentioned, one source of error in calculating E/P ratios is the determination of tissue hct. Another is grouping together the rather heterogeneous infected rabbits. It was hoped to divide the rabbits on the basis of their CxRP titres, but this was not found to be possible. The use of F-tests to determine significant differences between standard deviations (and hence a modified t-test) was felt to go some way in allaying this latter source of error.

The major omission in this study is the estimate of the extravascular space. This is usually done by the use of a third isotope labelling for example EDTA about 5 minutes prior to the end of the experiment. When the present experiments were started, only a rather primitive single channel gamma counter was available. Discrimination between 3 isotopes was very difficult. Another approach would have been to estimate the extravascular space in a separate group of animals and apply the results to the experimental groups. This was felt to be too animal intensive.

Because of the lack of estimation of extravascular space in the infected rabbits, it is not possible to draw conclusions about protein exclusion of the interstitium. Goodwin and Guy's (1973) data from subcutaneously implanted capsules was discussed in 2.4.1.3 and shown in fig. 2.4.

Capsular fluid is not representative of interstitial fluid though. No protein exclusion properties would be expected in the absence of the interstitial collagen and glycosaminoglycan matrix structure. Also, large volumes of fluid are not normally encountered in normal interstitium.
There is ample indirect evidence that the structure of the interstitium is modified during *T.b. brucei* infection. Goodwin et al (1973) demonstrated perivascular cuffing by mononuclear cells, regenerating tissue in wound-healing to be thicker and less well-organised, fragmented collagen and vacuolated fibroblasts containing abundant lipid. Electron microscopic studies show similar disorganisation of tissue (Edeghere, 1980; Goodwin and Hook, 1968; Goodwin, 1971; Rudin et al, 1984).

The present study shows that there is a marked increase in the rate of accumulation of labelled albumin (increased permeability) in several tissues, but particularly heart, cremaster muscle, abdominal wall muscle and ear. There is also some indication that there is physiological alteration of the interstitium such that a quasi-steady state is reached at a later time than in controls. This was not found to be due (except possibly in the case of cremaster muscle) to oedema formation and is consistent with the histopathological findings of interstitial tissue damage.

Certain features of the present data are difficult to resolve. It looks as though skeletal muscle provides the fluid for expansion of the plasma which is apparently of similar COP to that of controls. This implies net fluid flow out of the tissues into the bloodstream. Yet the likely increase in lymph flow and the extravascular accumulation of labelled albumin suggest the opposite. What could be happening is that there is a process of "damage limitation" such that the net effect seen here is the result of complex mechanisms to prevent large scale oedema in spite of microvascular damage and interstitial changes. This may be mediated via increased lymph flow and concentration making lymph "more efficient". There is some evidence that heart lymphatics are occluded in the later stage of the infection (Morrison et al, 1979, Poltera, 1980) which may be a general feature.
3. In vivo Observations of the Mesenteric Microvasculature

3.1 Introduction

The mesentery is essentially a two dimensional membranous tissue containing a branching network of blood vessels. It is widely used to study the microcirculation both in physiological and inflammatory conditions because it is easily accessible with minimal interference in vascular behaviour. Individual vessels can be cannulated or stimulated. A major advantage of using the mesentery is that it can be easily studied without sophisticated technology though quantitation is more difficult in such circumstances.

An in vivo capillarioscopic study of the mesentery was undertaken to observe directly the leakage of dye-labelled albumin and the behaviour of blood cells in infected and uninfected rabbits. This was to assess how severe changes were during the infection, to complement the tracer study (chapter 2).

Histologically, damage to the mesentery during trypanosomiasis includes leucocyte adherence to venular endothelium, rouleaux formation and platelet aggregation with progressive occlusion of the vascular lumen (Edeghere, 1980). Extravascularly, cellular infiltration and parasites were found (Edeghere, 1980). These changes were similar to those found by Goodwin (1971) in the ears of rabbits infected with T.b. brucei (ear chamber technique).

Physiologically, alterations to macromolecular permeability during trypanosomiasis is not as clear cut in mesentery as, for example, in heart tissue. Significant increases in the accumulation of $^{125}\text{I}$-albumin
were seen in infected mesentery in all but the 60 minute experiments (no tissue hct correction) and in the 90 and 120 minute experiments (corrected for tissue hct). This suggests little increase in the rate of $^{125}$I-alb accumulation during the infection but perhaps a greater available space for albumin in the extravascular tissue.
3.2 Materials and Method

3.2.1 Animals

The experiments were performed on the mesentery of male NZW rabbits weighing between 2 and 3 Kg. Eight of the rabbits were infected with T.b. brucei 427, as described in 2.2.2. These were used in the experiments after being infected for 12 days (2 rabbits), 19 days (1), 20 days (2), 31 days (1) or 32 days (2). Nine uninfected rabbits were used as controls.

3.2.2 Procedure

Anaesthesia was induced by sodium pentobarbitone (35-40 mg/Kg) given via a butterfly cannula in a marginal ear vein. Supplementary doses were given as required. The midline incision area was infused with local anaesthetic. The incision was made using diathermy to minimise bleeding. The rabbit was placed on its side on a heating blanket to maintain its temperature at 38°C. Temperature was monitored with a rectal thermometer.

With gentle pressure on the body, a loop of the small gut with its attached mesentery was slipped into a shallow perspex tray (approx. 12 x 15 x 2 cm) mounted on the microscope stage. The mesentery was superfused with warmed buffer solution (Tris buffer, Ring et al, 1978; see Appendix 1) at 38°C delivered by a perfusion system. A suitable mesenteric "window" was selected and, using cotton tipped sticks, was draped over a small heated stage and superfused with warmed buffer. The stage was made out of polished perspex and glued to a thinned and polished panel at the base of the tray (see fig. 3.1). The whole of the exposed tissue
The tray was screwed to the microscope stage. Care was taken to maintain all fluid and tissue at 38°C. The buffer inflow onto the mesentery and miniscus were at all times in contact with the base (glass coverslip) of the water immersion device.
was then covered in cling film except for the region of the mesentery under observation.

The mesenteric microvessels were observed through a microscope fitted with a water immersion adaptation of a long working range objective (courtesy of P.A. Fraser, Kings' College, London) at a magnification of X100-400. Any abnormalities such as leucocyte rolling, rouleaux stasis, petechiae, etc., were noted and photographed using a 35mm camera attached to the Wild Heerbrugg microscope via a phototube. This procedure was important because it allowed a comparison between experimental results and the initial untraumatised condition.

The mesentery was gently replaced in the abdomen and the rabbit rolled over onto its back. A small section of a carotid artery was exposed and two loose ties placed around it. By feeling for the arterial pulse, the artery supplying the selected section of mesentery was located. With great care, the overlying adipose tissue was teased out to expose the artery. Two loose ties were placed around it. A cannula with a 3-way tap was filled with heparinized saline (1000IU/ml) and quickly inserted into the carotid artery (towards the heart) and secured with the ties. This cannula was connected to the mesenteric cannula via a roller pump and another 3-way tap. The mesenteric cannula was inserted into the artery (away from the heart). Blood was then allowed to flow from the carotid artery via the pump to the mesenteric circulation. This allowed the injection of dye close-arterially to the section of mesentery under study but perfused with oxygenated blood at all other times. The rabbit was heparinised with 1000IU/Kg.

During the cannulations, only a very small part of the mesentery was exposed so that minimal trauma to the tissue occurred. After cannulation,
the abdomen was closed with 2 or 3 bulldog clips for 10 minutes to permit the tissue to recover from the surgery (Ring et al, 1978).

Meanwhile, a low dose of atropine (10-15 μg/Kg) was injected close arterially to reduce gut peristalsis and improve observation. Handling of the gut was kept to a minimum as this stimulated gut movement.

The mesentery was again set up for observation as described above. Further photographs were taken.

Evans blue dye (EB), 0.7% in saline and Evans' blue-albumin (EBA), 0.7% and 5% w/v, respectively, in saline were freshly prepared, filtered and injected as required.

Either EB or EBA were injected close arterially in 2-5ml boli at about the same flow rate as the blood. Dye was present in the vessels for about 10-15 seconds.

Photographs were taken using a 400 ASA Kodak Ektachrome slide film from which black and white prints were made. There were problems obtaining enough photographs of reasonable quality. Gut motility was minimised but the blood pulsation made the field of view go in and out of focus. Even though a fast film was used (400 ASA), the long exposure needed with the transillumination made many of the photographs rather blurred.

3.2.3 Precautions

As has already been mentioned, handling of the mesentery and surgical trauma were kept to a minimum. The initial observation allowed comparison between the pre- and post-operative integrity of the vascular bed. No differences were found between these two observations suggesting
that tissue was in a good physiological condition during the experiment.

This preparation deteriorates fairly rapidly so observations were made within an hour of surgery. The reason for this deterioration is partly because of the large surface area exposed (although protected with cling film and superfused). It was noticed that once the experiments were completed and the rabbit killed, the mesentery was wet to the touch but not "soapy" as is fresh mesentery. This is presumably due to the Tris buffer not being the same as peritoneal fluid. The other factor could be because passing the blood through tubing and a roller pump activates, amongst other things, complement (particularly C5a). This phenomenon is seen to a greater extent during the extracorporeal circulation necessary for cardiopulmonary by-pass surgery (Craddock et al, 1977). However, rabbits seem less prone to pulmonary dysfunction when infused with complement activated plasma than sheep and humans (Fountain et al, 1980). This may also be true of rabbit mesentery suggesting that the blood perfusion technique is acceptable, but the use of Tris buffer less so.
3.3 Results

3.3.1 General Observations

The vessels observed were primarily small arterioles, capillaries and small venules (diameter approximately 5-20\(\mu\)m).

In infected rabbits flow seemed more intermittent in the smallest vessels than in controls. Leucocyte rolling was frequently seen in the small venules but not in the arterioles of infected animals. Rouleaux were common in infected animals and venous stasis apparently occurred more readily. Parasites were never seen in the microvessels.

Figure 3.2 demonstrates the normal appearance of the mesenteric tissue in control rabbits. The tissue appears to have a loose fibrillar type structure with no evidence of cellular infiltration. A small lymphatic vessel (diameter approx. 23\(\mu\)m) can be seen between the two blood vessels (diameter approx. 14-15\(\mu\)m). The lymphatic vessels is approximately 60-65\% wider than the blood vessels. There are arcades between the blood vessels, a feature noted in rat mesentery by Zweifach (1954).

In infected rabbits it was apparent that pathological changes were progressive but focal. In the early stages of the infection (12 days duration) most areas of the mesenteric bed showed apparently normal blood flow, few rouleaux and only occasional instances of leucocyte rolling along the venular walls. Other areas showed some leucocyte margination and reduced flow. As the infection progressed these changes became widespread. The mesenteric tissue became infiltrated with leucocytes. Figure 3.3 is from a rabbit infected for 32 days. This shows the confluence of two venules, arrows indicating the direction of flow. A
Figure 3.2  Small venules with arcades in a control rabbit.
A lymph vessel (L) runs between the two venules.
Figure 3.3 The confluence of two venules in a rabbit infected for 32 days. Red cell rouleaux (r) and red cell aggregation (a) are evident. Note the large lymph vessel (L) and leucocytes in the tissue.
large lymphatic vessel (L) is over twice the diameter of the adjacent venule. A red cell aggregate is in the right hand venule and red cell rouleaux can be seen at the vessel junction. The left hand venule shows some degree of "knobiness", that is, leucocyte pavementing. This is more marked in figure 3.4.

Extravascularly, the tissue appears disorganised, fragmented and contains many cells. This is in marked contrast to the extravascular tissue of control rabbits (fig. 3.2).

Figure 3.4 is from another rabbit infected for 32 days. The interstitium is grossly disorganized but is slightly unfocussed making individual cells less easily seen. The lymphatic vessel is over twice the diameter of the adjacent vessel. The "knobbly" appearance of the venule (V) is indicative of leucocyte sticking to the vessel wall and is a cardinal sign of inflammation. This was never seen in arterioles. Red cell aggregation (or sludging) can be seen downstream from the branch.

Figure 3.5 shows a paired arteriole and venule from a rabbit infected for 19 days. The connective tissue is somewhat indistinct. The faster flow in the arterial vessel is obvious. The venule appears to have slower flow and margination of leucocytes can be seen along the vessel wall.

3.3.2 Permeability

Following bolus injection of EBA or EB alone, no dye leakage was seen in the mesenteric microcirculation either in infected or control rabbits. Leakage of the dye was apparent, however, in the wall of the ileum, manifest as generalized blueing of a small segment of gut wall.
Figure 3.4 The appearance of microvessels in the mesentery of a second rabbit infected for 32 days.

There is marked leucocyte sticking to the venule wall (V). The lymph vessel is denoted (L).
Figure 3.5 A paired arteriole (A) and venule (V) from a rabbit infected for 19 days.

Marked leucocyte margination can be seen in the venule.
3.4 Discussion

An intravital microscopy method was devised to observe the living, functioning microvasculature in the mesentery of rabbits infected with T.b. brucei. The method involved perfusion of the mesenteric bed via the carotid artery and superfusion of the tissue with a modified Ringer solution (Ring et al, 1978).

No difference was found between the condition of the mesentery before and after surgical procedures. Zweifach (1974) describes a normal, healthy mesenteric microvasculature as having no signs of vascular stasis, petechiae, leucocyte rolling or sticking to the vascular endothelium. None of the control rabbits in this study had any of these signs during the experiments. Observations were made shortly after the surgery because even healthy tissue can become infiltrated with leucocytes and exhibit leucocyte rolling and sticking in the venules and red cell stasis after a time (L.H. Smaje - personal communication). Lack of these signs in the control preparations indicates that the tissues are in a reasonable physiological condition. By way of contrast, the mesenteries of the infected rabbits showed all these signs. The leucocyte margination, rouleaux formation and cellular infiltration is therefore assumed to be due to the infection and not damage due to poor handling. These observations are consistent with previous histological studies on T.b. brucei infection in rabbits (Goodwin and Hook, 1968; Edeghere, 1980; Goodwin, 1971; Goodwin et al, 1973).

One of the difficulties with this study was the difference between what was observed during the experiment and what became apparent when the photographs were developed. For example, leucocyte infiltration of
of the tissue and lymphatic vessels were not observed during the experiment but leucocyte rolling, not very apparent from the photographs, was commonly seen in the venules and post-capillary venules of infected rabbits.

Historically, mesentery has long been used to study the microcirculation. In 1930, Landis prepared the mammalian mesentery to measure capillary blood pressure using a micro-injection method. Later definitive studies were undertaken to observe the topography and function of the microcirculation (Chambers and Zweifach, 1944; Zweifach, 1954). More recently, the mammalian mesentery has been used as a model to study fundamental aspects of microvascular transport of solutes and water. Using complex apparatus, it is possible to record, digitise and intensify the photographic images (Zweifach and Intaglietta, 1968; Smaje et al, 1980; Fraser et al, 1978; Lipowsky et al, 1978; 1980; Schmid-Schonbein et al, 1980a; b).

Curry and Michel (1980) used data from frog mesenteric capillaries to formulate their fibre matrix theory of capillary permeability. Curry and Huxley (1982) analysed experimental data from mammalian single vessel and whole organ studies in terms of the fibre matrix theory. They found, with the exception of dog gastric wall (fenestrated microvessels), that this data could be fitted to their theory. On the other hand, Spencer (1984), using data from salivary glands (fenestrated capillaries) and a computer model found that pore theory could still explain the experimental results in this tissue.

The mesentery is also a useful tool with which to study pathological changes. Most of these studies (Fox et al, 1980; Marchesi, 1962; 1964; Buckley and Ryan, 1969; Altura, 1970; Northover, 1967a; b; 1978;
Northover and Northover, 1970; Clough and Smaje, 1984b) involve the injection or topical application of inflammatory mediators or similar substances to an otherwise healthy tissue. This method has the advantage of being able to compare the "before and after" condition in the same tissue. In the present study, however, this was not possible because of the disease's chronicity.

Inflammatory changes observed in other vascular beds (hamster cheek pouch and rat cremaster muscle, in particular) under conditions of acute inflammation are often very similar. This suggests that such changes are fundamental. Certainly, vascular changes in two of the tissues extensively studied in rabbit T.b. brucei infection appear similar - ear (Goodwin and Hook, 1968; Goodwin, 1971) and cremaster muscle (Goodwin and Hook, 1968; Edeghere, 1980). Both show leucocyte accumulation along venular endothelium, red cell rouleaux, vascular stasis, swollen endothelial cells and intercellular gap formation, constricted arterioles (particularly in ears) and dilated, tortuous veins and venules. The present study concentrated on the smaller vessels. There was no evidence for particularly dilated venules though some of the lymphatic vessels appeared to be dilated in infected rabbits. The most consistent feature in the mesentery was leucocyte margination. This was fascinating to watch in vivo. Even when the blood was relatively free-flowing, the slow, jerky movement of leucocytes along the venular endothelium was evident. It was never seen in arterioles nor in control animals.

The involvement of immune complexes (IC) in the pathogenesis of trypanosomiasis seems very likely. Bjork and Smedgard (1984) studied the effect of IC in sensitized hamster cheek pouch microvasculature. They demonstrated that different antibody:antigen ratios (forming
different sized immune complexes) had quantitatively and qualitatively
dissimilar effects. Scherzer and Ward (1978) showed that different sized
IC induced lung injury of variable severity which was correlated with
the ability of the IC to activate complement. The immunological complexity
of trypanosome infection may provide for a variety of IC sizes. The
fluctuation in parasitaemia and the progressive nature of the disease
may produce different patterns of pathological changes (due to IC?) super-
imposed on each other as the infection progresses.

The implications of red cell aggregation and the behaviour of
leucocytes in the microcirculation has already been discussed in chapter
1.7.4.

Discrepancies between observations on cat mesentery and data
obtained in vitro led Lipowsky et al (1978) to suggest that in addition
to haematocrit shear stress and blood flow, the presence or absence of
leucocytes was important. In vivo studies suggest that flow inhomogeneities
may be due to "leucocyte plugging" (Braide et al, 1984; Bagge et al, 1980).
Leucocyte rolling or sticking is a feature of low flow states in general.
Goldsmith and Spain (1984) showed that as flow rate decreased, erythro-
cytes formed rouleaux which migrated towards the centre of the vessel,
displacing the leucocytes towards the wall. In trypanosomiasis, this
effect would be expected to greatly increase because of the increased
plasma concentrations of fibrinogen and large numbers of leucocytes.
In addition, the endothelial cell surface changes, becoming "sticky".
Edeghere (1980) showed projections between vascular endothelial cells
and leucocytes in infected animals. This is a well-known feature of
inflammation. Using washed blood, Goldsmith and Spain (1984) showed that
there was no leucocyte margination, even at very low shear rates when
there was no red cell aggregation. It would appear that the factors
augmenting red cell aggregation in trypanosomiasis are of crucial importance in continuation of the pathological processes.

It has been suggested that leucocytes (and/or substances released by them) are necessary for increases in macromolecular permeability. There is evidence for this in rabbit mesentery (Clough and Smaje, 1984b) and lung (Lloyd et al, 1984; Flick et al, 1981). Clough and Smaje (1984b) found that if rabbit C5a was added to the superfusate of healthy mesentery large numbers of leucocytes were attracted and their actions in increasing macromolecular permeability potentiated. This is very important in trypanosomiasis as there is much evidence showing the involvement of the complement, coagulation, kinin-kallikrein and fibrinolytic systems in the pathogenesis of the disease.

It was surprising not to see leakage of dye (either free or bound to albumin) out of the microvessels particularly in infected rabbits. Levick and Michel (1973), perfusing single capillaries of frog mesentery with EB and EBA, found that free dye on its own leaked out but not when complexed with albumin. Free dye could be seen outside within 30 secs, mostly within 5 seconds. If the microcirculation was more permeable in infected rabbits some free dye leakage would have been expected.

The reasons for not obtaining extravasation of free dye could be due to a difference in technique. Levick and Michel (1973) perfused frog capillaries whereas in the present study, dye was injected as a bolus close arterially. This allows a high dye concentration locally which is then markedly diluted before recirculation, permitting more than one injection. The leakage of EB alone (Levick and Michel, 1973) is likely to be due to capillaries becoming leaky when perfused with protein-free solutions (Mason et al, 1977). The bolus injection of dye in the present
study may be too rapid for the "protein-free effect" to occur. It was unknown how an infusion with a physiological solution would affect the microvascular endothelium, particularly as pathological changes were to be studied.

The results for the transport of $^{125}\text{I}$-albumin in mesentery (chapter 2) are somewhat equivocal. The rate of extravasation of the tracer does not appear to be greatly enhanced during the infection. On this basis, the lack of dye leakage is consistent.

However, the mesentery may have altered protein exclusion properties due to trypanosome infection. The appearance of the extravascular mesenteric tissue during the disease (fig. 3.3 and 3.4) implies breakdown of its structural integrity. Goodwin et al (1973) also found connective tissue changes during trypanosome infection of rabbits. Alterations in the collagen and glycosaminoglycan constituents of the interstitium can change protein exclusion properties (Granger, 1979; Aukland and Nicolaysen, 1981). This may allow the tissue to contain greater amounts of protein than in controls (Aukland and Nicolaysen, 1981). The significantly increased accumulation of $^{125}\text{I}$-alb in infected mesentery at 90 and 120 minutes (both calculation methods) is consistent with this. The fibre matrix theory allows for the fine fibres, characteristic of control states to condense into a coarser network in protein-free conditions (Curry and Huxley, 1982). A similar mechanism may occur during inflammatory conditions.

Measurement of the extravascular and protein exclusion spaces would provide more answers to the fundamental changes occurring in trypanosomiasis. Also, a fluorescent tracer would have been more appropriate in this set of experiments.
4. Effects of Trypanosome-derived Material in the Systemic Circulation

4.1 Introduction

There is much evidence that immune complexes (IC) play a role in the pathology of trypanosomiasis (see chapter 1.6). This role may be in part mediated by the kinin/kallikrein system. Kinins are released into the blood and urine during trypanosomiasis infections in several host species (Goodwin and Richards, 1960; Richards, 1965; Goodwin and Boreham, 1966). Kinin release is associated with increased levels of free kallikrein which reach a maximum just after the first parasitaemic peak in rabbit trypanosome infections (Boreham, 1968a; 1970; Parry, 1980).

Immune complexes (made from anti-trypanosome serum and live or disrupted trypanosomes) are capable of activating the kinin/kallikrein system both in vivo and in vitro (Parry, 1980).

When live trypanosomes complexed with antibody were injected into normal rabbits, a decrease in arterial blood pressure (aBP) occurred which could be prevented by the drug aprotinin (Trasylol) (Boreham and Wright, 1976).

Rabbits infected with T.b. brucei are hypotensive (Boreham and Wright, 1976; Yates, 1978). When disrupted trypanosomes were injected into infected rabbits, a further decrease in aBP occurred, similar to that resulting from IC injection in control rabbits (Boreham and Wright, 1976). This decrease in aBP was also inhibited by Trasylol. As hypotension was not seen in control rabbits injected with disrupted trypanosomes, it was felt not to be due to trypanosome material alone.
Boreham and Wright (1976) proposed that the chronic hypotension seen in trypanosomiasis was due to the release of endogenous, pharmacologically active substances triggered by the presence of IC. Since the reductions in aBP caused by both disrupted trypanosomes (in infected rabbits) and IC (in control rabbits) were inhibited by Trasylol, it was postulated that the kinin/kallikrein system was involved in both systems.

However, Trasylol inhibits the enzymatic action of serine proteins not only kallikrein but also plasmin, trypsin, chymotrypsin, tissue and leucocyte proteinases and may interfere with the activation of plasma factors in the preliminary stages of blood coagulation. It may also inhibit mitochondrial ATPase (Hagiwara et al, 1980). So to inhibit the action of kinins specifically, it is necessary to use carboxypeptidase B or bradykinin antiserum (Chao et al, 1981). Trasylol inhibits the enzymatic action of kallikrein in producing kinins but does not inhibit pharmacological actions of kallikrein itself (chapter 1.8.3.1).

At least two questions arise from the studies of Boreham and Wright, (1976) - what is the nature of the IC causing decreases in aBP and, secondly, what mediates the hypotension?.

The in vivo effects of IC depend on inter alia, antibody class (Haakenstad and Mannik, 1977). Immunoglobulin G and IgM are the main antibody classes and, in T.b. brucei 427 in rabbits, are increased 4-5 fold and 2 fold respectively (Parry, 1980). Therefore in this study trypanosome-specific IgG and IgM were isolated from immunized rabbits and used to make IC's. It was hoped thereby to uncover more about the acute hypotensive response, its trigger and its relation to the pathogenicity of T.b. brucei infections in rabbits.
4.2 Materials and Methods

4.2.1 Parasites

_Trypanosoma b. brucei_ 427 was obtained from the Lister Institute of Preventative Medicine and the parasite strain was maintained in male Cd-1 mice (Charles River UK Ltd) as described previously (chapter 2.2.1).

4.2.2 Parasite Hosts

All the rabbits used were male NZW weighing 2.5-5Kg. Twelve control rabbits were free from any previous contact with trypanosome material. Nine rabbits were infected as described previously (chapter 2.2.2) and were used in the experiments 9-36 days later, mean infection length 18.4 days. Table 4.1 gives the details of these rabbits indicating their clinical presentation. See also chapter 1.5.5.

In addition, three rabbits were used which had previously been injected with _T.b. brucei_ 427 but did not succumb to the infection. They were then used for the production of immune serum (by other members of the laboratory). These rabbits showed no outward signs of infection (Table 4.1) and were apparently cured.

4.2.3 Immunological Procedures

4.2.3.1 Preparation of Trypanosome Antiserum

Disrupted trypanosomes were prepared by repeated freezing and thawing of trypanosomes which had been prepared as described in chapter 2.2.2. One ml. of the resultant suspension + 1ml of Freund's Complete
<table>
<thead>
<tr>
<th>RABBIT</th>
<th>DAYS AFTER INFECTION</th>
<th>PRESENTATION AND COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1</td>
<td>9</td>
<td>No outward signs of infection</td>
</tr>
<tr>
<td>I2</td>
<td>11</td>
<td>No outward signs of infection</td>
</tr>
<tr>
<td>I3</td>
<td>12</td>
<td>Swollen face, ears starting to droop, mild erythema of genitals</td>
</tr>
<tr>
<td>I4</td>
<td>17</td>
<td>Mild outward signs of infection with swollen genitals</td>
</tr>
<tr>
<td>I5</td>
<td>18</td>
<td>Drooping ears, mild swelling of face, moderate swelling of genitals</td>
</tr>
<tr>
<td>I6</td>
<td>19</td>
<td>Moderate to severe signs of infection for a 19 day infection</td>
</tr>
<tr>
<td>I7</td>
<td>21</td>
<td>Moderate signs of infection consistent with a 3 week infection</td>
</tr>
<tr>
<td>I8</td>
<td>23</td>
<td>Typical appearance. i.e. droopy, heavy ears, swollen face, rhinitis, swollen erythemic genitals</td>
</tr>
<tr>
<td>I9</td>
<td>36</td>
<td>Eyes closed, ears, face and genitals swollen. A few scabs present on genitals</td>
</tr>
<tr>
<td>S1</td>
<td>sensit.</td>
<td>Infected 9 months previously and apparently cured. Immunized with trypanosome material &amp; FCA into lymph nodes</td>
</tr>
<tr>
<td>S2</td>
<td>sensit.</td>
<td>Infected 1 year previously and immunized similarly to rabbit 2</td>
</tr>
<tr>
<td>S3</td>
<td>sensit.</td>
<td>Infected 2 months previously and dosed with 5000IUTrasylol daily on days 5-13 inclusive</td>
</tr>
</tbody>
</table>

Table 4.1

Clinical presentation and history of infected and sensitized rabbits.

Control rabbits are indicated by prefix 'C'.

Adjuvant (FCA) was emulsified and injected into the rabbit gluteus maximus muscle. This was repeated on days 8, 15 and 29 in alternate legs. Two to three weeks after the last inoculation 3-4mls of blood were collected from the marginal ear vein and incubated at 37°C for 3 hours in a glass vial. Serum was removed by centrifugation and tested for trypanosome-agglutinating and -precipitating antibody (see 4.2.3.2 and 4.3.2.3). If the antibody titre was greater than 2048, 20-25mls of blood were taken from the immunised rabbit and the serum stored at -15°C in 1ml samples.

4.2.3.2 Agglutination Test

This method was based on that of Cunningham and Vickerman (1962). Two-fold serial dilutions of the test serum were made and a drop placed on a glass plate. Thawed stabilates or fresh diluted trypanosomes were dotted on the surface of the drop of diluted serum. After an hour at room temperature, agglutination was observed under a microscope at a magnification of X400. The antibody titre was the reciprocal of the highest dilution demonstrating agglutination.

4.2.3.3 Precipitation Test (crossover electrophoresis)

Sample wells were cut in agar coated slides. In an electrophoresis tank, a 0.2-0.3 amp current at 160-200 volts was passed through the slides. Serum samples and trypanosome antigen were added and electrophoresed for 15 minutes. After washing and gently drying, the slides were stained with brilliant croscein coomassie blue (Appendix 1). Precipitation arcs were clearly seen if antibody-antigen reaction occurred.
4.2.3.4 Preparation of IgG

This method is that of Stanworth (1960) as described in a Whatman Data Sheet (1968). Cooled, dialysed trypanosome-specific antisera (as prepared above) was mixed with wet, equilibrated DEAE DE52 cellulose and, after incubation at 4°C for 1 hour, filtered in a Buchner funnel. The resultant eluant (IgG) was dialysed, freeze dried and then resuspended to the original serum volume when required. Each batch was tested for the presence of both IgG and IgM using a double radial diffusion method (Hudson and Hay, 1980). Any sample exhibiting IgM activity was discarded.

4.2.3.5 Preparation of IgM

This method utilized the IgG-binding properties of Staphylococcal protein A. Dialysed trypanosome-specific serum was run through a column of Sepharose-protein A (courtesy of Dr. David Lane, Biochemistry Dept. ICST), using 0.01M phosphate buffer, pH 8.0 (Appendix 1). The eluant contained IgM and other plasma proteins but not IgG. The buffer was changed to 0.1, citrate buffer, pH 3.0 and the IgG eluted. These fractions were neutralized with IM Tris-Hcl, pH 8.8 as they were eluted. Each fraction was tested for both IgG and IgM using a double radial diffusion test (Hudson and Hay, 1980). Any fraction with neither or both antibodies was discarded.

4.2.4 Preparation of Immune Complexes

a) Crude Complexes These were prepared using the method of Boreham and Wright (1976). One ml. of trypanosome-specific antiserum and 1ml of disrupted trypanosomes were mixed and incubated for 1 hour at 37°C. A further incubation of 12 hours at 4°C was performed. The mixture was
then centrifuged at 3,000 rpm for 5 minutes and washed in saline or PSG three times. The pellet was resuspended to the original volume for immediate use.

b) IgG and IgM Complexes This method was the same as above, substituting the appropriate antibody class for antiserum. The supernatant solutions obtained during the washing procedure were retained.

c) Live Complexes These were prepared by mixing trypanosome-specific antiserum or IgG with freshly separated trypanosomes and incubated for 1 hour at 37°C. The mixture was centrifuged gently at 1,500 rpm for 3 minutes. The supernatant was reserved and the pellet resuspended in PSG.

d) Nature of the Immune Complexes (IC) The nature and precise make-up of these IC are unknown. The complexes are divided for convenience into soluble (supernatant) and insoluble (resuspended pellet) fractions.

4.2.5 Procedure

The rabbits were anaesthetised via the marginal ear vein with 30-40mg/Kg sodium pentobarbitone (Sagatal, May and Baker), supplemented when necessary.

The carotid artery was cannulated for continuous blood pressure measurement. The arterial cannula was filled with 1000IU/ml heparin, inserted towards the heart and connected to a pressure transducer (type 4-88, S.E. Laboratories, (EMI) Ltd). The signal was analysed by an Arterial Blood Pressure Module (SEM 422, S.E. Laboratories, (EMI) Ltd), and monitored with a single channel pen recorder (Bryans 28 000). A
foot-operated event marker was made from an ordinary doorbell.

The jugular vein was cannulated for injection. Occasionally, however, in infected rabbits fatty tissue and enlarged lymph nodes in the neck made surgery very difficult and a femoral vein was cannulated instead.

Mean arterial blood pressure (aBP) was determined as:

\[
\frac{2}{3} \text{ diastolic} + \frac{1}{3} \text{ systolic blood pressure}
\]

(A. Ungar - personal communication)

The challenges were administered intravenously over 15-30 seconds. Immune complexes with live trypanosomes were sometimes difficult to inject because of agglutination of the trypanosomes. If no response was obtained within 10 minutes, another challenge was administered. Otherwise subsequent challenges were made after aBP returned to pre-challenge levels (or stopped changing). Occasionally the rabbit died after the first or second challenge.

All rabbits were killed with an overdose of pentobarbitone at the end of the experiment.

Aprotinin (Trasylol) of 5,000IU to 200,000IU was used prior to some challenges. Boreham and Wright (1976) found 5,000IU to be adequate to block the hypotensive response to challenge with IC. High doses (200,000IU) are well tolerated by rabbits.

**Control Injections** The control preparations injected were the buffer solution used as injection vehicles (PSG and heparinized saline) and antiserum alone.
Vascular reactivity to vasodilatory stimuli in anaesthetised animals may be reduced if the animals are vasodilated already. To test this, small doses of histamine (5-10μg) were injected. These give reliable, dose-dependent decreases in aBP both in control and infected rabbits (unpublished observations), but these are only transient so allow vascular reactivity to be tested.

4.2.6 Statistics

The arterial blood pressure of infected rabbits was tested for a decrease compared with control rabbits by the use of a one-tailed t-test.

The aBP responses to in vitro-formed complexes in all rabbits were ranked for magnitude of response and duration of infection to obtain the coefficient of rank correlation (r). This procedure was also carried out for the putative in vivo-formed IC and the responses to challenge after injection of Trasylol.

\[
    r_{\text{rank}} = 1 - \frac{6 \sum D^2}{N(N^2-1)}
\]

where:

\[D = \text{differences between ranks of corresponding values of the 2 variables}\]

\[N = \text{number of pairs of variables}\]
4.3 Results

4.3.1 Arterial Blood Pressure

Initial arterial blood pressure was measured in infected, sensitized and control rabbits. The results are given in Table 4.2. There was a significant decrease ($p < 0.05$) in aBP in the infected rabbits compared to the controls, using a one-tailed t-test. The mean aBP in infected animals was also lower than that found in the three sensitized rabbits though not significantly lower. The aBP in sensitized rabbits was not significantly different from that recorded in the controls.

4.3.2 Reaction of Arterial Blood Pressure to Challenges

This data does not lend itself easily to statistical evaluation. However, as there are qualitative aspects of these experiments that are of interest, the data is presented in detail in categories of challenge type with accompanying figures (exact reproductions of aBP traces) followed by a summary of data and then the results of the statistical manipulations.

4.3.2.1 Disrupted and Partially Disrupted Trypanosomes (Table 4.3)

a) Control Rabbit

In one case trypanosomes were separated from rat blood and stored at 12°C for 12 hours. The sample was thawed and some parasite motility observed under a X40 objective. When $1.75 \times 10^9$ of these partially disrupted trypanosomes were injected in rabbit Cl, mean aBP decreased from 104 mmHg to 68 mmHg (a fall of 35%) (fig. 4.1). This strong reaction
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>mean aBP ± SEM</th>
<th>sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>12</td>
<td>95.75 ± 5.22</td>
<td></td>
</tr>
<tr>
<td>infected</td>
<td>9</td>
<td>78.44 ± 7.02</td>
<td>0.95</td>
</tr>
<tr>
<td>sensitized</td>
<td>3</td>
<td>91.33 ± 13.64</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 4.2. Comparison of initial mean arterial blood pressure (aBP, mmHg) in three groups of rabbits. n is the number of rabbits, and sig. is the level of significance for a decrease compared with control (ns = not significant).
Figure 4.1

The response of the arterial blood pressure of control rabbit Cl to injection of 1ml of partially disrupted trypanosomes. Note the relatively prolonged onset and duration of the response compared to other types of challenge.

Figure 4.2

The response of the aBP of rabbit II, infected for 9 days (see Table 4.1), to challenge with 1ml of disrupted trypanosomes. Note the low initial aBP and the marked hypertensive phase followed by the usual hypotensive response.
is in contrast to the results of Boreham and Wright (1976) who reported that injection of whole live or disrupted trypanosomes into control rabbits produced no response. (However, see 4.4.3).

When the aBP had stabilized, an injection of repeatedly freeze-thawed trypanosomes produced no change in blood pressure. Injection of trypanosome-specific antiserum provoked a 36% reduction in aBP (73 - 47mmHg). This is presumably due to in vivo formation of IC with trypanosome antigen already circulating.

b) Infected Rabbit

Injection of 1ml of disrupted trypanosomes caused a marked biphasic response in rabbit II, infected for 9 days (fig. 4.2). The initial aBP of 47mmHg (very low) increased to 60mmHg and then down to 30mmHg, an increase of 28% and a decrease of 36% from the initial aBP. A slight hypertension and then hypotension was often observed following challenge but was not usually so marked. The aBP then recovered to 69mmHg, indicating that the initial aBP of 47mmHg was rather low, characteristic of considerable vasodilatation. A further 1ml of disrupted trypanosomes only produced a 6% decrease in aBP.

c) Sensitized Rabbit

In sensitized rabbit S2, 1ml of disrupted trypanosomes caused mean aBP to fall from 73 to 53mmHg (a 27% decrease) (fig. 4.3a). Two ml of the same disrupted trypanosome injected 5 minutes after 5,000IU of Trasylol provoked a hypotensive response of only 3mmHg (a 4% decrease) (fig. 4.3b). A large dose of bradykinin (4.5µg) produced a transient hypotensive response of 54% (fig. 4.5, see section 4.3.2.2 below). This
Figure 4.3

The effect on the aBP of sensitized rabbit S2 of injection with 1ml of disrupted trypanosomes. It was necessary to give a supplementary dose of pentobarbitone during the observation period.
latter response is not affected by the prior injection of Trasylol. A further 2ml of disrupted trypanosomes provoked no response.

Few observations on control rabbits were carried out using trypanosome material alone, since little response was expected (Boreham and Wright, 1976). Just for comparison with injected disrupted trypanosomes in infected rabbits, live trypanosomes were tried. It is interesting to note that a more intense response is obtained with live trypanosomes (see 4.3.2.3 below).

The hypotensive response provoked by injection of trypanosome antigen into sensitized and infected rabbits is likely to be due to the formation of immune complexes in vivo.

4.3.2.2 Bradykinin (Table 4.3)

The response of aBP to bolus injection of bradykinin is rapid and transient. This contrasts in both onset and duration with the responses to complexes (whether formed in vitro and then injected, or formed in vivo) which are slower in onset and more prolonged.

a) Control Rabbits

Figure 4.4 shows the response of rabbit C2 to challenge with bradykinin (1.0µg and 1.5µg). The fall in aBP is transient, only lasting about 2 minutes. The small pulse pressure is probably due to a small amount of air trapped between the carotid artery and the pressure transducer producing a damping effect.
Figure 4.4

The effect in control rabbit C2 of bolus injection of bradykinin. a) 1.0μg and b) 1.5μg.

Figure 4.5

The effect of bolus injection of 4.5μg of bradykinin on the arterial blood pressure of sensitized rabbit S2.
b) Sensitized Rabbit

Figure 4.5 shows the response to 4.5μg of bradykinin (large dose) in rabbit S2. This is similarly rapid in onset but with a more prolonged recovery. Such a large response may also provoke baroreceptor reflexes.

Another dose of bradykinin (1.5μg) was injected into rabbit C5 - see 4.3.2.4.

The extended duration of the IC-induced hypotension compared to that of injected bradykinin points to the involvement of factors other than kinins. Kinins in general have a very short biological half-life in vivo and are largely broken down in one complete blood circulation time. The complex interplay of a variety of inflammatory mediators (see chapter 1.8) make it unlikely that only kinins are involved.

4.3.2.3 Live Trypanosomes

a) Infected Rabbits

Live trypanosomes provoked a hypotensive response in most cases.

Rabbit I2 (11 day infection) had an initial mean aBP of 105mmHg which was reduced to 43mmHg (a decrease of 59%) on challenge with 1.86 x 10^9 live trypanosomes.

In rabbit I4 (17 day infection), 1ml of live trypanosomes produced a decrease in aBP of 43% (from 74 to 42mmHg) as shown in fig. 4.6a). One minute after 5,000IU of Trasyrol, 1ml of live trypanosomes produced
<table>
<thead>
<tr>
<th>Rabbit Condition</th>
<th>aBP₀</th>
<th>Challenge</th>
<th>aBP₁</th>
<th>ΔaBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl control</td>
<td>104</td>
<td>1.75x10⁹ partially disrupted tryps (fresh frozen)</td>
<td>68</td>
<td>-36</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>disrupted tryps</td>
<td>76</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>1ml tryp. imm. serum</td>
<td>47</td>
<td>-26</td>
</tr>
<tr>
<td>I1 infected</td>
<td>47</td>
<td>1ml disrupted tryps</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>day 9</td>
<td>69</td>
<td>1ml disrupted tryps</td>
<td>30</td>
<td>-17</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>1ml tryp imm. serum</td>
<td>67</td>
<td>-</td>
</tr>
<tr>
<td>S2 sensitized</td>
<td>73</td>
<td>1ml dis. tryps</td>
<td>53</td>
<td>-20</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>2ml dis. tryps 5 mins later</td>
<td>74</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>4.5µg bradykinin</td>
<td>40</td>
<td>-48</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>2ml disrupted tryps</td>
<td>90</td>
<td>-</td>
</tr>
<tr>
<td>C2 control</td>
<td>87</td>
<td>1µg bradykinin</td>
<td>68</td>
<td>-19</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>1.5µg bradykinin</td>
<td>64</td>
<td>-22</td>
</tr>
</tbody>
</table>

Table 4.3. The effects of disrupted and partially disrupted trypanosomes and bradykinin on rabbit mean arterial blood pressure (aBP, mmHg). aBP₀ and aBP₁ are the values before and after the challenge, respectively, and ΔaBP is the change in aBP (and percentage decrease).
Figure 4.6

The response of the aBP of rabbit I4 infected for 17 days (see Table 4.1) to challenge with (a) live trypanosomes and (b) live trypanosomes 1 minute after 5000IU of Trasylol.
a decrease in mean aBP from 67 to 54 mmHg (19%), less than half the original response (fig. 4.6b).

In rabbit 15 (18 day infection), challenge with live trypanosomes produced a fall in mean aBP from 70 to 42 mmHg (40% decrease). When a further challenge of live trypanosomes was administered, the rabbit died.

b) Sensitized Rabbit

In rabbit SI, initial challenge with live trypanosomes reduced mean aBP from 188 to 65 mmHg (45% decrease) with a further decrease in aBP 15 minutes after injection (fig. 4.7). Treatment with 5,000 IU of Trasylol reduced the response to a second challenge with live trypanosomes to only a 15% drop in aBP. Twenty eight minutes after the injection of Trasylol, a further challenge dose of live trypanosome reduced aBP by 19%.

Table 4.4 summarizes the results to challenge with live trypanosomes.

4.3.2.4 Crude Complexes (see Table 4.5)

a) Control Rabbits

Rabbit C3 produced a 28% decrease in aBP (from 53 to 38 mmHg) when challenged with 1 ml of crude complexes. Three to four minutes after 5,000 IU of Trasylol, a further 1 ml of the crude complexes produced a 10% hypotensive response, less than half the response observed before Trasylol.
Figure 4.7

The effect on aBP of sensitized rabbit Sl of challenge with live trypanosomes. Note the second phase of hypotension. This was not seen in any other response. It was not due to any mechanical alteration of the blood pressure cannula.

Figure 4.8

The response of injection of 1ml of crude immune complexes into control rabbit C4. Note that whilst the magnitude of the response was not great, the reaction of the aBP is distinctive.
<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Condition</th>
<th>( aBP_0 )</th>
<th>Challenge</th>
<th>( aBP_1 )</th>
<th>( \Delta aBP )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I2</td>
<td>infected day 11</td>
<td>105</td>
<td>( 1.86 \times 10^9 ) live tryps</td>
<td>43</td>
<td>-62 (59%)</td>
</tr>
<tr>
<td>I4</td>
<td>infected day 17</td>
<td>74</td>
<td>( 5 \times 10^8 ) live tryps</td>
<td>42</td>
<td>-32 (43%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5000 IU Trasylol, then</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>67</td>
<td>( 5 \times 10^8 ) live tryps</td>
<td>54</td>
<td>-13 (19%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 min later</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I5</td>
<td>infected day 18</td>
<td>70</td>
<td>( 7 \times 10^8 ) live tryps</td>
<td>42</td>
<td>-28 (40%)</td>
</tr>
<tr>
<td>S1</td>
<td>sensitized (see table 4.1)</td>
<td>118</td>
<td>( 4 \times 10^8 ) live tryps</td>
<td>65</td>
<td>-53 (45%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5000 IU Trasylol, then</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>93</td>
<td>( 4 \times 10^8 ) live tryps</td>
<td>79</td>
<td>-14 (15%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 mins later</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>84</td>
<td>( 4 \times 10^8 ) live tryps</td>
<td>68</td>
<td>-16 (19%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28 mins after Trasylol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4. The effects of live trypanosomes on mean arterial blood pressure (\( aBP \), mmHg) in infected and sensitized rabbits. \( aBP_0 \) and \( aBP_1 \) are the values before and after the challenge, respectively, and \( \Delta aBP \) is the change in \( aBP \) (and percentage decrease).
One ml of crude complexes injected into rabbit C4 resulted in a slightly biphasic response (fig. 4.8), mean aBP of 112mmHg increased to 118mmHg and then decreased to 100mmHg (+5% and -11%). Five minutes after 5,000IU of Trasylol, two ml of crude complexes produced a 24% decrease in aBP (from 112 to 85mmHg).

These apparently conflicting results suggest that 5,000IU of Trasylol may not completely inhibit the enzymatic effect of kallikrein or that the larger dose of crude complexes initiates other hypotension-producing mechanisms.

Live trypanosome complexes were used for comparison with other controls. In rabbit C5, 1ml of PSG produced no change in aBP. One ml of live complexes reduced mean aBP by 48% (96 to 50mmHg) (fig. 4.9a). A smaller dose of live complexes (0.35ml) reduced aBP by 17% (from 89 to 74mmHg) (fig. 4.9b). Live complexes seem to produce a bigger response in controls than crude complexes and appear to exhibit some dose-dependence. In this rabbit, 1.5µg of bradykinin provoked a 27% decrease in aBP (see 4.3.2.2).

b) Infected Rabbits

Challenge with 1ml of crude complexes in rabbit I8 (23 days after infection) decreased aBP by 21% (from 92 to 73mmHg) (fig. 4.10). A further 1ml of crude complexes 6 minutes after 1,500IU of Trasylol provoked a 12% decrease in aBP (from 86 to 76mmHg). Twenty-five minutes after the Trasylol, 1ml of crude complexes produced a decrease in mean aBP of 27% (from 82 to 60mmHg). Ten minutes later, a further challenge with 1ml of crude complexes produced a fairly marked biphasic response. This was not the more usual hyper- then hypotensive response, but
The response of the aBP of control rabbit C5 to injection with a) 1ml of live crude complexes and b) 0.35ml of live crude complexes. Note that the paper speed is three times faster than all other traces.
Figure 4.10 The response of the aBP of infected rabbit I8 (infected for 23 days) to challenge with 1ml of crude IC.

Figure 4.11 The response of sensitized rabbit S3 to challenge with 1ml of crude complexes. A second challenge killed this rabbit.
hypotension followed by a further phase of hypotension (from 88 to 72mmHg and then to 54mmHg - decreases of 18% and then 39% of pre-injection aBP). The effect of Trasylol in abrogating the response to challenge appears to wear off with time.

In rabbit 16 (day 19 infection), 1ml of crude complexes reduced aBP by 42% (from 77 to 45mmHg). Seven minutes after 5,000IU Trasylol, a further 1ml of crude complexes produced a biphasic response, an increase in aBP of 15% (from 61 to 70mmHg) followed by a decrease in aBP of over 50% from which the rabbit died. Trasylol had no apparent effect in this rabbit.

Rabbit 17 (21 day infection) was curiously unresponsive to any challenges. Injection of half the usual volume of crude complexes only produced a very slight response (from 48 to 51mmHg then down to 47mmHg). This was followed by 1ml of crude complexes which again produced a small biphasic response (from 45 to 48mmHg and then a decrease to 40mmHg). Five µg of histamine followed by a further 10µg produced a decrease in aBP of only 3mmHg on both occasions. Histamine in these doses usually produces a rapid, transient dose-dependent decrease in aBP similar to that produced by bradykinin. Such a small response in this rabbit suggests vascular unresponsiveness in the presence of significant vasodilatation. This may be related to the infection, but also the latter may have been aggravated by the anaesthetic.

c) Sensitized Rabbit

Rabbit S3 produced no response to 1ml PSG. However, this rabbit displayed a marked hypotensive response to injection with 1ml of crude complexes (fig. 4.11). The first IC challenge caused mean aBP to fall
<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Condition</th>
<th>( aBP_0 )</th>
<th>Challenge</th>
<th>( aBP_1 )</th>
<th>( \Delta aBP )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>control</td>
<td>53</td>
<td>1ml crude IC</td>
<td>38</td>
<td>-15 (28%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5000IU Trasylol, then</td>
<td>45</td>
<td>-5 (10%)</td>
</tr>
<tr>
<td>C4</td>
<td>control</td>
<td>112</td>
<td>1ml crude IC</td>
<td>118</td>
<td>6 (-5%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5000IU Trasylol, then</td>
<td>100</td>
<td>-12 (11%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>112</td>
<td>2ml crude IC 5 mins later</td>
<td>85</td>
</tr>
<tr>
<td>C5</td>
<td>control</td>
<td>99</td>
<td>1ml PSG</td>
<td>99</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>1ml live IC</td>
<td>50</td>
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<td>89</td>
<td>0.35ml live IC</td>
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<tr>
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<td>95</td>
<td>1.5(\mu g) bradykinin</td>
<td>69</td>
</tr>
<tr>
<td>I8</td>
<td>infected</td>
<td>92</td>
<td>1ml crude IC</td>
<td>73</td>
<td>-19 (21%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>day 23 5000IU Trasylol, then</td>
<td>76</td>
<td>-10 (12%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HR=288 beats/min 86</td>
<td>60</td>
<td>-22 (27%)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>82</td>
<td>1ml crude IC</td>
<td>72</td>
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<td>88</td>
<td>1ml crude IC</td>
<td>54</td>
</tr>
<tr>
<td>I6</td>
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<td>1ml crude IC</td>
<td>45</td>
<td>-32 (42%)</td>
</tr>
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<td>day 19 5000IU Trasylol, then</td>
<td>70</td>
<td>9 (-15%)</td>
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<tr>
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<td></td>
<td></td>
<td>61</td>
<td>1ml crude IC 6 mins later</td>
<td>30</td>
</tr>
<tr>
<td>I7</td>
<td>infected</td>
<td>48</td>
<td>0.5ml crude IC</td>
<td>51</td>
<td>3 (-6%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>day 21 47</td>
<td>48</td>
<td>-1 (2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45</td>
<td>1ml crude IC</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51</td>
<td>5(\mu g) histamine</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>47</td>
<td>10(\mu g) histamine</td>
<td>44</td>
</tr>
<tr>
<td>S3</td>
<td>sensitized</td>
<td>83</td>
<td>1ml PSG</td>
<td>83</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(see table 4.1)</td>
<td></td>
<td>83</td>
<td>1ml crude IC</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>70</td>
<td>1ml crude IC</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 4.5. The effects of crude complexes on mean arterial blood pressure (\(aBP, \text{mmHg}\)) in control, infected, and sensitized rabbits. \( aBP_0 \) and \( aBP_1 \) are the values before and after the challenge, respectively, and \( \Delta aBP \) is the change in \( aBP \) (and percentage decrease).
from 83 to 30mmHg (64%). Blood pressure recovered to 70mmHg. A second challenge reduced aBP to 18mmHg (74%) from which it did not recover.

A summary of the results for crude and live complexes is given in Table 4.5

4.3.2.5 IgG Complexes (IgGIC)

Injections of soluble IgGIC refers to the first supernatant obtained in the washing procedure. This obviously contained a greater concentration than supernatant 2 which, in turn, was more concentrated than supernatant 3. These latter 2 supernatants are denoted as such.

Bearing in mind the uncertain composition of these complexes, the results are as follows:

a) Control Rabbits

In rabbit C6, 1ml of soluble IgGIC provoked a biphasic response, raising aBP by 3.5% (113 to 117mmHg and reducing it by 12% (down to 99mmHg)) with associated hyperventilation. One ml of insoluble IgGIC (resuspended pellet, similar to crude complexes) reduced aBP by 36% (from 108 to 69mmHg) also with associated tachycardia and hyperventilation. A lower concentration of soluble IgGIC (supernatant 2) caused aBP to increase by 3.5% (113 to 117mmHg) and then fall by 3% (to 110mmHg).

In rabbit C7, 2ml of soluble IgGIC caused aBP to decrease by 32% (109 to 74mmHg) (fig. 4.12a). Two mls of supernatant 2 reduced aBP by 27% (94 to 69mmHg), whilst 2mls of supernatant 3 provoked virtually no response (104 to 103mmHg). This indicates that response is to some
Figure 4.12

The effects of IgG complexes in control rabbit C7. In a) the effect of 2ml of SOLUBLE IgGIC and b) 1ml of INSOLUBLE IgGIC.
degree dose-dependent. The rabbit was then injected with 1ml of insoluble IgGIC which produced only a 13% decrease in aBP (112 to 98mmHg) (fig. 4.12b). A further 1.7ml of soluble IgGIC decreased aBP by 34% (from 114 to 75mmHg). This indicates that unless the hypotension is produced by different mechanisms (depending on whether the IC are soluble or insoluble), refractoriness does not seem to occur.

Seventeen minutes after 200,000IU of Trasylol was infused (over 4 minutes), 2ml of soluble IgGIC caused a 38% decrease in aBP (104 to 64mmHg). The Trasylol did not have any effect here at all. A further 0.75ml of insoluble IgGIC reduced aBP by 25% (111 to 83mmHg).

In rabbit C8, 1ml of soluble IgGIC caused a 16% decrease in mean aBP (104 to 87mmHg). A further 2mls of the same solution of IgGIC produced a biphasic response, a 10% increase (98 to 108mmHg) and then a 16% decrease (98 to 82mmHg). Finally 1ml of insoluble IgGIC caused aBP to reduce by 14% (86 to 74mmHg).

When rabbit C9 was challenged with 1ml of soluble IgGIC there was a gradual but profound hypotensive response associated with a high pulse pressure and hyperventilation from which the rabbit did not recover.

Two mls of live trypanosome-IgG complexes injected into rabbit C10 produced an 18% decrease in aBP (from 90 to 74mmHg) (fig. 4.13). The washing supernatant produced a 6% decrease in aBP. A further challenge with 2ml of the live trypanosome complexes produced a 23% decrease in aBP (96 to 73mmHg). Two mls of the washing supernatant decreased aBP by 5%.
The response of the aBP of control rabbit C10 to injection of 2ml of live trypanosome-IgG complexes. The long injection time is because these complexes tend to agglutinate and may block the cannula if injected too quickly.

Figure 4.13

The effects of IgG complexes on the aBP of rabbit I9 (infected for 36 days), a) the effect of 1ml of INSOLUBLE IgGIC and b) of SOLUBLE IgGIC.

Figure 4.14
b) Infected Rabbits

In rabbit 19 (day 36 infection) 1ml of insoluble IgGIC caused a biphasic response (fig. 4.14a). From a mean aBP of 97mmHg there was an 8% increase (105mmHg) followed by a decrease of 2% (95mmHg). Two mls of soluble IgGIC caused a decrease in mean aBP from 104 to 73mmHg (30%) (fig. 4.14b). A weaker solution (supernatant 2) reduced aBP by 12% (90 to 79mmHg). Three minutes after 40,000IU of Trasylol, 1.8ml of soluble IgGIC caused a 26% decrease in mean aBP (89 to 66mmHg). Challenge with a weaker solution of IgGIC (supernatant 2) reduced aBP by 18% (77 to 63mmHg). Trasylol again had no effect on the hypotensive response.

One ml of soluble IgGIC injected into rabbit 13 (day 12 infection) caused a very large decrease in mean aBP from 96 to 32mmHg (67%) from which the rabbit did not recover (fig. 4.15).

Table 4.6 is a summary of the results for IgG-containing complexes.

4.3.2.6 IgM Complexes (IgMIC)

The responses to injected IgM complexes were quite different to those from other challenges.

Rabbit C11 reacted to 1ml of soluble IgMIC with a slight decrease in aBP from 99 to 92mmHg (7%). This was associated with very laboured breathing. A further injection of 1ml soluble IgMIC lowered aBP by 9% (102 to 93mmHg) (fig. 4.16a). One ml of insoluble IgMIC caused a 4% decrease in aBP (96 to 92mmHg) associated with coughing (fig. 4.16b).
<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Condition</th>
<th>aBP₀</th>
<th>Challenge</th>
<th>aBP₁</th>
<th>ΔaBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>control</td>
<td>113</td>
<td>1ml sol. IgIC</td>
<td>111</td>
<td>4 (-4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>99</td>
<td>-14 (12%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>108</td>
<td>1ml insol. IgIC</td>
<td>69</td>
<td>-39 (36%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>113</td>
<td>2ml sol. IgIC (supernat. 2)</td>
<td>117</td>
<td>4 (-4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>110</td>
<td>-3 (3%)</td>
</tr>
<tr>
<td>C7</td>
<td>control</td>
<td>109</td>
<td>2ml sol. IgGIC</td>
<td>.74</td>
<td>-35 (32%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94</td>
<td>2ml sol. IgGIC (supernat. 2)</td>
<td>69</td>
<td>-25 (27%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>104</td>
<td>2ml sol. IgGIC (supernat. 3)</td>
<td>103</td>
<td>-1 (1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>112</td>
<td>1ml insol. IgGIC</td>
<td>98</td>
<td>-14 (13%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>114</td>
<td>1.7ml sol. IgGIC</td>
<td>75</td>
<td>-39 (34%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200,000IU Trasylol, then</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>104</td>
<td>2ml sol. IgGIC 17 mins later</td>
<td>64</td>
<td>-40 (38%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>111</td>
<td>0.75ml insol. IgGIC</td>
<td>83</td>
<td>-28 (25%)</td>
</tr>
<tr>
<td>C8</td>
<td>control</td>
<td>104</td>
<td>1ml sol. IgGIC</td>
<td>.74</td>
<td>-17 (16%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>98</td>
<td>2ml sol. IgGIC</td>
<td>.108</td>
<td>10 (-10%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86</td>
<td>1ml insol. IgGIC</td>
<td>.82</td>
<td>-16 (16%)</td>
</tr>
<tr>
<td>C9</td>
<td>control</td>
<td>108</td>
<td>1ml sol. IgGIC</td>
<td>.74</td>
<td>-12 (14%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>maximal (died)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>control</td>
<td>90</td>
<td>2ml LIVE IgGIC</td>
<td>74</td>
<td>-16 (18%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>89</td>
<td>2ml supernat.</td>
<td>84</td>
<td>-5 (6%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96</td>
<td>2ml LIVE IgGIC</td>
<td>73</td>
<td>-23 (24%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94</td>
<td>2ml supernat.</td>
<td>89</td>
<td>-5 (5%)</td>
</tr>
<tr>
<td>I9</td>
<td>infected</td>
<td>97</td>
<td>1ml insol. IgGIC</td>
<td>105</td>
<td>8 (-8%)</td>
</tr>
<tr>
<td>day 36</td>
<td></td>
<td></td>
<td></td>
<td>95</td>
<td>-2 (2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>104</td>
<td>2ml sol. IgGIC</td>
<td>73</td>
<td>-31 (30%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90</td>
<td>2ml sol. IgGIC (supernat. 2)</td>
<td>79</td>
<td>-11 (12%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400,000IU Trasylol, then</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>89</td>
<td>1.8ml sol. IgGIC after 3 mins</td>
<td>66</td>
<td>-23 (26%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77</td>
<td>2ml sol. IgGIC (supernat. 2)</td>
<td>63</td>
<td>-14 (18%)</td>
</tr>
</tbody>
</table>

Table 4.6. The effects of IgG complexes on mean arterial blood pressure (aBP, mmHg) in control and infected rabbits. aBP₀ and aBP₁ are the values before and after the challenge, respectively, and ΔaBP is the change in aBP (and percentage decrease).
Figure 4.15 The response of rabbit I3 (12 day infection) to injection with 1ml of soluble IgGIC.
Note the scale is 5mm/minute.

Figure 4.16
The response of control rabbit Cll to injection with
a) 1ml of SOLUBLE IgMIC and b) 1ml of INSOLUBLE IgMIC.
In rabbit Cl2, 1ml of soluble IgMIC decreased aBP by 3% (from 71 to 69mmHg). However this was 3 minutes after injection whereas the response to other types of immune complexes generally occurred within 30 seconds to 2 minutes. One ml of insoluble IgGIC also caused a 3% decrease in aBP (from 70 to 68mmHg). This was associated with a great increase in pulse pressure and laboured breathing. This response was transient (about 10 seconds). This contrasts with the responses to IgGIC, crude complexes and trypanosome antigen which, as the figures show, were often of several minutes duration. Table 4.7 summarizes the data for challenge with IgM-containing IC.

4.3.2.7 Trasylol

A summary of the effects of Trasylol are shown in Table 4.8. There was no correlation between dose of Trasylol, nature of challenge or length of infection.
Table 4.7. The effects of IgM complexes on mean arterial blood pressure (aBP, mmHg) in control rabbits. \( aBP_0 \) and \( aBP_1 \) are the values before and after the challenge, respectively, and \( \Delta aBP \) is the change in aBP (and percentage decrease).

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Condition</th>
<th>( aBP_0 )</th>
<th>Challenge</th>
<th>( aBP_1 )</th>
<th>( \Delta aBP )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C11</td>
<td>control</td>
<td>99</td>
<td>1ml sol. IgMIC</td>
<td>92</td>
<td>-7 (7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96</td>
<td>1ml insol. IgMIC</td>
<td>92</td>
<td>-4 (4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>102</td>
<td>1ml sol. IgMIC</td>
<td>93</td>
<td>-9 (9%)</td>
</tr>
<tr>
<td>C12</td>
<td>control</td>
<td>71</td>
<td>1ml sol. IgMIC</td>
<td>69</td>
<td>-2 (3%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>1ml insol. IgMIC</td>
<td>68</td>
<td>-2 (3%)</td>
</tr>
</tbody>
</table>
Table 4.8 The effects of Trasylol on arterial blood pressure changes induced by injected trypanosome-derived material. No correlation was found between the effect of Trasylol and infection length (days) or type of challenge.

* slightly lower dose in 2nd challenge.
4.4 Discussion

The main difficulties arise in this study because of the qualitative rather than quantitative nature of the data. The method was based on that of Boreham and Wright (1976) although it could be considered to be somewhat crude.

4.4.1 Blood Pressure Measurements

Arterial blood pressure was significantly reduced in infected rabbits compared to uninfected control rabbits, but did not correlate with the disease duration. This agrees with the findings of Boreham and Wright (1976) and Yates (1978), shown with the present data in Table 4.9.

Yates (1978) also measured heart rate (HR) and cardiac output (CO), and found that as the infection progressed, hypotension was accompanied by increased CO and decreased HR (or bradycardia) and hence decreased total peripheral resistance (TPR) calculated as BP/CO (Table 4.10). Heart rate was measured in only one rabbit (day 23), but was found to be 288 beats/min which agrees with Yates (1978). Arterial blood pressure and HR in Boreham and Wright's (1976) infected rabbits were particularly depressed. Though this could possibly be related to the fact that these were rabbits with late infections (25-67 days), even the aBP and HR for controls were very low (Tables 4.9 and 4.10). Other workers have found normal rabbits aBP to be in the region of 90mmHg (e.g. Intaglietta et al, 1970) and HR as 223-300 beats/min (Stinnett et al, 1976).

The results from the present study and Yates (1978) are consistent with values in the literature.
<table>
<thead>
<tr>
<th>Control</th>
<th>Infected</th>
<th>L</th>
<th>sig.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12) 62.2</td>
<td>(12) 27.13</td>
<td>25-67 (39)</td>
<td>sig Boreham &amp; Wright (1976)</td>
<td></td>
</tr>
<tr>
<td>(15) 113 ± 2.9</td>
<td>(23) 80.88</td>
<td>12-26 (19.3)</td>
<td>0.5 Yates (1978)</td>
<td></td>
</tr>
<tr>
<td>(12) 95.75 ± 5.22</td>
<td>(9) 78.44 ± 7.02</td>
<td>9-36 (18.4)</td>
<td>Present study</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.9. Measurements of arterial blood pressure (aBP) in control and infected rabbits, in the form (n) mean ± SEM (where data is available). The column headed L gives the length of infection (in days) on the first line, with the mean duration of infection in the second. The column headed sig. gives the level of significance for the decrease in aBP with infection. For Boreham & Wright (1976), the difference was significant, but the level was not given.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Infected</th>
<th>L</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>(12) 116.3 ± 15.9</td>
<td>(12) 120.5 ± 24.2</td>
<td>25-67</td>
<td>Boreham &amp; Wright (1976)</td>
</tr>
<tr>
<td></td>
<td>(15) 321 ± 5.3</td>
<td>(23) 272.94</td>
<td>12-26</td>
<td>Yates (1978)</td>
</tr>
<tr>
<td></td>
<td>(1) 288</td>
<td>(23) 262.68</td>
<td>23</td>
<td>Present study</td>
</tr>
<tr>
<td>Cardiac output (ml/min)</td>
<td>(15) 205.4 ± 10.3</td>
<td>(23) 262.68</td>
<td>12-26</td>
<td>Yates (1978)</td>
</tr>
<tr>
<td>Total peripheral resistance</td>
<td>(15) 0.58 ± 0.04</td>
<td>(23) 0.32</td>
<td>12-26</td>
<td>Yates (1978)</td>
</tr>
</tbody>
</table>

Table 4.10. Cardiovascular parameters during *T.b.bruceti* infection in rabbits (see also table 4.9). Control and infected values are given in the form (n) mean ± error (the form of the error, i.e. SD or SEM, is not specified in either Boreham & Wright (1976) or Yates (1978)). The column headed L gives the length of infection (in days). Total peripheral resistance is blood pressure (mmHg) divided by cardiac output (ml-kg/min).
When microvascular flow velocities are reduced to less than 0.5 mm/sec, leucocytes adhere to the endothelium of the venules (Mayrovitz et al., 1977; Atherton and Born, 1973). Edeghere (1980) showed that in the cremaster muscle of rabbits infected with T.b. brucei leucocytes adhered to the venular endothelium completely blocking some venular lumens. Such blockages may lead to recruitment of other channels particularly preferential high flow channels or shunts (Schmid-Schonbein et al., 1980b; see chapter 1.7.3.2). By-passing the capillaries and capillary venules, which have the highest pressure gradients in the circulation (Lipowsky et al., 1978; Caro et al., 1978) will cause increased pressure in the venules. This may in part explain Goodwin's (1971) reports of venous congestion during the infection.

Attempts to measure venous pressure during the present study were not very successful. A high central venous pressure of 40 mmHg was found in one infected rabbit and the presence of a venous pulse in another.

Experiments using labelled albumin and red cells (chapter 2) indicated that blood volume increased (due to increased plasma volume) during the infection. This is consistent with the increased CO and decreased HR found by Yates (1978). The concurrent arterial hypotension suggests peripheral vasodilatation, as indicated by the decreased TPR (Yates, 1978). The hypotension could be a reflex-mediated response to increased blood volume. However, the conditions of low flow observed by Goodwin and Hook (1968) and present study, chapter 3, lead to the build up of metabolites. This in turn provokes local regulatory mechanisms to promote arteriolar dilatation. The release of vasoactive substances in response to the infection may also occur (Boreham and Wright, 1976; Boreham, 1977; Parry, 1980). The parasites themselves may also release vasoactive agents when dead or dying (Tizard et al., 1979) as the
injection of the partially disrupted trypanosomes in rabbit Cl showed.

The possible interactions of cardiovascular parameters during trypanosome infections is shown in figure 4.17.

Another well known feature of *T. b. brucei* in rabbits is the physical deterioration of large arteries and veins. They become exceedingly fragile, difficult to cannulate and may become less responsive to vaso-active stimuli. Boreham (1977) suggested that reaction to infusion into control rabbits of *in vitro* prepared IC was more severe than the reaction in the infected rabbits. In the present experiments, though limited in number, a significant negative rank correlation was found between length of infection and intensity of response to injected *in vitro* formed IC (crude, live and IgG). It is interesting to speculate that reduced vascular responsiveness is a general feature of the disease.

4.4.2 The Involvement of the Kinin/kallikrein System

Use of kallikrein-inhibitor Trasylol was intended to clarify the role, if any, of kinins in the hypotensive response to challenge with IC. Though Boreham and Wright (1976) found complete abolition of the hypotensive response to IC challenge, the present results indicated variable responses. The involvement of the kinin-kallikrein system cannot be inferred by the use of Trasylol only, because it inhibits the enzymatic action of other serine proteases (see 4.1) and does not inhibit the pharmacological actions of kallikrein itself.

The vasodilator effect of kallikrein (not inhibited by Trasylol) is mediated in part by prostacyclin (PGI$_2$) and is independent of kinin formation (Morita et al, 1984). As PGI$_2$ is secreted by vascular
FIG 4.17: Some cardiovascular parameters and their interaction during T. b. brucei infection in rabbits.

- Hyperviscosity
- Rouleaux formation
- Low microvascular blood flow
- Leucocyte adherence to venules
  - INFLAMMATION
  - Build up of metabolites

- Microvascular damage
  - Selective lumen blocking
  - Arterial hypotension
  - Local vasodilatation

- Fluid recruited from skej. muscle
- Tissue COP
- Protein exclusion
- Volume in interstitium
- BV
- PV
- CO
- HR
- Reflex mediated?
- BP (venous)
- Microvascular hypertension?
- Lymph flow
- Outflow of water and solutes into the tissues

- ? COP changes

- TPR
endothelium and is a potent vasodilator, it is possible that endothelial cell damage in trypanosomiasis (Edeghere, 1980) may alter the production or effect of PGI₂. This may have a role in the acute hypotension observed.

The observed differences in response to injection of bradykinin and challenge with IC may arise because:

a) the response to IC challenge is not due to activation of the kinin/kallikrein system.

b) other mediators may also play a role in the hypotension.

c) the response may reflect the difference between bolus injection and formation at discrete sites in the body.

Injection into conscious rabbits (via the marginal ear vein) of crude trypanosome IC caused severe constriction of the vasculature of both ears (Parry, 1980). This vasoconstriction is interesting in the light of work done on the response of rabbit's blood vessels to kinins. Kinins mediated vasoconstriction in rabbit ear, but vasodilatation in other vascular beds (Barabe et al, 1979). As trypanosome-derived IC can activate the kinin/kallikrein system both in vitro and in vivo (Parry, 1980), it may be inferred that there is some kinin/kallikrein involvement when IC challenge is administered. This would rule out (a) above.

The main reason for the difference in response to injection and IC challenge is likely to be due to the release of other endogenous mediators (b) in response to challenge. The effect of (c) is unknown.

The postulated lack of vascular reactivity as the infection progresses may be a facet of immunosuppression. Immunosuppression appears to be mediated via T cells, B cells and macrophages (see chapter 1.6.2)
but may also affect the mechanisms involved in the inflammatory response, for example, the HF dependent pathways, as suggested by Boreham and Wright (1976).

4.4.3 The Formation and Nature of the Complexes

The response to injection of trypanosome antigen alone into rabbits with previous exposure to the antigen (infected or sensitized) is presumably due to the formation of IC in vivo (Boreham and Wright, 1976; Boreham, 1977). No response was seen to injection of disrupted or freshly separated live trypanosomes in control rabbits (Boreham and Wright, 1976). However, a rapid transient fall in aBP (not more than 15%) was occasionally seen. This was attributed to vascular reflexes caused by blockage of small vessels. If this is so, then such a mechanism would be expected to occur when IC were injected or formed in vivo.

The large response to the partially disrupted parasites (in rabbit C1) is likely to be mainly due to release of phospholipase A, (forming vasoactive lipids) or other factors, shown in vivo (Hambrey et al, 1980) and in vitro (Tizard et al, 1979). The particulate nature of the disrupted trypanosomes may also be a factor in triggering in vivo vascular reactions. Colloidal carbon, a commonly used indicator of changes in microvascular permeability has been shown to increase permeability itself. This is effected by the particulate carbon (Williamson et al, 1984).

During an infection, after at least some destruction of trypanosomes in vivo, the rabbit would be expected to have antibody to both types of antigen (surface and common). Both infected and sensitized rabbits react strongly to injected trypanosome antigen (live organisms or disrupted dead ones) suggesting that in vivo complexes are formed. However, the
trypanosomes themselves may also have some effect.

When the antiserum (or separated IgG or IgM) was incubated with the disrupted trypanosomes the various complementary antibodies and antigens form IC. The mixture was crudely divided into soluble and insoluble fractions. The (insoluble) crude IC are similar to those used by Boreham and Wright (1976). These and both fractions of the IgGIC provoke hypotensive responses in control rabbits. When live trypanosomes make up the IC, only surface antigens would be expected to complex with the antibody. Some of the surface antigen may detach and form free complexes in solution, though as expected these latter have only a small effect (see rabbit C10, Table 4.6).

Because the antigen (Ag) was not titrated against antibody (Ab), the ratio of Ab:Ag could not be determined. Though care was taken to be consistent, the complexes are fairly heterogenous so no conclusions may be drawn as to the efficacy of different sized complexes in mediating hypotension.

The relative lack of response to IgM complexes was surprising in view of the importance of IgM in protective immunity to trypanosomes (Mitchell and Pearson, 1983; Longstaffe and Terry, 1982). Though the rabbits did not produce a hypotensive response to challenge with IgMIC, there was laboured breathing, coughing and increased pulse pressure.

The similarity of the in vivo response to injected crude IC and IgGIC resembles that of the in vivo formed IC. This suggests that the acute hypotensive response is mediated by IgG-containing complexes in vivo.
As already outlined above, there appears to be two major groups of immunogenic molecules in the trypanosome - surface variable antigens and the common or internal antigens (Gray, 1967). The results of agglutination and precipitation tests with the antiserum demonstrate that antibody to both types of antigen have been produced. With hindsight, it would have been useful to check the separated IgG and IgM for precipitation and agglutination activity. It is not certain that the IgM obtained was trypanosome-specific. It has also been suggested that IgM may decrease in activity when frozen (Herbert et al., 1980).

The mechanism involved in the acute hypotension provoked by IC is not necessarily the same as that causing the chronic hypotension during the disease. The chronic reduction in aBP may be due to physiological reflexes and/or inflammatory mechanisms.

Parry (1980), using a number of drugs (Trasylol, indomethacin, penicillamine, hydrocortisone, dexamethasone and cyclophosphamide) was unable to establish conclusively that kallikrein was directly activated by immune complexes during trypanosomiasis infection in vivo and was a significant feature of the disease.

The presence of circulating complexes (e.g. Lindsley et al., 1981; Parry, 1980; Rose et al., 1982) and IC deposition (e.g. Facer et al., 1978; Nagle et al., 1980; Murray et al., 1975) implies an immune complex mediated pathogenesis (see also chapter 1.6.1.2; 1.6.1.3 and 1.9).

T.b. brucei is predominantly a tissue parasite associated with generally low but intermittent parasitaemia, and it would be expected that antibody and antigen are largely compartmentalised in blood and extravascular space respectively. Complexes would then be formed.
whenever trypanosomes enter the bloodstream and also whenever antibody enters the tissue spaces and lymphatics (favouring IgG in the latter case as it is much smaller than IgM).
5. Erythropoietin

5.1 Introduction

The cause of anaemia in T. b. brucei infection is unclear and may be multifactorial (Jenkins et al, 1980). Haemolysis occurs (Jennings et al, 1974; Jenkins et al, 1980) and this may be immunological in nature (Amole et al, 1982; Herbert and Inglis, 1973). In view of the gross splenomegaly seen in the disease, non-specific activation of the splenic reticulo-endothelial is thought to have a role (McCrorie et al, 1980). Marked hyperplasia of erythroid tissue has been noted (Jenkins et al, 1980; Jenkins, 1980). Appearance of marked reticulocytosis during the infection would indicate a positive haemopoiesis (Jennings et al, 1974; Jenkins et al, 1980) but evidence is indirect.

Noyes et al (1982) measured erythropoietin levels during malarial infections. Anaemia in T. b. brucei infections is less severe than in malaria. However, studies on erythropoiesis are usually indirect so it was felt to be useful to obtain some direct measurements during a trypanosome infection.

5.2 Erythropoietin Assay

Erythropoietin is an asymmetrical glycoprotein hormone with a molecular weight which is undetermined but thought to be in the range 26,000 - 66,000. It is thought to be secreted by the kidney in response to an anoxic stimulus which may result from a reduction of circulating red cell concentration or direct oxygen deficiency. The generally accepted view is that erythropoietin exerts its controlling influence on erythropoiesis primarily by directing the differentiation of a
multipotential stem cell into erythroid precursors (Alpen and Cranmore, 1959).

The assay used to estimate erythropoietin (Ep) in the present study was based on that of Dunn et al (1975) and Napier et al (1977). Further modification were made to the basic technique by Napier and Evans (1980). The method involves the stimulation of erythroblast proliferation by Ep, measured by incorporation of $^{125}$I-deoxyuridine. The "Ep unit" is defined as the amount of Ep that would produce a response in the starved male Sprague-Dawley rat assay equivalent to that produced by 5 M cobaltous chloride (Goldwasser et al, 1958). The term milliunit (mu) used here is equivalent to 0.001 unit of the International Reference preparation of Ep supplied by the World Health Authority.

The assay was carried out by Dr. S.H. Reynolds of the National Blood Transfusion Service in Cardiff. The method she used appears in Appendix 1.

5.3 Animals

Six rabbits were injected with T.b. brucei 427 as described previously. Three rabbits acted as controls. Blood samples were obtained via the marginal ear vein. One ml. of blood was placed in a 2ml heparinized tube, the remainder used to obtain at least 5mls of serum or plasma for the assay.
5.4 Results

We found a moderate but significant decrease in haematocrit in infected rabbits compared to controls (see 2.3.1.1). Figure 5.1 shows the data obtained from the assay. The Ep levels in infected animals were not significantly different from those of controls. However, three of the infected animals which were examined more than once showed Ep levels which increased with duration of the infection.

5.5 Discussion

This was a pilot study carried out with an experimental assay. Although it was developed to detect human Ep, there did not appear to be any fundamental difference in the sensitivity of the assay for human and rabbit Ep (S.H. Reynolds - personal communication).

The results are inconclusive but together with the more indirect evidence of haemopoietic hyperplasia and reticulocytosis, it is possible that Ep increases to compensate for the anaemia. The marked erythroid hyperplasia seen in T.b. brucei infections in rabbits was seen to increase to a maximum at 18 days and decrease by 24 days (Jenkins, 1980) but sustained to at least 33 days in another study (Van den Ingh, 1976). The possible increase in Ep may reflect the progressive anaemia in the later part of the infection rather than the earlier part. As mentioned earlier, with the rise in blood viscosity, the anaemia may to some degree allow better perfusion of the tissues.

The main causes of anaemia appear to be haemolysis and sequestration in the spleen of the red cells. The response of the host is partly to increase stimulation of erythropoiesis. Late in the infection, the anaemia is probably that of "chronic disorders" (Jenkins, 1980).
Fig 5.1: Erythropoietin titres against duration of infection (days). Letters indicate individual rabbits. Joined points indicate observations from the same rabbit over the course of the infection.
6. Concluding Remarks

It is clear that the pathogenesis of trypanosomiasis in general and T.b. brucei infection in particular is exceedingly complex. Obviously, more information about the disease process can be gathered from experimental animal models than humans. Though the extrapolation from experimental animals to humans presents the usual problems of species differences, the overall pathogenesis of the disease in Rhodesian sleeping sickness appears similar to that in rabbits, dogs and monkeys.

There is abundant information on histological, immunological and clinical biochemical changes during trypanosomiasis, but relatively little on the pathophysiology of the disease. Poltera (1985) underlined the need for further work to unravel the pathogenesis of the disease, particularly of the cerebral stages. This is because of the toxicity of the drugs needed to combat the disease once CNS involvement has occurred. Vaccination would reduce the need for chemotherapeutic drugs and sophisticated molecular immunological techniques may yet provide us with a trypanosomiasis vaccine, as they appear to have done for the great scourge, malaria. But, the antigenic variation of trypanosomes is still a major problem.

By investigating some pathophysiological aspects of trypanosomiasis in the rabbit, I hope to contribute to the growing understanding of disease processes in general and trypanosomiasis in particular.

The progressive increases in vascular permeability to albumin during the infection were found to be most marked in heart, muscle, kidney and ear (tissues with continuous type microvessels) rather than being a generalized feature throughout the body. Lung, mesentery, aorta and
scrotal skin appeared less affected physiologically but the wide variation between rabbits may obscure potential changes.

A significant increase in albumin E/P of infected tissues at 30 minutes suggests that the rate of transport of albumin across the microvascular endothelium (permeability) is increased. After 120 minutes, significant increases in E/P in the infected tissues also tends to indicate that less protein is excluded from the interstitial matrix than in controls, allowing more tracer per unit weight of tissue.

The lack of peripheral oedema in the tissues studied (with the exception of the cremaster muscle) was surprising. However, there are mechanisms whereby the body can protect itself from oedema (Aukland and Nicolaysen, 1981). It is possible that in the first 2 stages of the disease, the increased permeability occurring in some body tissues is compensated for by increased lymph flow and concentration.

In the two tissues with discontinuous vascular endothelium (liver and spleen), macromolecular permeability may actually decrease during the infection. The extravascular accumulation of macromolecules is partly dependent on the permeability but also on lymph flow and the protein exclusion properties of the interstitium. With T.b. brucei being largely a tissue parasite and histological observations indicating often severe alterations in many tissues during the infection (as was demonstrated in mesentery), it is reasonable to propose that protein exclusion properties are changed. The parasites themselves may take up host albumin (Fairlamb and Bowman, 1980) which would cause retention of the tracer in the interstitium. This problem should be further investigated as it may provide a link between physiological transport processes in the host and biochemical aspects of the parasites. This particular pathway may
be useful for a novel drug delivery system (Gutteridge, 1985). In addition, an extensive study of regional tissue interstitium ought to be pursued. In the microcirculation field, many workers are investigating the role of interstitial properties in transport and homeostatic mechanisms.

Evidence provided by Barry and Emery (1984) and Bijovsky et al (1984), underlines the importance of the lymphatic system in the initial dissemination of trypanosomes within the host. The vascular endothelium, interstitium and lymphatic system are closely inter-related and are not easily viewed in isolation.

The emphasis in the development of the disease tends to be on how the body's own defense mechanisms appear to be detrimental. Whilst this is undoubtedly true, the effects of the parasites themselves must not be underestimated. Though the parasitaemia is often very low in Gambian trypanosomiasis and in the present model of T.b. brucei in rabbits, this does not reflect the total body parasite burden. The parasite metabolic products and proteolytic enzymes released during parasite breakdown are likely to be major features of host damage (Tizard et al, 1979; Hambrey et al, 1980; Huan et al, 1975; Richards et al, 1983). Production of vasoactive metabolites may account for the large decrease in arterial blood pressure found when partially disintegrated trypanosomes were injected into a control rabbit (chapter 4).

Parasite-derived substances may conceivably affect the osmotic balance across the endothelial wall providing a stimulus for solvent transport. The significant increase in plasma volume during the disease in rabbits may reflect osmotic changes. Calculated colloid osmotic pressure (COP) from the data of Facer (1976) and Goodwin and Guy (1973)
shows fluctuation over the course of the infection. Though the major
determinant of the normal plasma COP is usually albumin, other substances
present during trypanosome infection would be expected to contribute to
COP.

The finding of expanded plasma volume in the infected rabbits comp­
licates the interpretation of lowered plasma concentrations of various
constituents. Decreased concentration may be a passive result of the
plasma expansion. Conversely, increases would be underestimated.

Increases in plasma immunoglobulins are a consistent feature of
trypanosome infections. In rabbits, IgG increases 3-4 fold (Parry, 1980)
and appears to be the class of antibody involved in the acute hypotension
(see chapter 4) resulting from injection of trypanosome-derived IC
(Boreham and Wright, 1976). Though it appears that such hypotension is
in part mediated by the activation of the kinin/kallikrein system, the
chronic hypotension is likely to be of different aetiology. Baroreceptor
reflexes in response to increased plasma volume may also play a role.

Categorizing the severity of the infection proved a difficult task.
It was hoped that Cx-reactive protein would be of use in this respect
as the rabbits were largely between two and four weeks of the infection.
However, CxRP titre did not correlate with infection duration or E/P
ratios. The accumulation of extravascular albumin did correlate with
the infection duration though would not be practical in assessing severity.

The response of aBP to in vitro formed trypanosome-derived complexes
was negatively correlated with infection length suggesting lower vascular
responsiveness. This is also an interesting finding. It may be related
to the generalized hypotension but as the results were expressed in
terms of percentage decrease of aBP rather than actual decrease in mmHg, it may be a genuine finding of hyporesponsiveness.
Appendix I

Buffers and Solutions

Citrate buffer (pH 3.0)
0.1M Citric Acid (21.01g/l)
0.1M Na$_2$HPO$_4$ (di-hydrate - 17.80g/l)

At pH below 5, titrate citric acid with phosphate.

Evans Blue (T-1824)
0.14g Evans blue
made up to 20 mls with saline.

Evans Blue-Albumin
1.0g bovine serum albumin
0.14g Evans blue
made up to 20 mls with saline.

Mesenteric Buffer (Ring et al., 1978) (pH 7.4 at 37°C)
NaCl 8.01g
KCl 0.201g
CaCl$_2$ 0.265g
Tris base 2.25g
(Tris(hydroxymethyl)aminomethane) - SIGMA
made up to 1 litre with distilled water.
**Phosphate buffer (pH 8.0)**

\[
\text{Na}_2\text{HPO}_4 \quad 0.5\text{M - stock solution}
\]

\[
\text{Na}_2\text{HPO}_4 \quad 0.5\text{M - stock solution}
\]

Mix the 2 solutions to obtain the required pH then adjust to desired molarity.

**Phosphate-buffered Saline (PBS) (pH 7.2)**

\[
\text{NaCl} \quad 8\text{g}
\]

\[
\text{KCl} \quad 0.2\text{g}
\]

\[
\text{Na}_2\text{HPO}_4 \quad 0.008\text{M}
\]

\[
\text{KH}_2\text{PO}_4 \quad 0.2\text{g}
\]

Dissolve in 1 litre distilled water. Adjust to pH 7.4.

**Phosphate-glucose-saline (PGS) (pH 8.0)**

0.85% NaCl \hspace{1cm} 300ml

2.5% α-D-glucose \hspace{1cm} 400ml

0.2M Na₂HPO₄ \hspace{1cm} 285ml

0.2M NaH₂PO₄ \hspace{1cm} 15ml

to give 1 litre
Tris-HCl (pH 8.8)
Tris(Trizma) base 45.51g
1 M HCl ~ 66ml

Dissolve Tris in 100 ml of distilled water. Titrate to pH 8.8 with
1M HCl and adjust final volume to 250ml.

Tissue Culture Medium for erythropoietin assay.
Eagles MEM containing:

26.2m mole NaHCO₃
10.0µ mole ferric citrate
Benzylopenicillin 200µg/ml
Streptomycin 100µg/ml
6% serum

Foetal mouse liver cells at 2 x 10⁶/ml.

8.33% of a dilution of test or standard samples.
**Brilliant crescein, coomassie blue (stain)**

Brilliant crescein 250mg  
Coomassie blue 15mg

Brilliant crescein was dissolved in 100 ml 5% acetic acid and 3% trichloracetic acid at 60°C, cooled to room temperature and then the coomassie blue added.

**Erythropoietin Assay**

The serum/plasma samples were acidified and boiled (briefly) to denature the protein, erythropoietin being heat stable. After centrifugation (to remove denatured protein), dialysis and concentration, 2-fold serial dilutions were made. These dilutions were assayed using a foetal mouse liver cell culture (100% erythroid culture of $3 \times 10^6$ cells/ml) against a known standard (high potency serum of human origin) diluted similarly. Microtitre culture plates of well volume 150 μl were employed, with each dilution of standard or sample plus cell culture, occupying a separate well. After incubation at 37°C in 5% CO$_2$ in air for 20 hours, 0.05 μCi $^{125}$I-deoxyuridine was added and left for a further 4 hours. Cells were washed and harvested semi-automatically using a Skatron Harvester (Flow Labs.) then counted on an auto-logic γ counter (Abbot Labs.). The potency of the test sample was calculated using a parallel line assay method (Colquhoun, D. - Lectures in Biostatistics, Clarendon Press, Oxford, 1971) and expressed in μu/ml.
Figure A. The plasma levels of $^{125}\text{I}$-albumin in three groups of rabbits. The first reading is taken as 100%. Where error bars are shown, the data is mean ± SEM at that particular time. Single points without error bars are individual readings.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Infected</th>
<th>t</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>heart</td>
<td>7.29 ± 0.32</td>
<td>7.40 ± 0.25</td>
<td>0.27</td>
<td>109</td>
</tr>
<tr>
<td>abdominal muscle</td>
<td>0.89 ± 0.08</td>
<td>1.14 ± 0.07</td>
<td>2.37</td>
<td>100</td>
</tr>
<tr>
<td>ear</td>
<td>1.93 ± 0.21</td>
<td>2.27 ± 0.21</td>
<td>1.01</td>
<td>99</td>
</tr>
<tr>
<td>scrotal skin</td>
<td>1.38 ± 0.12</td>
<td>1.56 ± 0.09</td>
<td>1.20</td>
<td>99</td>
</tr>
<tr>
<td>cremaster muscle</td>
<td>3.08 ± 0.41</td>
<td>3.03 ± 0.32</td>
<td>-0.10</td>
<td>96</td>
</tr>
<tr>
<td>mesentery</td>
<td>6.51 ± 0.57</td>
<td>8.08 ± 0.54</td>
<td>1.82</td>
<td>98</td>
</tr>
<tr>
<td>aorta</td>
<td>15.27 ± 1.36</td>
<td>16.59 ± 0.70</td>
<td>0.95</td>
<td>86</td>
</tr>
<tr>
<td>lung</td>
<td>18.81 ± 0.64</td>
<td>21.31 ± 0.78</td>
<td>2.14</td>
<td>109</td>
</tr>
<tr>
<td>kidney</td>
<td>8.16 ± 0.38</td>
<td>7.30 ± 0.29</td>
<td>-1.82</td>
<td>130</td>
</tr>
<tr>
<td>liver</td>
<td>9.48 ± 0.71</td>
<td>8.93 ± 0.37</td>
<td>-0.76</td>
<td>105</td>
</tr>
<tr>
<td>spleen</td>
<td>20.45 ± 1.80</td>
<td>31.81 ± 3.10</td>
<td>2.38</td>
<td>85</td>
</tr>
</tbody>
</table>

Table B. Tissue blood fractions, assuming $H_t = H_b$. The values are given as percentages, and are in the form $(n)$ mean ± SEM. The $t$ values and the associated degrees of freedom are given on the right.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Infected</th>
<th>t</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>heart</td>
<td>(41) 11.67 ± 0.52</td>
<td>(70) 11.84 ± 0.40</td>
<td>0.27</td>
<td>109</td>
</tr>
<tr>
<td>abdominal muscle</td>
<td>(40) 1.21 ± 0.11</td>
<td>(62) 1.55 ± 0.09</td>
<td>2.37</td>
<td>100</td>
</tr>
<tr>
<td>ear</td>
<td>(35) 2.84 ± 0.31</td>
<td>(66) 3.32 ± 0.31</td>
<td>1.01</td>
<td>99</td>
</tr>
<tr>
<td>scrotal skin</td>
<td>(34) 2.17 ± 0.19</td>
<td>(67) 2.47 ± 0.15</td>
<td>1.20</td>
<td>99</td>
</tr>
<tr>
<td>cremaster muscle</td>
<td>(34) 4.09 ± 0.54</td>
<td>(64) 4.02 ± 0.43</td>
<td>-0.10</td>
<td>96</td>
</tr>
<tr>
<td>mesentery</td>
<td>(34) 9.62 ± 0.84</td>
<td>(66) 11.94 ± 0.81</td>
<td>1.82</td>
<td>98</td>
</tr>
<tr>
<td>aorta</td>
<td>(30) 21.49 ± 1.92</td>
<td>(58) 23.35 ± 0.99</td>
<td>0.95</td>
<td>86</td>
</tr>
<tr>
<td>lung</td>
<td>(39) 23.29 ± 0.79</td>
<td>(72) 26.38 ± 0.97</td>
<td>2.14</td>
<td>109</td>
</tr>
<tr>
<td>kidney</td>
<td>(48) 24.96 ± 1.16</td>
<td>(84) 22.32 ± 0.87</td>
<td>-1.82</td>
<td>130</td>
</tr>
<tr>
<td>liver</td>
<td>(36) 16.93 ± 1.27</td>
<td>(71) 15.95 ± 0.65</td>
<td>-0.76</td>
<td>105</td>
</tr>
<tr>
<td>spleen</td>
<td>(27) 33.14 ± 2.92</td>
<td>(60) 51.56 ± 5.02</td>
<td>2.38</td>
<td>85</td>
</tr>
</tbody>
</table>

Table C. Tissue blood fractions, with the correction for $H_\tau$. The values are given as percentages, and are in the form (n) mean ± SEM. The $t$ values for the difference between control and infected and the associated degrees of freedom are given on the right of the table. Note that the correction for $H_\tau$ does not alter the $t$ values, i.e. the values here are the same as in table B.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>$H_c$</th>
<th>±</th>
<th>SEM</th>
</tr>
</thead>
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<td>0.2485</td>
<td>±</td>
<td>0.0149</td>
</tr>
<tr>
<td>abdominal wall muscle</td>
<td>0.2950</td>
<td>±</td>
<td>0.0278</td>
</tr>
<tr>
<td>ear</td>
<td>0.2710</td>
<td>±</td>
<td>0.0132</td>
</tr>
<tr>
<td>scrotal skin</td>
<td>0.2509</td>
<td>±</td>
<td>0.0113</td>
</tr>
<tr>
<td>cremaster muscle</td>
<td>0.2974</td>
<td>±</td>
<td>0.0073</td>
</tr>
<tr>
<td>mesentery</td>
<td>0.2701</td>
<td>±</td>
<td>0.0195</td>
</tr>
<tr>
<td>aorta</td>
<td>0.2825</td>
<td>±</td>
<td>0.0173</td>
</tr>
<tr>
<td>lung</td>
<td>0.3220</td>
<td>±</td>
<td>0.0230</td>
</tr>
<tr>
<td>kidney</td>
<td>0.1333</td>
<td>±</td>
<td>0.0165</td>
</tr>
<tr>
<td>liver</td>
<td>0.2234</td>
<td>±</td>
<td>0.0163</td>
</tr>
<tr>
<td>spleen</td>
<td>0.2478</td>
<td>±</td>
<td>0.0224</td>
</tr>
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</table>

Table D. Tissue haematocrits calculated from the three control experiments of 30 minutes duration.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Duration</th>
<th>Control</th>
<th>Infected</th>
<th>t</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>heart</td>
<td>30</td>
<td>(11) 4.91 ± 0.28</td>
<td>(23) 8.07 ± 0.24</td>
<td>7.78</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>(12) 6.47 ± 0.29</td>
<td>(25) 9.45 ± 0.44</td>
<td>4.45</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>( 9) 9.02 ± 0.40</td>
<td>( 9) 11.76 ± 0.38</td>
<td>4.94</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>( 9) 8.64 ± 0.40</td>
<td>(13) 15.14 ± 1.05</td>
<td>4.97</td>
<td>20</td>
</tr>
<tr>
<td>abdominal muscle</td>
<td>30</td>
<td>( 9) 0.36 ± 0.03</td>
<td>(18) 0.55 ± 0.03</td>
<td>4.65</td>
<td>25</td>
</tr>
<tr>
<td></td>
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<td>(12) 0.46 ± 0.04</td>
<td>(23) 1.07 ± 0.13</td>
<td>3.27</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>(10) 0.66 ± 0.04</td>
<td>( 9) 1.22 ± 0.20</td>
<td>2.86</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>( 9) 0.65 ± 0.04</td>
<td>(12) 0.95 ± 0.07</td>
<td>3.50</td>
<td>19</td>
</tr>
<tr>
<td>ear</td>
<td>30</td>
<td>( 9) 1.27 ± 0.13</td>
<td>(20) 2.48 ± 0.43</td>
<td>1.85</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>(12) 1.53 ± 0.29</td>
<td>(25) 3.37 ± 0.31</td>
<td>3.75</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>( 6) 1.79 ± 0.13</td>
<td>( 9) 2.85 ± 0.38</td>
<td>2.21</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>( 8) 2.34 ± 0.39</td>
<td>(12) 6.39 ± 1.58</td>
<td>2.04</td>
<td>18</td>
</tr>
<tr>
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Table E. Extravascular $^{125}$I-albumin ratios (x10$^2$) for control and infected tissues, assuming $H_t = H_b$. The data is given in the form (n) mean ± SEM, with the tissue type and duration of experiment (in minutes) given at the left, and t values and degrees of freedom at the right.
Table E (cont.). Extravascular $^{125}$I-albumin ratios (x10^2).

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Table F. Extravascular $^{125}$I-albumin ratios (x10²) for control and infected tissues, correcting for $H_i$. The data is given in the form (n) mean ± SEM, with the tissue type and duration of experiment (in minutes) given at the left, and t values and degrees of freedom at the right.
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<th>Tissue</th>
<th>Duration</th>
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<th>Infected</th>
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Table F (cont.). Extravascular $^{125}$I-albumin ratios (x10²).
References


Landis, E.M. (1927). Microinjection studies of capillary permeability; relation between capillary pressure and rate at which fluid passes through the walls of single capillaries. Am.J.Physiol 82, 217-238.


