

A study of the mechanisms of immunity to Pasteurella  
haemolytica infection.

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## DECLARATION

The work reported in this thesis was part of a larger project concerned with investigations of respiratory diseases of sheep, and consequently some of the experimental findings were obtained in collaboration with my colleagues at the Moredun Research Institute. Nevertheless, most of the work presented in this thesis was carried out by myself and where conjoint experiments were necessary, a full role was played in design of the experiments and the interpretation of the results.

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Immunity to Pasteurella haemolytica was studied using models of P. haemolytica infection in both lambs and mice.

Immunisation of specific pathogen-free lambs with a vaccine containing a sodium salicylate extract of P. haemolytica type A1 protected against challenge with P. haemolytica type A1, superimposed upon infection with parainfluenza type 3 virus. Lymphocyte transformation responses of peripheral blood leucocytes from vaccinated lambs increased markedly after challenge. The passive transfer of hyperimmune serum, produced by repeated vaccination of lambs with the same vaccine, did not protect recipients from challenge. Serum from vaccinated lambs had a bacteriostatic effect upon P. haemolytica in vitro, although this serum failed to facilitate phagocytosis of P. haemolytica by alveolar macrophages in vitro.

Two models of P. haemolytica infection in mice were investigated. Intranasal infection of mice with P. haemolytica caused lesions in the lung 3 days later. Bacteria were cleared from the lung by 48 hours after infection, and because similar lesions were produced by the introduction of



heat-killed P. haemolytica, this model was not investigated further.

P. haemolytica introduced to mice intraperitoneally in gastric mucin multiplied in the liver and spleen. The effect of vaccination with various extracts of P. haemolytica upon growth in the liver was investigated. Vaccines containing sodium salicylate extracts of P. haemolytica types A1, A6, A8, A11 and T10 protected against challenge with homologous strains, and little cross-protection between the various strains was observed.

The model of intraperitoneal challenge of mice was used to investigate the commercial Pasteurella vaccine, Carovax. When given 12 or 24 hours before challenge, the vaccine protected against challenge with P. haemolytica type A1, and also Escherichia coli, indicating that protection was non-specific. When the vaccine was given 1 hour, 48 hours, 7 days or 14 days before challenge with P. haemolytica type A1, no such protection was observed.

An enzyme-linked immunosorbent assay was developed for detection of antibodies to P. haemolytica in the sera of vaccinated mice. There was considerable cross-reaction

between sera from mice immunised with sodium salicylate  
extracts of the different types of P. haemolytica.

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Abbreviations used in text

AM	= alveolar macrophage
BA	= bovine albumin
C	= control
CFA	= complete Freund's adjuvant
cfu	= colony forming unit
CMI	= cell-mediated immunity
DW	= distilled water
E	= absorbance
ELISA	= enzyme-linked immunosorbent assay
gelatin-Hanks	= Hank's balanced salt solution, containing 0.1% gelatin
HBSS	= Hank's balanced salt solution
H.E.	= haematoxylin and eosin
HKO	= heat-killed organisms
HuRBC	= human red blood cells
IFA	= incomplete Freund's adjuvant
IHA	= indirect haemagglutination
IN	= intranasal
IP	= intraperitoneal
IT	= intratracheal
IV	= intravenous
MIF	= macrophage migration inhibition factor
MV	= missing value

NB	=	nutrient broth
ND	=	not done
n.t.	=	not treated
OD	=	optical density
PBS	=	phosphate-buffered saline
PBS/Tween	=	phosphate-buffered saline containing 0.05% Tween 20
PI3	=	parainfluenza type 3
PVP	=	polyvinyl pyrrolidone
RBC	=	red blood cells
SC	=	subcutaneous
SE	=	standard error
SED	=	standard error of difference of means
S.I.	=	stimulation index
SPF	=	specific pathogen free
SSE	=	sodium salicylate extract
V	=	vaccinate

Pneumonia is an important lesion in a high proportion of sheep necropsied in the United Kingdom (Veterinary Investigation Diagnosis Analysis II Report, 1977). Although the figures quoted in the report may not accurately reflect the total losses to the industry from this cause, it is likely that the disease is of some considerable economic consequence. The pneumonia, at least in its terminal stages, is commonly associated with bacteria, and the organism which may be isolated from the lungs with the greatest frequency is Pasteurella haemolytica. Organisms with similar characteristics may be isolated from a variety of other clinical conditions of sheep, the most important of which is a septicaemic disease. P. haemolytica is not restricted to sheep, and the organism is also associated with pneumonia or "transit fever" of cattle in both North America and Europe (Carter, 1954).

The characteristics of P. haemolytica have been extensively studied and the species is subdivided into types on the basis of certain biochemical and antigenic features. Particular types of the organism are predominantly associated with pneumonia and other types

with septicaemia in sheep. This has been extensively reviewed recently (Carter, 1967; Thompson, 1973) and only the salient points will be considered at a later point in this introduction.

Vaccines aimed at prevention of pasteurellosis are available in this country\*, but nevertheless P. haemolytica is still implicated in many outbreaks of disease. This indicates that either the use or uptake of the vaccine is inadequate, or that the immunising powers of current vaccines are insufficient to protect against the field disease. The latter possibility is supported by the general unsatisfactory nature of vaccine trials (Pyke, 1966).

Development of P. haemolytica vaccines has been hampered in the past by the absence of a suitable system to reproduce the disease either in sheep or in laboratory

\*Carovax, Wellcome Foundation Ltd., Beckenham, England;  
Ovivac-P, Pasticidin, Hoechst Pharmaceuticals, Hounslow,  
Middlesex, England.

animals. This has not only hindered progress towards satisfactory immunisation, but it has also prevented development of a full understanding of the immune mechanisms involved in protection against the organism. These aspects will also be discussed in this introduction.



Characterisation of *P. haemolytica*

The taxon, *P. haemolytica* was introduced by Newsom and Cross (1932) to designate an organism associated with disease in cattle, although the first description of the organism had appeared eleven years earlier (Jones, 1921). In a study of bacteria isolated from cases of bovine pneumonia Jones distinguished three groups of organisms by the criteria of colonial morphology, biochemical and serological reactions. Group I organisms were short, non-motile, gram-negative bipolar cocco-bacilli. Colonies on blood agar were flat and translucent, 3-5 mm in diameter after 48 hours incubation, and were haemolytic. Cultures were indole-negative. These organisms, and Group II and III organisms, which were indole-positive and non-haemolytic, were referred to as *Bacillus boydii*. Similar organisms, isolated from pneumonic lambs were named *Pasteurella ovisepitum* (Spray, 1923). In a study of gram-negative cocco-bacilli isolated from sheep and cattle, Newsom and Cross (1932) defined a "typical" and an "atypical" group. The "typical" group was similar to Groups II and III of Jones (1921). The "atypical" haemolytic group resembled

Jones' Group I and was designated Pasteurella haemolytica.

(The "typical" group has since been classified as Pasteurella multocida).

P. haemolytica is defined as a gram-negative, non-motile rod, which is aerobic and facultatively anaerobic. It is oxidase-positive and attacks sugars by fermentation, but does not produce gas (Cowan and Steel, 1974). Cultural reactions of P. haemolytica are diverse, and in view of this, certain criteria for identification of the species were adopted by Biberstein, Gills and Knight (1960). These are listed here, in diminishing order of importance.

1. Haemolysis on blood agar. The extent of haemolysis varies considerably from strain to strain, and sometimes is only detectable directly underneath the colonies. However, haemolysis is a useful feature by which P. haemolytica and P. multocida can be distinguished, as the latter is non-haemolytic.

2. Absence of indole production. This is a uniform characteristic of P. haemolytica, in contrast to P. multocida, which is indole-positive.
3. Growth on McConkey's agar. P. haemolytica grows well on McConkey's agar, whereas P. multocida does not.
4. Failure to reduce methylene blue milk.

In addition to these characteristics, P. haemolytica is oxidase-positive, a useful feature which distinguishes it from most of the Enterobacteriaceae, which are oxidase-negative.

Satisfactory fermentation reactions with these bacteria are difficult to achieve, as growth on many fermentation media is poor, and results obtained are often inconclusive (Thompson, 1973). Smith (1959 B) classified strains of P. haemolytica from sheep into two biotypes, designated A and T. Biotype A strains appeared to be associated with pneumonia, and biotype T strains

with septicaemia of lambs. These biotypes were later characterised further on the basis of colonial morphology, fermentation reactions, growth curves and sensitivities to antibiotics (Smith, 1961). Biotype A strains formed small grey colonies on sheep blood agar, and fermented arabinose within 7 days, but not trehalose by 10 days, while biotype T strains formed larger colonies with brownish centres and fermented trehalose within 2 days, but not arabinose within 10 days. Biotype A strains lost viability rapidly in ageing broth cultures and were more sensitive to penicillin and tetracycline than biotype T strains.

A variety of methods have been used in attempts to subdivide P. haemolytica serologically. Early studies established that P. haemolytica was antigenically distinct from P. multocida (Jones, 1921; Newsom and Cross, 1932), whereas studies concerned mainly with P. haemolytica revealed one (Tweed and Edington, 1930), two (Florent and Godbille, 1950), or three serotypes (Montgomerie, Bosworth and Glover, 1938), by means of agglutination reactions. Biberstein, Gills and Knight (1960) attempted to subdivide the species on the basis of somatic antigens

by direct agglutination tests, using autoclaved bacterial cells. Strains from sheep and cattle derived from a variety of clinical conditions, pneumonia, septicaemia, arthritis and mastitis, and from the nasopharynx of healthy individuals, were examined by this method. These strains, which would be expected to cover a wide spectrum of types of P. haemolytica, fell into two main groups (I and II) on the basis of these and additional fermentation tests.

P. haemolytica possesses a soluble, heat-stable capsular component, which has been used successfully to subdivide the species into several serotypes. An indirect haemagglutination (IHA) test, using this as antigen was developed by Biberstein, Gills and Knight (1960). The test involved sensitisation of bovine red blood cells (bovine RBC) with soluble antigens from P. haemolytica and their agglutination with antisera raised in rabbits against selected strains. This IHA reaction divided the species into eleven sharply defined types, and cross-reactions between types were minimal.

A variety of other IHA tests have also been developed

to subdivide P. haemolytica. Carter (1956) attempted to classify 51 strains of P. haemolytica isolated from cases of shipping fever in Canada. The soluble polysaccharide from these strains was adsorbed onto type O human red cells (HuRBC) which were then agglutinated in the presence of dilutions of specific immune sera raised against the test strains in rabbits. All strains were found to be serologically homogeneous and totally distinct from P. multocida in this study. This test was modified by Cameron (1966). Guinea pig red cells were used instead of HuRBC, and it was found that clearer agglutination patterns were obtained if the red cells, after treatment with antigen, were suspended in dilute normal rabbit plasma. In this study, the IHA test was employed to determine the titres of sera from sheep immunised with a known strain of P. haemolytica, rather than to type unknown strains.

The IHA test of Biberstein, Gills and Knight (1960) has been widely used. As larger numbers of strains were typed, it became apparent that occasionally a batch of antigen prepared from a particular strain of P. haemolytica would be agglutinated by 2 or 3 antisera (Biberstein, 1965).

In some cases this was due to a mixture of types in the original culture, but in others there were cross-reactions between different types. It was demonstrated that these were due to minor antigens, which did not interfere with the typing procedure. The technique was modified by Biberstein and Thompson (1966) who substituted Perspex trays of 1 ml capacity for the Wasserman tubes previously used. A 12th serotype was discovered in this study. Later, the test was adapted to a microtitre system (Shreeve, Biberstein and Thompson, 1972).

Smith's discovery of the biotype A and biotype T strains of P. haemolytica seemed to relate to the two divisions of the species later suggested by Biberstein, Gills and Knight (1960). To confirm this, a selection of strains of P. haemolytica, representing each serotype was classified as either biotype A or T on the basis of fermentation tests (Biberstein and Gills, 1962). A reasonable degree of correlation was found between the two methods of classification. In general, Smith's biotype A (1959 B) correspond to Group I of Biberstein, Gills and Knight (1960) and biotype T corresponded to Group II. An exception was serotype 2, which was classified as biotype A by Smith, and is included in Group II of .

Biberstein, Gills and Knight (1960). Serotypes 1, 2, 5, 6, 7, 8, 9, 11 and 12 are now considered to belong to biotype A and serotypes 3, 4 and 10 to biotype T.

An investigation of nucleic acid homologies between biotypes A and T of P. haemolytica showed a high degree of homology between two biotype A strains, but the homology between a biotype A and a biotype T strain (30-50%) was less than that often obtained for interspecies hybridisations (Biberstein and Francis, 1968). Electrophoresis in polyacrylamide gels of the constituent proteins of each serotype of P. haemolytica gave patterns which could be used to subdivide the species into two groups which broadly corresponded to the biotypes A and T (Thompson and Mould, 1975). The available evidence suggests that the subdivision of P. haemolytica into these two groups is a valid one, and some workers feel that there are grounds for regarding the biotypes as separate species (Smith and Thal, 1965). This has not been resolved, so for the sake of brevity strains of P. haemolytica will be referred to as "type A1" and "Type T3", etc.



The distribution of *P. haemolytica* types amongst healthy  
sheep

The recognition of different types has assisted the study of epidemiology of *P. haemolytica* in healthy flocks of sheep. *P. haemolytica* has been isolated frequently from the upper respiratory tract of apparently healthy sheep (Biberstein and Thompson, 1966; Biberstein, Shreeve and Thompson, 1970). A survey of normal flocks showed that the same types occur in the USA and Great Britain, with the possible exception of type A12, which was not identified in the USA (Biberstein and Thompson, 1966). Type A2 strains were predominant in Great Britain, whereas some types well represented in the USA, such as 5, 8 and 9 were apparently rare in Great Britain. Type A strains outnumbered type T strains in the nasopharynx of healthy sheep. In contrast, *P. haemolytica* was isolated from the tonsils of 95 of 100 adult sheep, 65% of these isolates being type T (Gilmour, Thompson and Fraser, 1974). Sixty-four of 100 nasopharyngeal swabs from these sheep yielded *P. haemolytica* and only 6% of these were of type T.

A 12 month survey showed that the proportion of sheep

carrying P. haemolytica intranasally increased markedly in late autumn and spring (Biberstein, Shreeve and Thompson, 1970). Prevalence of the different types varied from month to month, and flock to flock. There was no simple relationship between incidence of carriers and weather changes. It is interesting that, although the flocks in this survey remained healthy, the two peak periods of nasal carriage of P. haemolytica correlated with the known pattern of enzootic pneumonia in the region.

Young lambs reared with their ewes acquired an increasing number of types as they grew older (Shreeve and Thompson, 1970). The first typable colonies were detected 48 hours after birth. In contrast, few typable strains were recovered from lambs removed from their dams at birth, and it appears likely that intimate and prolonged contact with adult sheep may facilitate colonisation of the lamb with P. haemolytica.

Diseases of sheep associated with *P. haemolytica*

1. Septicaemia

Fatal septicaemia of lambs, in which death is sudden and often not preceded by other symptoms, was first described by Stamp, Watt and Thomlinson (1955). Although no quantitative epidemiological study of septicaemic pasteurellosis has been made, this disease is an important cause of death.

Post-mortem findings include congestion of the trachea and bronchi with blood-stained, frothy fluid, distension of the capillaries and venules of the lung, with oedema in the alveoli and interlobular septa. However, the lungs are seldom pneumonic. Lymph nodes are enlarged and haemorrhagic. Haemorrhagic exudative inflammation is found on the abomasal mucosa, and there is blood splashing on the visceral peritoneum, in the neck and beneath the parietal pleura. *P. haemolytica* can be cultured in large numbers from the heart blood, lung, liver, kidneys and spleen. Generalised septicaemia of lambs under three months of age is predominantly associated with type A strains (Smith, 1960 A; Biberstein and Thompson, 1966). In contrast,

100% of septicaemias in lambs over three months of age are due to type T strains (Biberstein and Thompson, 1966).

Type T strains of P. haemolytica are normally found as commensals in the tonsils of healthy sheep (Gilmour, Thompson and Fraser, 1974), and no convincing explanation has yet been offered as to why this organism should suddenly become pathogenic. The syndrome occurs in all ages of sheep, at all times of the year, but would appear to be more common in hoggs during September, October and November (Gilmour, 1978). This period coincides with movement of sheep from high to low ground pastures for the winter or the start of folding on rape or turnips. The first deaths often occur within a few days of a flock being moved. Deaths are sporadic and stop after several days. The mortality rate may be as high as 20% and, surprisingly, deaths tend to be amongst sheep in the best of condition. The factors which predispose sheep to this condition have yet to be identified. The primary site of invasion of P. haemolytica is not known. It has been suggested that P. haemolytica may enter the blood via the respiratory tract, perhaps as a secondary invader, but Stamp, Watt and Thomlinson (1955) found no evidence to support this. In several cases where pneumonic lesions

were present, it was considered that these were secondary to bacterial thrombi in the pulmonary vessels.

## 2. Pneumonia

One of the earliest outbreaks of pneumonic pasteurellosis of sheep to be described was in Wales (Montgomerie, Bosworth and Glover, 1938). These authors referred to the disease as "enzootic pneumonia", which by strict definition means a pneumonia of low incidence, but which is constantly present in a given community, so the term is not a specific one. However, in this country "enzootic pneumonia" has come to be the term applied to pneumonia of sheep associated with P. haemolytica.

Outbreaks of acute pneumonic pasteurellosis usually begin suddenly, with sheep dying or becoming severely ill with obvious respiratory disease. Other sheep may exhibit signs of mild respiratory involvement, such as coughing and oculonasal discharge. Mortality during such acute outbreaks rarely exceeds 10% (Gilmour, 1978). In the acute phase of the disease, pleurisy and pericarditis are usually present, with a greenish gelatinous exudate over the pericardium, extending into the anterior mediastinum.

Also, large volumes of straw-coloured pleural fluid with fibrin clots are observed. In hyperacute cases the lungs are enlarged and oedematous with large bright red or purple areas. Such areas are solid, oedematous and haemorrhagic.

Histologically, the lungs show necrosis of the alveoli and the alveolar spaces are filled with fluid and abundant gram-negative cocco-bacilli. The interlobular septa are widened by oedema, and the capillaries are distended with fluid and red blood cells. Spindle-shaped cells with basophilic nuclei, called "oat cells" are present in the lung lesions and are a characteristic feature of this pneumonia. Large numbers of these cells surround areas containing necrotic alveoli.

Pneumonic pasteurellosis is almost exclusively associated with type A strains of P. haemolytica (Smith, 1961), which are found in numbers exceeding  $10^7$  per gram of tissue in the lung lesions.

The production of experimental pneumonia in sheep has proved to be difficult. Dungal (1931) produced typi-

cal pneumonia in three sheep by intratracheal (IT) inoculation of 9 hour cultures of P. haemolytica. One animal showed symptoms, while pneumonia in all three was demonstrated at necropsy. Salisbury (1957) failed to reproduce pneumonia by inoculation of infected material by a variety of routes. Partial success in transmitting pneumonia with infected chorioallantoic suspension along with P. haemolytica culture was achieved by Downey (1957). Intrabronchial inoculation of large numbers of P. haemolytica type A, which had been cultured in the peritoneal cavities of mice, produced pulmonary infections resembling the acute form of pneumonic pasteurellosis (Smith, 1964). Intrabronchial inoculation of similar numbers of heat-killed P. haemolytica did not produce lesions.

The strains which cause disease normally reside as commensals in the nasopharynx. Therefore, there must be factors which predispose sheep to disease. These are not fully characterised, but one suggestion is that prior infection with Parainfluenza type 3 (PI3) virus may render sheep more susceptible to invasion of the lungs with P. haemolytica. Experimental pneumonic pasteurellosis in specific pathogen free (SPF) lambs has been successfully

produced by the establishment of a PI3 virus infection prior to exposure to an aerosol of P. haemolytica (Sharp, Gilmour, Thompson and Rushton, 1978). This finding lends support to the theory that PI3 virus infection might be an important predisposing factor in the etiology of pneumonic pasteurellosis. However, it is recognised that other factors may well exist which play an important part in rendering sheep susceptible to P. haemolytica infection. Synergism between respiratory viruses and bacteria in the respiratory tract is well documented, and has been reviewed by Loosli (1968).

The question of whether a single factor or many factors predispose sheep to pasteurellosis is important. If one factor only compromises the host and allows P. haemolytica to invade, there would be little place for a P. haemolytica vaccine. Control of the disease could be more effectively achieved by vaccination against the primary infecting agent, or, if an environmental factor were involved, by its exclusion by management. However, if there were many factors which predispose sheep to pasteurellosis, an effective pasteurella vaccine would be most useful, as control of the disease by vaccination to a single agent would be easier than attempting to exclude



a multiplicity of other factors.

Requirements for a suitable vaccine against P. haemolytica  
and methods for study of vaccine efficacy

Commercial vaccines against pasteurellosis are available, but these are of questionable value (Gilmour, 1978). Despite their use, pasteurellosis is widespread. These vaccines contain only a limited number of types of P. haemolytica, and there is as yet no evidence to suggest that cross-protection between the different types occurs. Field trials of vaccine preparations are difficult to assess, due to the sporadic nature of the disease. Many attempts have been made to reproduce pneumonic pasteurellosis experimentally (Dungal, 1931; Salisbury, 1957; Downey, 1957), and it is only recently that this has been consistently successful. Due to the ubiquitous distribution of the organism, the vast majority of sheep have prior experience of it, and have antibodies in their serum to P. haemolytica detectable by the IHA test (Gilmour, personal communication). Such sheep may therefore be unsuitable for experimental purposes. Laboratory animals are not susceptible to P. haemolytica infection which has made it difficult to use these species either for initial vaccine evaluation or for determination of the immunological features of the disease in these animals.

Experimental reproduction of pasteurellosis in sheep

1. Septicaemia

Successful reproduction of P. haemolytica septicaemia in lambs was achieved by the intravenous (IV) injection of cultures derived from clinical cases of septicaemia (Stamp, Watt and Thomlinson, 1955). Intraperitoneal (IP) injection of P. haemolytica type A cultures into three week old lambs resulted in death within 12 hours from acute fibrinous peritonitis (Smith, 1960 A). Adult sheep were not susceptible to doses which killed young lambs.  $10^{9.9}$  colony forming units (cfu) of P. haemolytica type T given IV to both 6 month old lambs and adult sheep resulted in death (Smith, 1960 B). Lambs which survived challenge with smaller numbers of organisms had positive blood cultures at 24 hours, whereas P. haemolytica was recovered from only one adult sheep. However, the number of organisms required to produce septicaemia is very large especially in adult sheep, and is only slightly less than the number of dead bacteria which would cause death. This tends to suggest that hitherto undefined factors are involved in the development of the field disease. As septicaemia often follows a change of

pasture, it was thought that acidosis, caused by the sudden change of diet, may be a predisposing factor. For this reason, a state of acidosis was induced in sheep by dietary manipulation, prior to challenge with P. haemolytica. These sheep were no more susceptible to the disease, implying that acidosis alone is not responsible (Gilmour, personal communication).

## 2. Pneumonia

Early attempts to reproduce pneumonia in sheep with P. haemolytica achieved only limited success (Dungal, 1931; Salisbury, 1957; Downey, 1957). The most promising method was that of Smith (1964) which involved intrabronchial inoculation of large numbers of P. haemolytica type A. Pulmonary infections similar to acute pneumonic pasteurellosis were produced.

Most conventionally reared sheep have circulating antibodies to P. haemolytica, which may affect the consistent reproduction of the disease. Serum immunoglobulin levels in newborn lambs prior to sucking are generally very low, indicating that there is little or no transfer of immunoglobulin across the placenta (Smith, Wells, Burrells

and Dawson, 1976). Therefore, lambs which have not received colostrum might be expected to give reproducible results in challenge experiments. Five of ten SPF lambs exposed to an aerosol of P. haemolytica type A1 developed pneumonia indistinguishable from natural pneumonic pasteurellosis (Gilmour, Thompson, Smith and Angus, 1975).

PI3 virus has been associated with ovine respiratory disease and after experimental inoculation, produces a transient mild disease (Hore and Stevenson, 1969). SPF lambs inoculated with both PI3 virus and P. haemolytica consistently develop severe clinical disease in a high proportion of animals (Sharp, Gilmour, Thompson and Rushton, 1978). The illness and lung lesions in lambs which received both agents were more severe than in lambs inoculated with either agent alone. Previous combined infections with PI3 virus and P. haemolytica were less severe and less consistent (Biberstein, Shreeve, Angus and Thompson, 1971). The success of the method of Sharp, Gilmour, Thompson and Rushton (1978) was attributed to the use of SPF lambs. Lambs not hysterectomy-derived and reared in isolation may well have encountered P. haemolytica before inoculation. The time interval between PI3 virus infection and challenge may also be important. Three days elapsed

between viral and bacterial challenge in the method of Biberstein, Shreeve, Angus and Thompson (1971) whereas the interval was 7 days in the method of Sharp, Gilmour, Thompson and Rushton (1978).

This method has been successfully used for the investigation of P. haemolytica vaccine preparations (Gilmour, Martin, Sharp, Thompson and Wells, 1979). In a series of experiments, the immunogenicity of sodium salicylate extracts (SSE) of P. haemolytica was tested. These experiments will be discussed more fully elsewhere in this thesis. Briefly, protection was demonstrated against challenge with the homologous strain when the antigen was an extract of either P. haemolytica type A1 or A6. A heat-killed preparation (HKO) of P. haemolytica type A2 afforded some protection in one experiment, but in another, where the vaccine contained extracts of types A1 and A6, and HKO of type A2 no protection against challenge with P. haemolytica type A2 was demonstrated. This model of infection has proved extremely useful for the study of P. haemolytica vaccines. Later in this thesis, experiments are described in which this model of infection was used to investigate the mechanisms of immunity to P. haemolytica in sheep pneumonia.

Models of infection with *P. haemolytica* in  
laboratory animals

Experiments with sheep, in particular SPF lambs, are costly and the number of animals available is limited and seasonal. A model of *P. haemolytica* infection in a laboratory animal would have the advantages of relative low cost and ready availability of greater numbers of experimental animals throughout the year. Unfortunately, infection of small animals with *P. haemolytica* has proved difficult.

Many workers have tried in vain to infect small animals by various routes. These include cats, dogs, hens, pigeons, rabbits and guinea pigs (Dungal, 1931; Lovell and Hughes, 1935; Beveridge, 1937; Montgomerie, Bosworth and Glover, 1938). *P. haemolytica* is also relatively non-pathogenic for mice, unless very large numbers of organisms are administered, and the lethal effects may well be due to the direct action of the endotoxin present in such a large inoculum (Smith, 1958).

In contrast, the related organism, *P. multocida* is exceptionally virulent for mice. When introduced by

various routes, the organism grows rapidly and causes fatal septicaemia (Collins, 1973). The  $LD_{50}$  of P. multocida strain 5A was shown to be 1-2 organisms when given IV, IP or by the subcutaneous (SC) route. Mice vaccinated IV with two doses of a heat-killed P. multocida suspension were fully protected against challenge 7 days later by IV, IP and SC routes. Partial protection against aerogenic challenge was also achieved (Collins and Woolcock, 1976). Passive immunisation studies showed that protection was humorally mediated. The manner by which the immune host eliminates this extremely virulent organism has been studied by following the rate of growth of P. multocida in mice given various treatments and challenged by a variety of routes.

Another member of the genus Pasteurella, Pasteurella pneumotropica, is a natural infectious agent of mice and is often isolated from the respiratory tract. Its incidence in mouse colonies is high, but mice can carry the organism and show no signs of clinical disease (Hoag, Wetmore, Rogers and Meier, 1962). However, P. pneumotropica is a potential pulmonary pathogen, and is frequently implicated in outbreaks of pneumonia in mouse colonies (Brennan,



Fritz and Flynn, 1965; Jawetz, 1950). Mice challenged by aerosol with P. pneumotropica alone eliminated the organism from the lungs within 7 hours (Jakab and Dick, 1973). In contrast, intrapulmonary killing of P. pneumotropica was delayed in mice which had been previously infected with Sendai virus, the maximum effect being achieved when the interval between virus infection and challenge with P. pneumotropica was 6 days. It was shown that previous immunisation with Sendai virus completely prevented subsequent virus infection, and thus the synergistic effect. This model has proved useful in the investigation of synergism between viral and bacterial infections.

Smith (1958) developed two successful methods of challenge with P. haemolytica in mice. Intracerebral inoculation of organisms in casein hydrolysate solution resulted in multiplication of the organisms and death. The bacteria were usually confined to the brain and meninges, and the susceptibility of individual mice varied considerably. Brain trauma induced in mice by intracerebral inoculation of starch-saline solution, in conjunction with IV infection with P. haemolytica gave 100% mortality, provided a suitable challenge dose was used.

To establish infection in mice with P. haemolytica given by the intraperitoneal route, very large numbers of organisms are needed. This can be considerably reduced if gastric mucin is incorporated into the inoculum (Smith, 1958).

It is accepted that mucins can enhance the virulence of organisms which are weakly or non-pathogenic (Reviewed by Olitzki, 1948). Examples include type III pneumococcus and Klebsiella pneumonia injected IP into mice, Bacillus anthracis injected IP into rats (Nungester, Jourdonais and Wolf, 1936) and Candida albicans injected IP into mice (Scherr, 1953). Although the viscosity of mucin evidently has a physically protective role towards the bacteria, other factors which act upon the host, rather than upon the organism, are probably of equal, if not greater importance. It has been suggested that mucin depletes serum of properdin, a protein which plays an important role in the alternative pathway of complement activation, thus lowering the resistance of the animal to infection. However, an investigation by DeWitt (1958) showed that there was no direct correlation between anti-properdin activity in vitro and the infection-promoting ability of mucins.

The IP infection of mice with P. haemolytica incorporated into gastric mucin has proved to be a most valuable model for the study of this organism. Deaths occur within 48 hours of infection, after the development of septicaemia. Smith (1958) demonstrated that mice could be passively protected against infection with rabbit antisera, and this was later developed into the "passive immunity test". By injecting mice IP with sera from sheep which have been vaccinated against P. haemolytica and then challenging them, some measure of the protective capacity of the sera can be determined (Gilmour, personal communication). Mice can also be protected against challenge by active immunisation (Smith, 1959 A). Biberstein and Thompson (1965) used this model to investigate the roles of capsular and somatic antigens in immunity to P. haemolytica. Mice were vaccinated with bacterins from strains of P. haemolytica which possessed various combinations of somatic and capsular antigens, and were subsequently challenged with homologous and heterologous strains. The resulting pattern of protection suggested that capsular antigens play the major role in determining the specificity of the immunity conferred, with the somatic antigens only secondarily involved.

However, a later report (Knight, Biberstein and Allison, 1969), in which similar experiments were undertaken with a larger number of animals, cast doubt upon some of these original conclusions. The importance of the capsular antigens was confirmed, but anomalies were found. In two experiments, the highest degree of protection was shown when the immunising and challenging strains shared no demonstrable antigenic factors. It was suggested that there may exist factors other than the capsular and somatic antigens demonstrable by simple laboratory typing, which determine the specificity of immunity.

A model for intranasal (IN) infection of mice with P. haemolytica has recently been developed (Rushton, 1978). Three days after IN inoculation of  $10^{6.6}$ - $10^{7.6}$  cfu, under anaesthesia, a non-fatal pneumonia developed. Investigations using this model will be described later in this thesis.

The pathology and bacteriology of both septicaemia and pneumonia of sheep associated with P. haemolytica are well characterised. However, the immune mechanisms of the sheep to this organism are at present ill-defined, and an effective vaccine against pasteurellosis has yet to be

produced. The work to be described in this thesis is an investigation into some aspects of this problem, using models of infection with P. haemolytica in both sheep and mice, in an attempt to elucidate the important features of the immune response to P. haemolytica.

Bacteriological techniquesStrains of *P. haemolytica*

All strains of *P. haemolytica* used in this study were of ovine origin. Strains of types A1, A2, A5, A6, A7, A8, A9 and A11 had been isolated from cases of sheep pneumonia. Strains of types T3, T4 and T10 were from cases of septicaemia in lambs, and the strain of type A12 was originally isolated from a case of mastitis in a ewe. These strains were stored either freeze-dried, or in 0.5 ml aliquots of broth culture at  $-70^{\circ}\text{C}$ . When required, an aliquot was removed from storage and inoculated into 50 ml nutrient broth (NB - Oxoid\* CM67) and incubated overnight at  $37^{\circ}\text{C}$ . The culture was streaked onto sheep blood agar (Oxoid\* CM55, with 15% citrated ovine blood) to check purity, and single colonies were used as inocula for further broth cultures.

Strain of *Escherichia coli*

The strain of *E. coli*, B188, was kindly provided by Dr. H. Williams Smith. It was of bovine origin,

\*Oxoid Ltd, Basingstoke, Hampshire, England.

serotype O78:K80:H<sup>-</sup>, and was invasive but did not produce enterotoxin (Smith and Huggins, 1976). E. coli was stored in a similar manner to P. haemolytica.

#### Enumeration of bacteria

Viable counts were carried out by the method of Miles, Misra and Irwin (1938). Appropriate 10-fold dilutions were made in peptone water (Oxoid L37) and duplicate 20 µl samples of these spotted onto sheep blood agar plates. Viable counts were read after incubation overnight at 37°C.

A method was devised whereby the number of organisms in a suspension could be estimated by measuring absorbance in a colorimeter (Vitatron UPS Photometer). Each strain of P. haemolytica was inoculated into 300 ml of NB and incubated at 37°C for 18 hours, washed twice in 0.15 M NaCl, and a range of dilutions were made which gave optical densities (OD) to cover the range of the colorimeter. The OD of the suspensions at 400 nm were measured. Viable counts were also performed on these preparations and standard curves of numbers of viable organisms against OD were plotted (Figs. 1-4). These graphs allowed inocula

Figure 1 Standard curves of P. haemolytica types  
A6, A7 and A8 against OD at 400nm

Figure 2 Standard curves of P. haemolytica types  
A1, A2 and A5 against OD at 400 nm



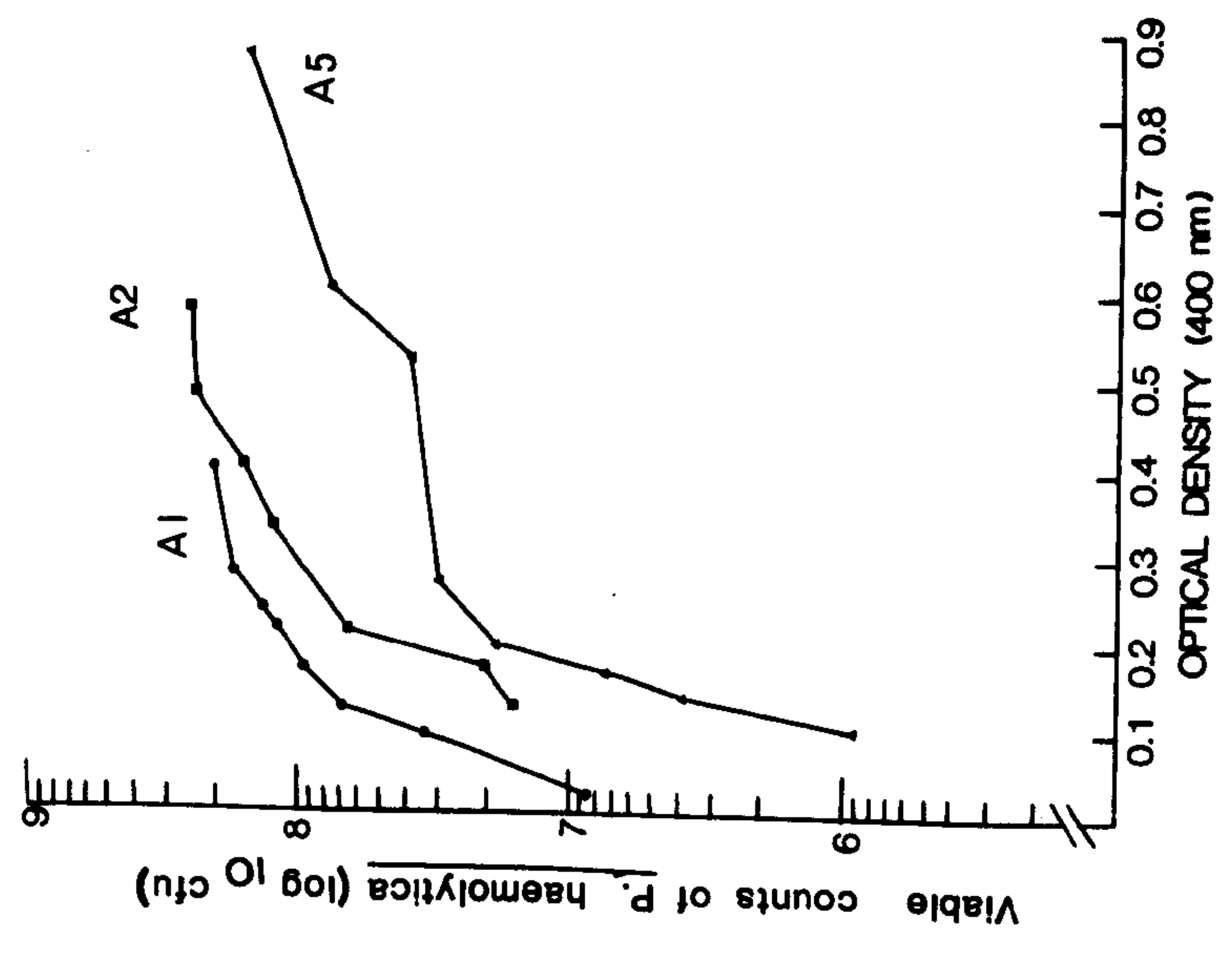
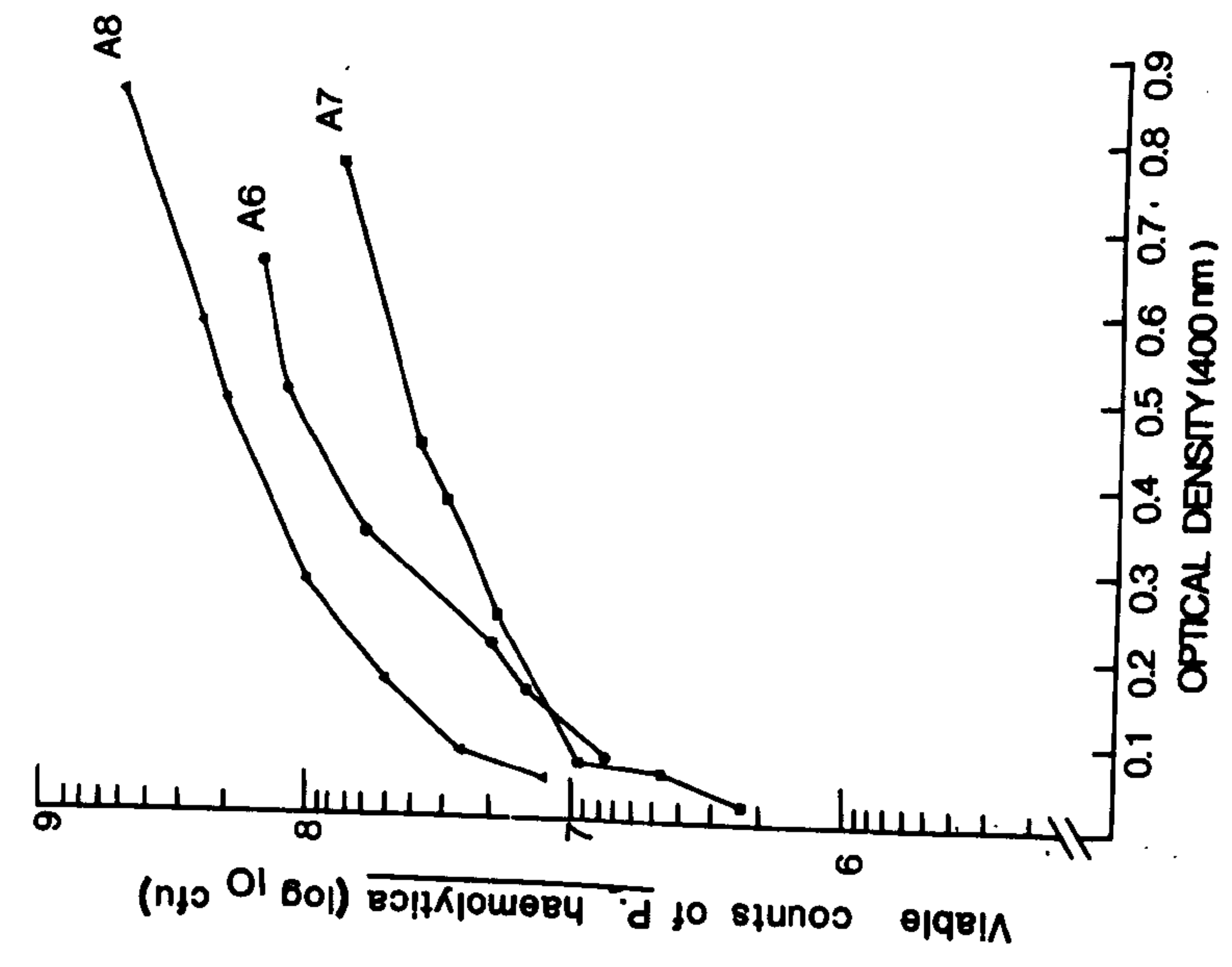
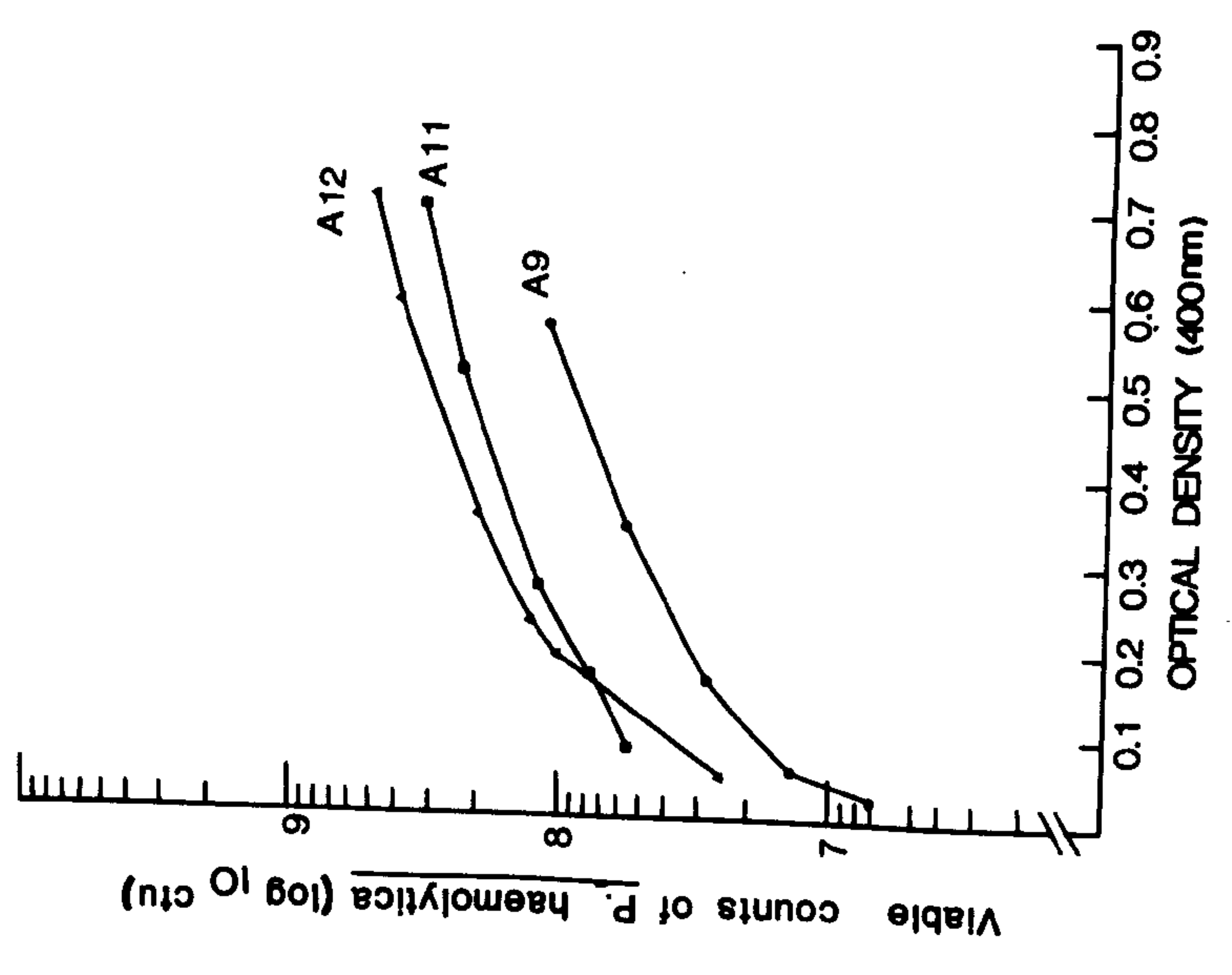
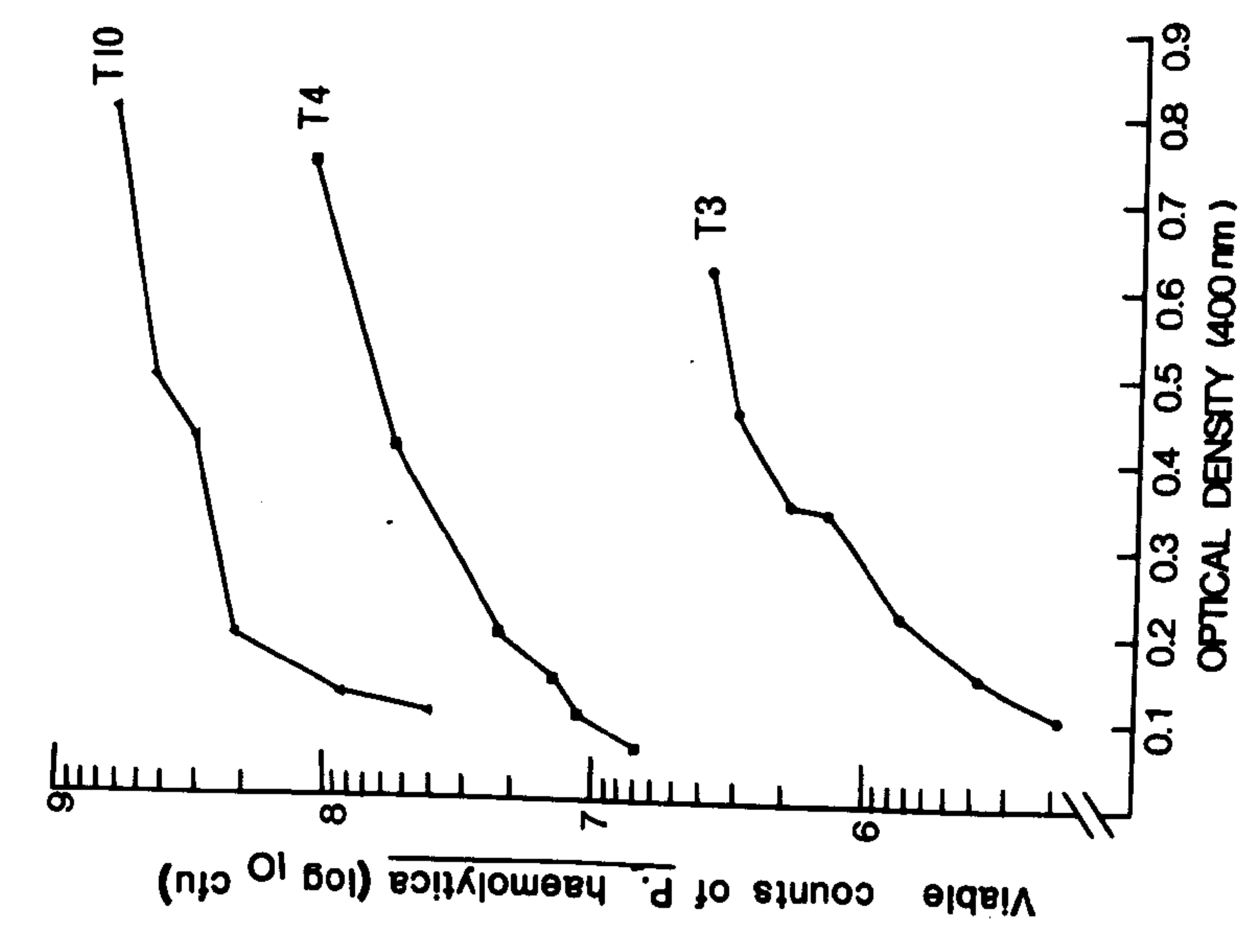


Figure 3 Standard curves of P. haemolytica types  
T3, T4 and T10 against OD at 400nm

Figure 4 Standard curves of P. haemolytica types  
A9, A11 and A12 against OD at 400 nm



to be adjusted to a determined density prior to administration; viable counts were however obtained for all bacterial cultures standardised in this manner.

## Laboratory animal techniques

### Mice

Unless otherwise stated, mice used in experiments were of the C57 black strain, bred at the Moredun Institute. Mice were of both sexes, and were 4-6 weeks old at the start of experimentation. Ten mice were housed in each cage. They were fed Modified Diet 41B (Laboratory Animal Diets, Herbert C. Styles (Bawdley)Ltd., for Oxoid Ltd.), and given water ad lib.

### Inoculations

#### 1. Subcutaneous

SC inoculations into mice were performed without anaesthesia. Mice were injected under the loose skin of the flank, using a 25 gauge needle. When more than one injection per mouse was required, the first was given on the right flank, and the second on the left.

#### 2. Intraperitoneal

Mice were injected IP without anaesthesia, using a 26 gauge needle. Injection was into the abdomen, slightly

to the left of the umbilicus.

### 3. Intranasal

Narcosis in mice was usually induced with halothane (Fluothane, ICI Ltd., Alderley Park, Cheshire), inhaled from saturated cotton wool beneath a false floor in a bell jar. When the level of narcosis was correct, mice were recumbent, insensitive and breathing evenly and shallowly. Mice were removed from the bell jar, were held upright and droplets of culture or saline (0.05 ml), which were inhaled rapidly, placed on the external nares. The mice recovered from anaesthesia within a few minutes.

## Vaccines

The experimental P. haemolytica vaccines used in much of this work were prepared from either SSE or HKO of various strains of P. haemolytica.

## Antigens

### 1. Preparation of P. haemolytica SSE

The extracts used in Chapters 1 and 2 were kindly provided by Dr. N. J. L. Gilmour. An aliquot of the required strain of P. haemolytica was removed from storage, inoculated into 50 ml nutrient broth and incubated at 37°C overnight. The culture was checked for purity and serotype identity at this stage. Two pre-warmed 1.5 l volumes of nutrient broth were each inoculated with 15 ml of this culture and incubated with agitation at 37°C for 6 hours. Viable counts were performed and the bacteria were sedimented by centrifugation at 12000 g for 20 minutes at 4°C. The cells were resuspended in 300 ml sterile 1.0 M sodium salicylate solution and agitated at 37°C for 3 hours. This procedure removed from the cells the outer layers.

The bacterial cells were removed by centrifugation at 28000 g for 40 minutes at 4°C. The supernate was further clarified by centrifugation at 40000 g for 30 minutes at 4°C (Spinco Model L, SW24 rotor). The extract was then dialysed for 48 hours against phosphate-saline buffer (0.02 M sodium phosphate, 0.03 M sodium chloride; pH 7.6) at 4°C, concentrated by ultrafiltration through a Diaflo X100A membrane (Amicon Corp., Lexington, Mass., USA) to approximately 20 ml, then freeze-dried and stored until required.

The method of sodium salicylate extraction was modified slightly in vaccines used in Chapters 4 and 5. The organisms were grown, and sedimented by centrifugation. Thereafter the pellet was resuspended in 0.15 M saline and stored overnight at 4°C. The following morning, the cells were sedimented and resuspended in 300 ml M sodium salicylate solution and shaken at 37°C for 3 hours. The extract was clarified as before, and concentrated to approximately 20 ml by ultrafiltration. A gelatinous deposit often present on the surface of the filter was removed by gently scraping with a glass rod, and added to the concentrate. The concentrated SSE, and an aliquot of the filtrate from ultrafiltration were each dialysed for



48 hours at 4°C against dilute phosphate-saline buffer and then dialysed against distilled water (DW) for 72 hours. The dry weight of each preparation was determined, and the extract was adjusted to 1.5 mg dry matter / ml, using the dialysed filtrate as diluent. Preparations were stored in small aliquots at -20°C until required.

## 2. Preparation of heat-killed *P. haemolytica*

Two 1.5 l volumes of *P. haemolytica* were grown in the same manner as employed in the preparation of SSE. The organisms were sedimented by centrifugation, resuspended in a minimal volume of phosphate-buffered saline (PBS), pH 7.2 and incubated at 60°C in a water bath for 90 minutes. The preparation was then checked for sterility and freeze-dried.

### Preparation of vaccines

The antigenicity of SSE of *P. haemolytica* type A6 in five adjuvants has been compared (Wells, Gilmour, Burrells and Thompson, 1979). Sheep were inoculated with antigen in either incomplete Freund's adjuvant (IFA), complete

Freund's adjuvant (CFA), IFA containing water-soluble extract of Mycobacterium tuberculosis, an aluminium hydroxide gel adjuvant, or a vaccine in which the aluminium hydroxide gel was incorporated into IFA. The last-named vaccine induced significantly higher titres of antibody against P. haemolytica type A6 as measured by an IHA test than did any of the other vaccines. In addition, local reactions at the site of inoculation with this vaccine were not severe. For these reasons, most of the vaccines used in this study were prepared by adsorption of the antigen onto aluminium hydroxide, followed by emulsification in mineral oil.

#### Adsorption of antigens onto aluminium hydroxide

SSE (1.5 mg/ml) and HKO (10 mg/ml) were adsorbed onto an aluminium hydroxide gel adjuvant (Alhydrogel, Superfos, Copenhagen, Denmark) after determination of the optimal dilution of Alhydrogel, according to the manufacturer's recommendations. When antigen is mixed with Alhydrogel at the optimal dilution, flocculation occurs. For each antigen a titration was carried out using a range of Alhydrogel concentrations with a standard amount of antigen (Table 1). The Alhydrogel was diluted 10-fold

TABLE 1

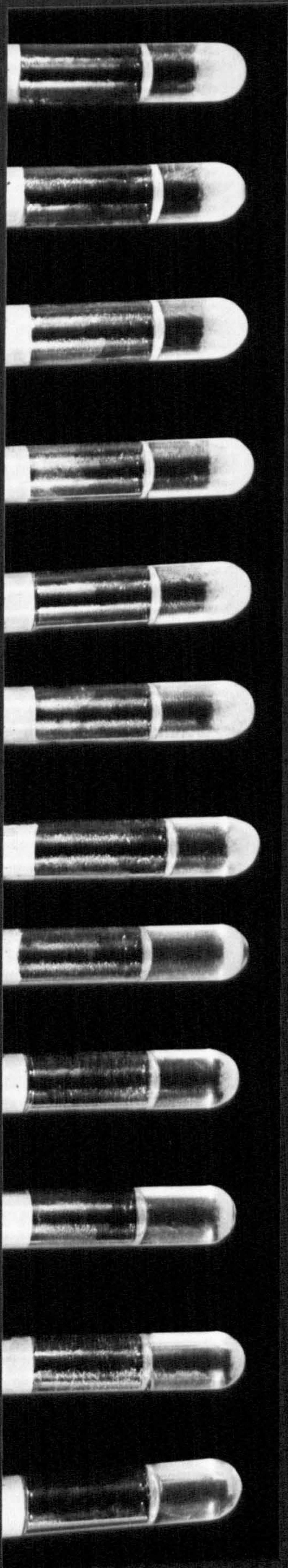
DETERMINATION OF OPTIMAL CONDITIONS FOR ADSORPTION OF ANTIGENS

TUBE	1	2	3	4	5	6	7	8	9	10	11	12
ANTIGEN (mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DW (mL)	0.55	0.5	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.05	-
1/10 ALHYDROGEL (mL)	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5	0.55	0.6

in DW for this purpose and added to antigen in Wasserman tubes. After rapid mixing, the tubes were left at room temperature for 20-30 minutes. The first tube in the series in which a marked flocculation occurred, with a clear supernate, was taken as having the optimal concentration of Alhydrogel for that antigen (Plate 1).

Emulsification of adsorbed antigen with mineral  
oil

The required volume of antigen adsorbed onto Alhydrogel at the optimal concentration was prepared, and left at room temperature for 30 minutes to ensure adequate flocculation. This was forced from a syringe fitted with a fine gauge needle into an equal volume of sterile Bayol F (Esso Ltd., New Jersey, USA) containing 10% Arlacel A (Sigma Chemical Company, St. Louis, USA). The vaccine was emulsified in a tissue homogeniser. To check that the resultant emulsion was a water-in-oil emulsion, and not an oil-in-water emulsion, vaccine was dropped from a pipette onto water. If the drop remained discrete, the emulsion was of the desired type. Vaccines were kept at 4°C until required. On occasion, the emulsion separated during storage, and it was necessary to re-homogenise the



preparation.

Bovine albumin vaccine

In several experiments, it was necessary to use a placebo vaccine which did not contain any P. haemolytica antigen. This was made by adsorbing a solution of 0.1% bovine albumin (BA) (Bovine albumin (Fraction V) Miles Laboratories Ltd., Slough, England) onto Alhydrogel, at the optimal Alhydrogel concentration, and emulsifying this with an equal volume of sterile Bayol F containing 10% Arlacel A.

Techniques employed by collaborators

Lymphocyte transformation responses to *P. haemolytica*  
antigens

Lymphocytes were obtained by layering a volume of heparinised (Heparin BP, Evans Medical, Liverpool, England) sheep blood onto an equal volume of a Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden)/Triosil (Triosil 440 - Nyegaard & Co., Oslo, Norway) mixture (specific gravity 1.075) and centrifuging the unmixed fluid at 500 g for 30 minutes at room temperature. The lymphocytes, which formed a band at the Ficoll/Triosil-plasma interface, were removed and washed 3 times with Hank's Balanced Salt Solution (HBSS) containing 5 iu/ml of heparin before resuspension at  $1 \times 10^6$  cells / ml in RPMI 1640 medium (Gibco-Biocult, Paisley, Scotland) supplemented with  $5 \times 10^{-5}$  M 2-mercaptoethanol and 20% autologous plasma. Twenty microlitres of a dilution of *P. haemolytica* type A1 SSE was added to wells of sterile flat-bottomed microtitre plates, and 20  $\mu$ l of sterile medium added to wells used for non-stimulated control cultures. 200  $\mu$ l of lymphocyte suspension was added to each well (ie.  $2 \times 10^5$  cells/well). All cultures were set up in triplicate. Plates were incubated at 37°C in a moist atmosphere for

5 days in a sealed box. Eighteen hours before termination of the cultures, 1  $\mu\text{Ci}$  of methyl  $^3\text{H}$ /thymidine (specific activity 5 Ci/mole) (Radio-Chemical Centre, Amersham, England) in 20  $\mu\text{l}$  of RPMI medium was added to each well. At the end of the culture period, cells were adsorbed onto glass-fibre filter discs with a semi-automatic cell harvester (Cryotech, Abingdon, England). Dried discs were immersed in 1 ml scintillation fluid in vials and counted in an automatic liquid scintillation counter. Lymphocyte proliferative responses to P. haemolytica type A1 were expressed as stimulation indices (S.I.)

$$\text{S.I.} = \frac{\text{Counts per minute due to incorporation of } ^3\text{H}\text{-thymidine into lymphocyte culture containing } \underline{\text{P. haemolytica}} \text{ antigen}}{\text{Counts per minute due to incorporation of } ^3\text{H}\text{-thymidine into unstimulated lymphocyte cultures}}$$

#### Histological examination of mouse lungs

Blocks were prepared from formol-fixed tissues, dehydrated in alcohols and embedded in paraffin wax. Sections were prepared and stained with haematoxylin and eosin (H.E.).



IHA test for *P. haemolytica* antibodies in sheep sera

Serum samples from sheep were tested for antibody against *P. haemolytica* by an IHA test (Shreeve, Biberstein and Thompson, 1972).

Antigen was prepared from nutrient broth culture, incubated at 37°C with agitation for 18 hours. The culture was heated at 56°C for 30 minutes. Bovine RBC, fixed in 1% gluteraldehyde (Shirai, Dietel and Osterman, 1975), were added to a final concentration of 0.5% and incubated at 37°C for 1 hour. The bovine RBC were then washed 3 times in buffered formalised saline (0.3% formalin), and resuspended at 5% in the same solution. Doubling dilutions of test sera were made in buffered formalised saline in U-bottomed microtitration plates (Cokes Engineering Company, Alexandria, Virginia, USA). Equal volumes (0.025 ml) of sensitised bovine RBC were added to each well, the test was left at room temperature for 2 hours, then at 4°C overnight, and read the following morning.



Challenge of SPF lambs with PI3 virus and

P. haemolytica

This method of challenge infection consistently induced pneumonia in a high proportion of lambs and has been reported in detail elsewhere (Sharp, Gilmour, Thompson and Rushton, 1978).

SPF lambs were delivered and maintained in isolation as described by Hart, Mackay, McVittie and Mellor (1971). Lambs were inoculated IT and IN with PI3 virus (Wells, Sharp, Burrells, Rushton and Smith, 1976). Seven days after infection with PI3 virus, the lambs were exposed to an aerosol of P. haemolytica (Gilmour, Thompson, Smith and Angus, 1975).

Lambs were examined clinically for 2 weeks after inoculation with PI3 virus. To avoid any subjective influence, the clinician was unaware of the treatments given to each group of lambs prior to challenge, and at the same time each day following the aerosol of P. haemolytica, a score for the degree of illness was awarded to each lamb. Dullness, pyrexia ( $> 40.6^{\circ}\text{C}$ ) and abnormal respiration were assigned one point each, and

death four points. Lambs were killed if they showed signs of severe respiratory disease.

#### Pathology and Bacteriology

Lambs which died, or were killed when ill were examined at necropsy. Also, all surviving animals were killed 7-10 days after challenge with P. haemolytica and were submitted for post-mortem examination. Lesions on the dorsal and ventral aspects of the lung were recorded on lung diagrams, measured with a planimeter, and expressed as a percentage of the total lung surface area (Wells, Sharp, Rushton, Gilmour and Thompson, 1978). Lesion scores were allocated on the basis of area affected, thus:-  
no lesions = 0, < 10% = 5, 11-25% = 10, > 25% = 20.  
At the end of the experiment the daily clinical scores for each animal were added together. Statistical differences between the total scores (clinical and lesion scores) of the groups were determined by means of the Mann-Whitney test (Snedecor and Cochran, 1967).

Portions of lung were fixed in formol-saline and sectioned for histopathological examination. Further samples of lung tissue from lesions and "normal" lung

tissue were macerated with peptone water in a homogeniser (Colworth) and the number of viable P. haemolytica counted by the method of Miles, Misra and Irwin (1938).

Quantitative estimation of antibody to P. haemolytica in sheep sera by a micro-enzyme-linked immunosorbent assay (ELISA)

The technique of this serological test (Burrells, Wells and Dawson, 1979) was basically a microplate modification (Voller and Bidwell, 1975) of the ELISA technique of Engvall and Perlmann (1972). A 1/100 dilution of P. haemolytica type A1 SSE in 0.05 M carbonate buffer, pH 9.6, was used to sensitise the wells of polystyrene microtitre plates (type M129A, Dynatech Laboratories Ltd., Billinghamurst, Sussex). Individual wells were filled with 300 µl of antigen solution, and the plates held at 4°C overnight. Plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS/Tween), shaking out excess fluid between each wash. Reference and test sera were not pretreated prior to testing and 1/1000 dilutions of these in PBS/Tween containing 0.02% sodium azide were added in 300 µl volumes to the wells of antigen-coated plates, and incubated at room temperature for 2 hours.

Serum dilutions were then aspirated from the wells, the plates washed 3 times with PBS/Tween, and 300  $\mu$ l of alkaline phosphatase-conjugated anti-sheep IgG diluted 1/1000 in PBS/Tween added. After incubation for 3 hours at room temperature, the wells were emptied and washed again with 3 changes of PBS/Tween. Enzyme substrate (p-nitrophenyl phosphate, Sigma Chemical Company, Poole, Dorset) at a concentration of 1 mg/ml in 10% diethanolamine (B.D.H. Chemicals Ltd., Poole, Dorset) buffer, pH 9.8, containing 0.5 mM magnesium chloride was added to the wells (300  $\mu$ l/well). After 1 hour at room temperature, the reaction was stopped by the addition of 50  $\mu$ l of 3.0 M sodium hydroxide.

Absorbance of individual samples was read at a wavelength of 400 nm in a colorimeter (Vitatron UPS Photometer) fitted with a micro flow-through cuvette. Results were recorded on a chart as peaks, the co-ordinates of which were translated into digital form on a punch-tape by means of an X-Y plotter. Five standard reference sera were included at the beginning of each test, and a further reference serum at the end of each run. The data tape was processed in a computer whose programme adjusted the values to allow for drift of the baseline during reading of

the samples, measured by variations in the results of the reference sera. The results were finally expressed in relation to a calibration curve provided by the standards.

P. haemolytica

Introduction

The problems associated with establishing and evaluating experimental infection of mice with P. haemolytica have been discussed (see General Introduction). The work described in this chapter was aimed at the development of a method of challenge infection in mice with P. haemolytica, which could be used for investigation of the factors involved in immunity to P. haemolytica and screening of potential vaccines. Ideally, a model of P. haemolytica infection should have features similar to the field disease of pasteurellosis, with multiplication of the organisms within the host, resulting in a true infection.

Two models of infection are described in this chapter. In the first, P. haemolytica was given intranasally, and the second is a modification of the IP challenge method of Smith (1958).

## Materials and Methods

### Strain of *P. haemolytica*

The strain of *P. haemolytica* used in these experiments is the *P. haemolytica* type A1 strain described in General Materials and Methods.

### Intranasal challenge procedure

Anaesthesia and IN inoculation of mice are detailed in General Materials and Methods. Each mouse received 0.05 ml of *P. haemolytica* suspension IN. Control mice received 0.05 ml sterile PBS IN.

### Preparation of inocula for intranasal challenge

Nutrient broth cultures were incubated at 37°C for 5 hours, washed twice in PBS by centrifugation and resuspended in PBS to give an estimated concentration of bacteria of between  $10^8$ - $10^9$  cfu/ml. This suspension comprised the inoculum for mice.

### Enumeration of viable *P. haemolytica* in lungs of infected mice

At selected time intervals after challenge, infected



and control mice were killed by cervical dislocation and their lungs removed aseptically prior to maceration with 9 ml sterile peptone water in a homogeniser (Colworth Stomacher, Seward Laboratory, London). Appropriate 10-fold dilutions were made in peptone water, and viable counts performed by the method of Miles, Misra and Irwin (1938). In Experiments 1, 3 and 4, a small sample of lung was removed from the diaphragmatic lobe for histological examination, before all the remainder was macerated for enumeration of bacteria.

Experiment 1 - Intranasal infection of mice with  
varying numbers of *P. haemolytica*

Design of experiment

The challenge inoculum was prepared as described above. A series of 10-fold dilutions in PBS was made, and 7 groups each consisting of 6 C57 black mice, were infected IN with these dilutions. Table 1.1 shows the number of organisms received by each group.

It has been shown that Swiss white mice challenged IN with *P. haemolytica* develop lesions in the lung which are maximal 3 days after challenge (Rushton, 1978). On the basis of this evidence, mice were killed on the third day. The lungs of these mice were removed and samples taken for histological examination (see General Materials and Methods). In this experiment, the lungs from each group of mice were assessed, and no attempt was made to score lungs individually. Each group was given a score (3+, 2+, 1+, <sup>+</sup>-, -) according to the extent and severity of the lesions present.

Table 1.1

<u>Group No</u>	<u>No of P. haemolytica type A1 given IN</u>	<u>Degree of severity of lesions in the lungs 3 days after challenge</u>
1	$10^{7.3}$	2+
2	$10^{6.3}$	2+
3	$10^{5.3}$	1+
4	$10^{4.3}$	1+
5	$10^{3.3}$	-
6	$10^{2.3}$	-
7	$10^{1.3}$	-

## Results

The severity of the lesions observed in each group of mice 3 days after infection is shown in Table 1.1. The lesions have been described by Rushton (1978). Plate 1.2 shows the lung of a mouse which scored 1+ degree of pneumonia. When compared to the lung of a sham-inoculated mouse (Plate 1.1) it can be seen that the alveolar air spaces are reduced in size and partially collapsed, and that the interalveolar septa are swollen. In contrast, a severely affected lung which scored 3+, is shown in Plate 1.3. A large area of the lung is pneumonic, with focal consolidation and hyperplasia of the bronchiolar epithelium with focal sloughing. Some of the features of this pneumonia are illustrated at higher magnification in Plates 1.4 - 1.8. Plate 1.4 shows detail from Plate 1.2, and illustrates swollen alveolar septa infiltrated by mononuclear cells, characteristic of lungs with 1+ severity of pneumonia. More severe pneumonia is seen in Plate 1.5, with focal consolidation, and an exudate of macrophages and neutrophils. The features of the most extreme pneumonia are shown in Plates 1.6 - 1.8, in which consolidation, bronchioles with hyperplastic epithelium, and a necrotic small artery are illustrated.

Plate 1.1 Lung from C57 black mouse given saline IN. Note expanded alveoli with thin septa.

H.E. X76

Plate 1.2 Lung from C57 black mouse given  $10^{4.3}$  cfu P. haemolytica type A1 IN (Experiment 1). The alveolar spaces are reduced in size, partly due to collapse, but also to swelling of interalveolar septa. This lung rates 1 + degree of severity.

H.E. X76

Plate 1.3 Lung showing 3 + degree of severity. The main changes are pneumonia with focal consolidation, and hyperplasia of bronchiolar epithelium with focal sloughing (arrows).

H.E. X76

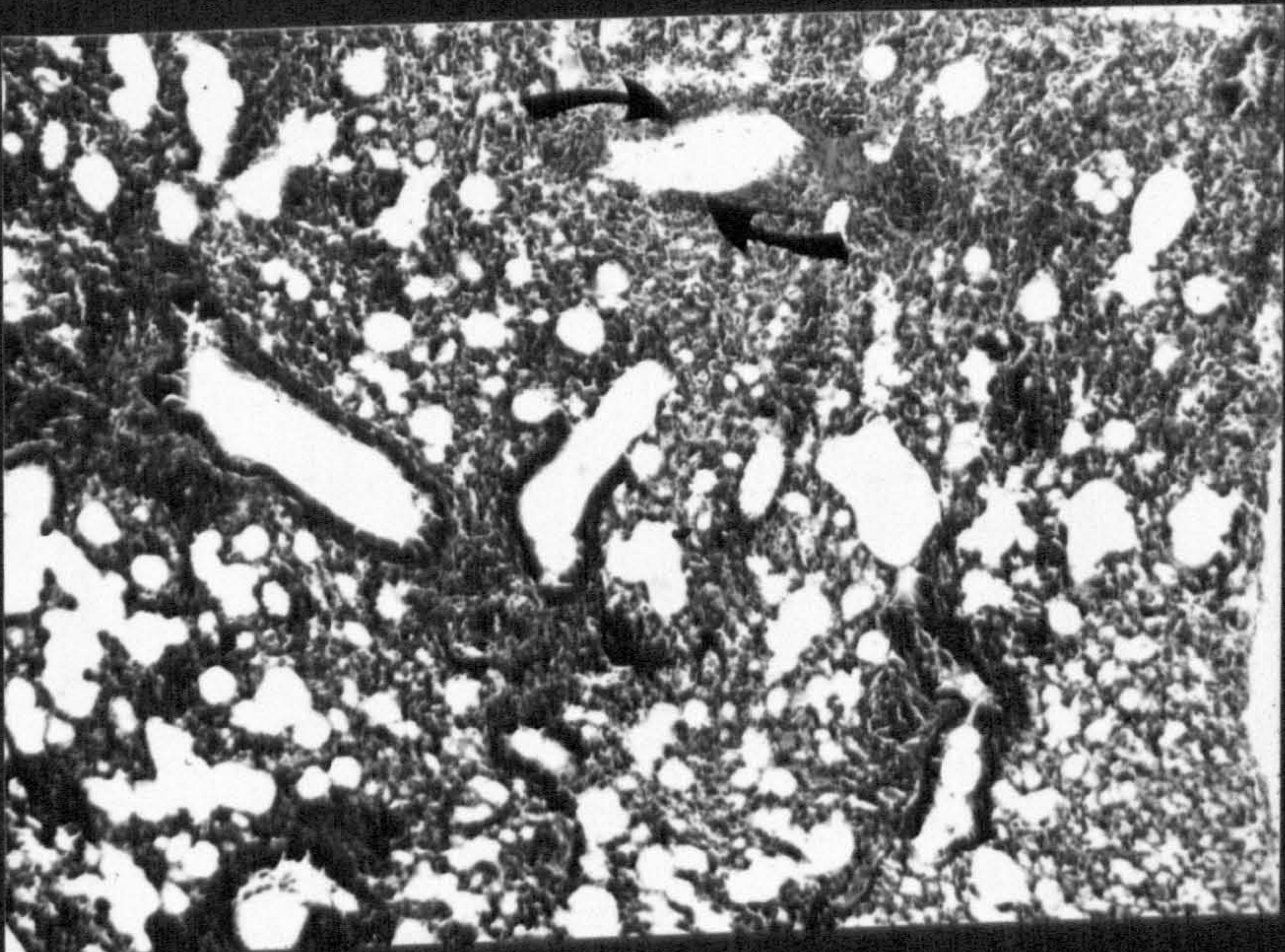
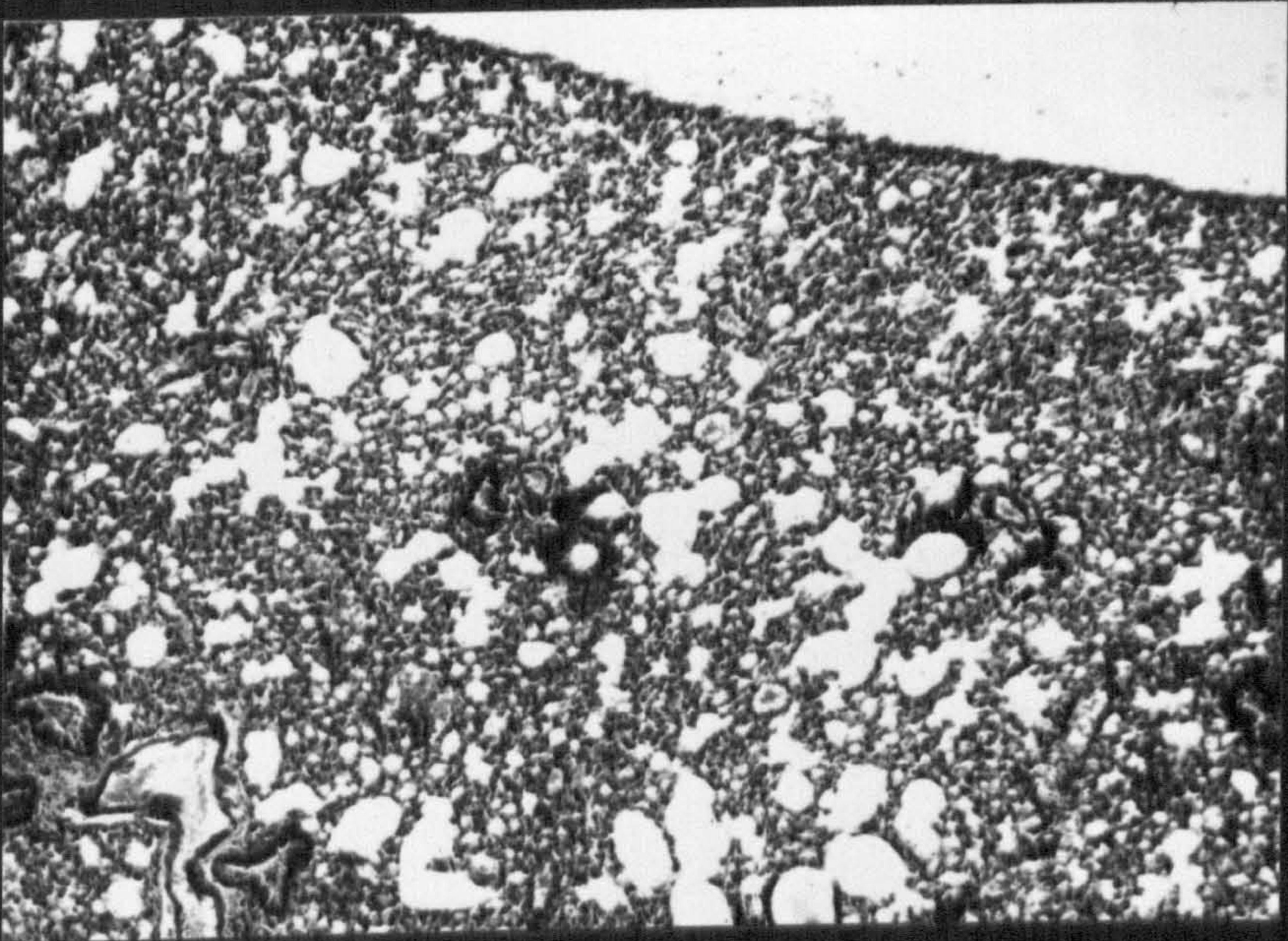
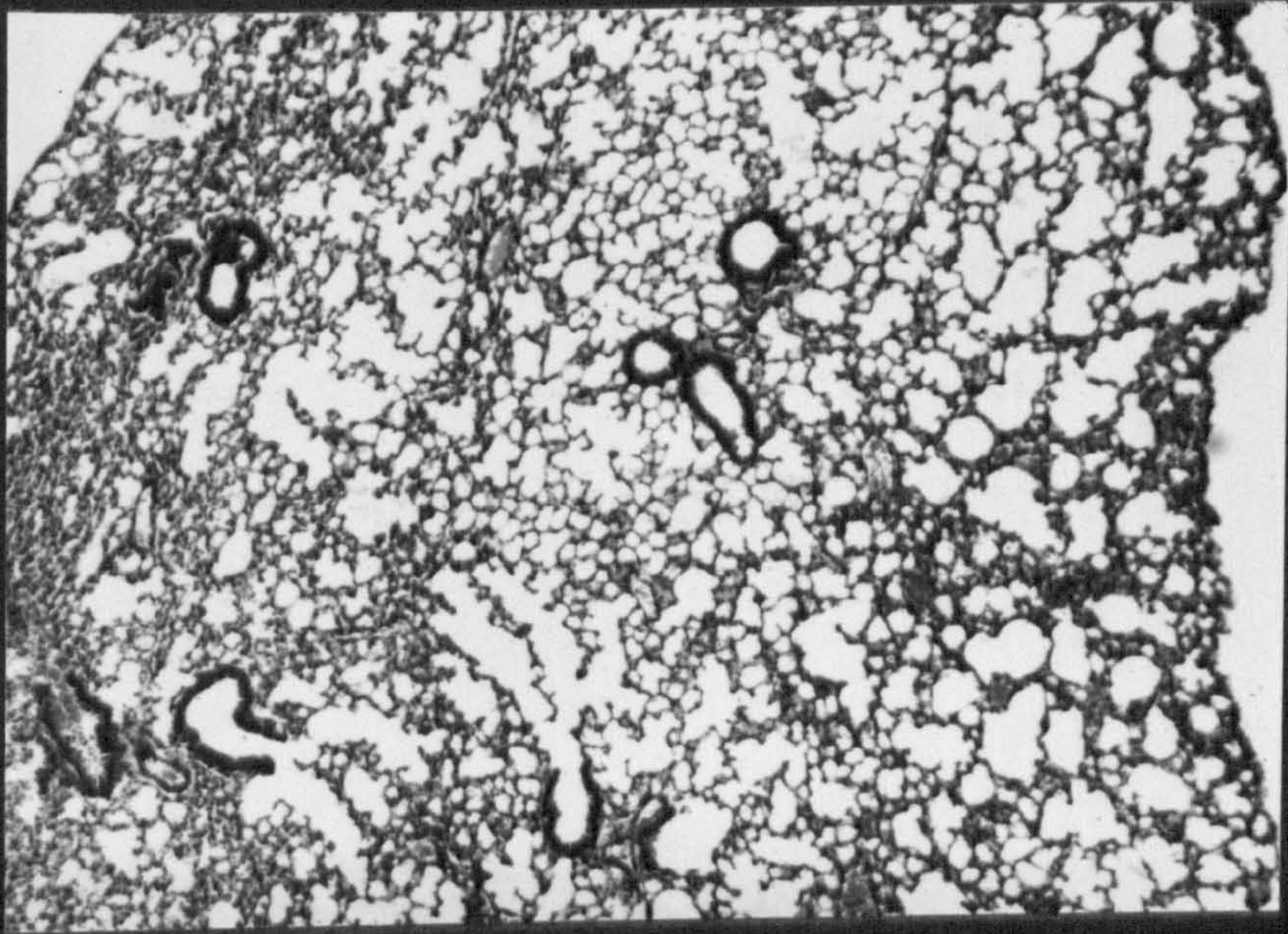


Plate 1.4 Higher magnification of section illustrated in Plate 1.2, showing swollen interalveolar septa infiltrated by mononuclear cells. Some hypertrophied lining cells, possible type II alveolar cells, are also present (arrows)

H.E. X875

Plate 1.5 Pneumonia associated with  $10^{6.8}$  cfu P. haemolytica type A1 given IN (Experiment 3). There is focal consolidation, with an exudate of macrophages and neutrophils

H.E. X875

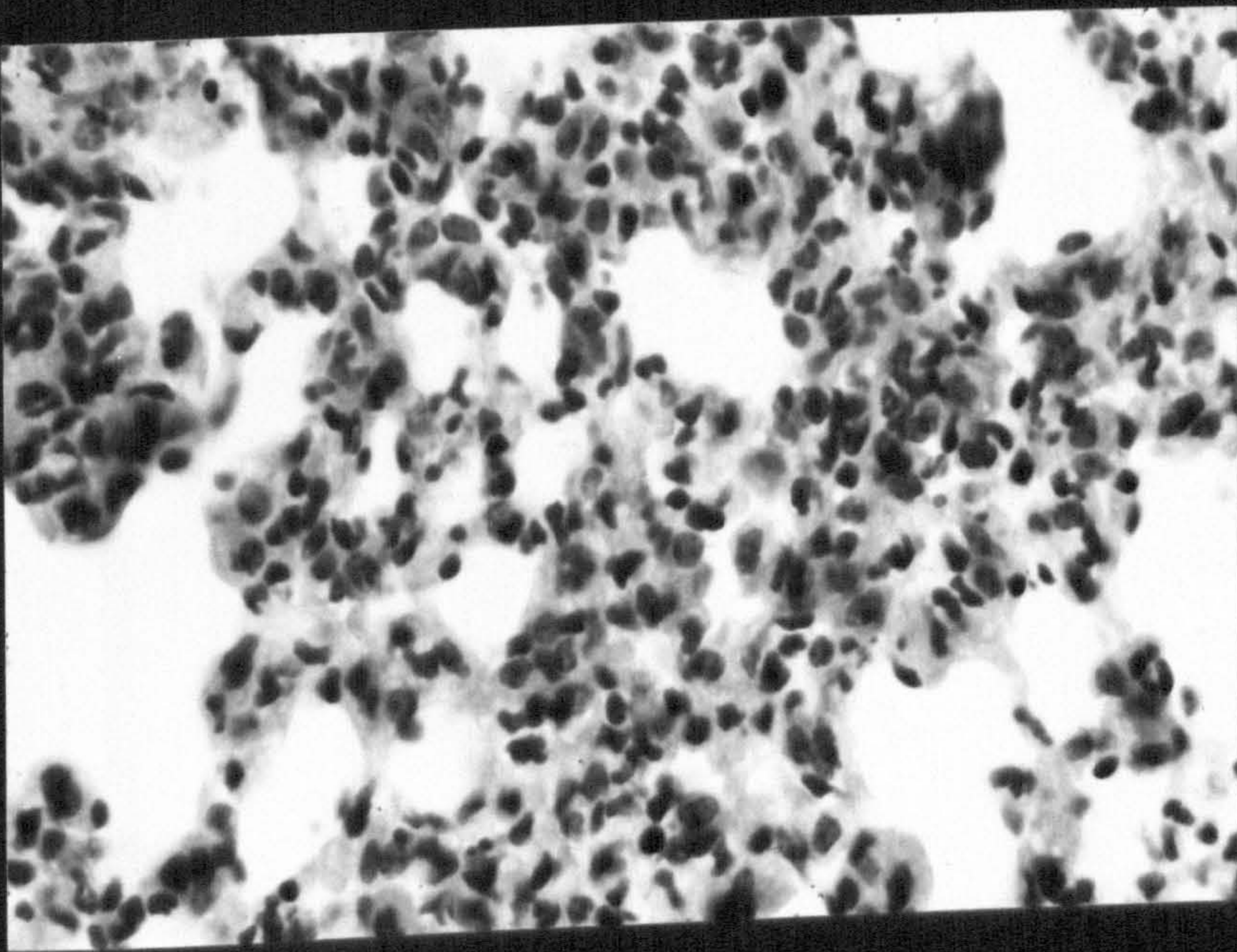
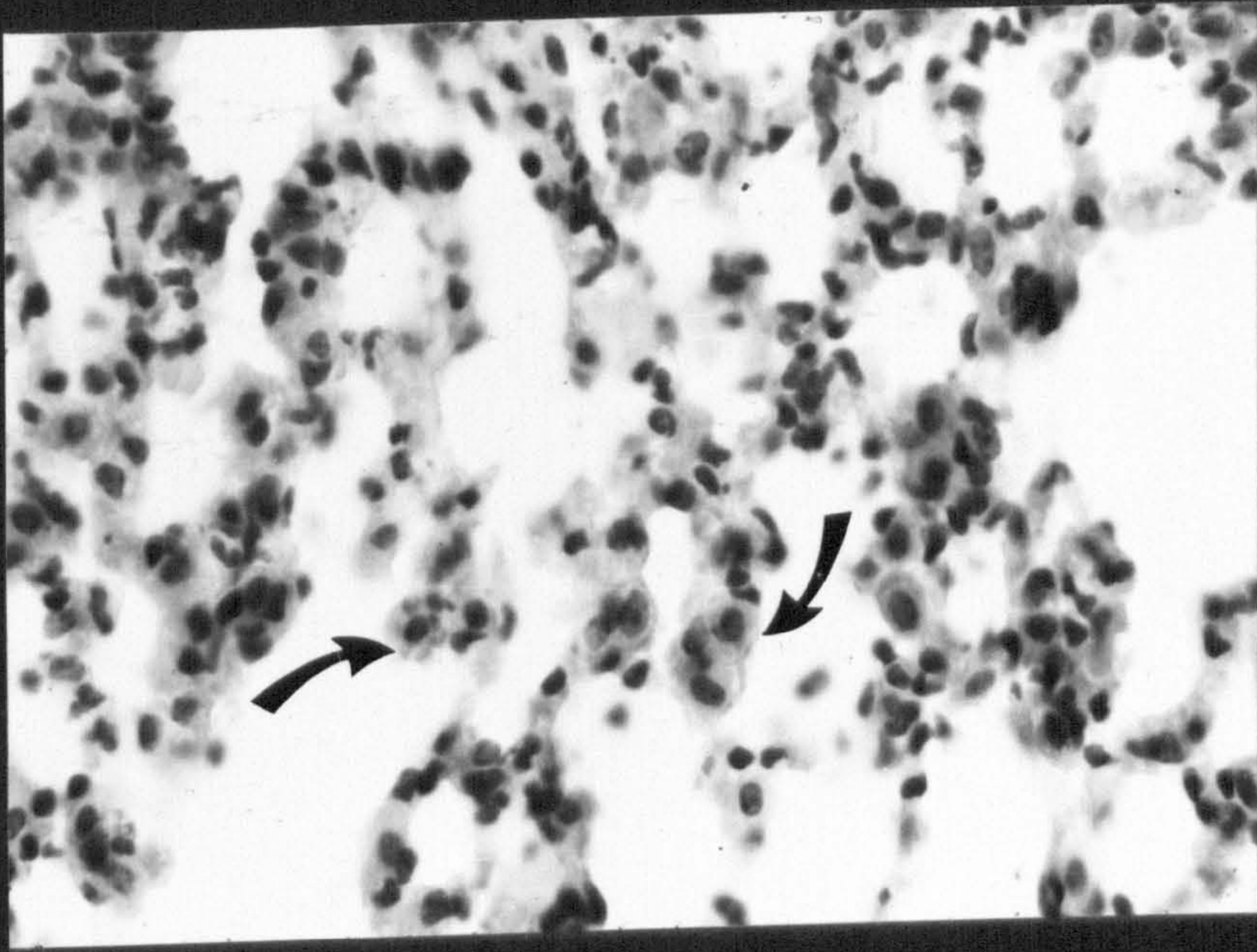




Plate 1.6 Severe consolidation with cellular exudate. A respiratory bronchiole (left) has a hyperplastic lining in the process of sloughing

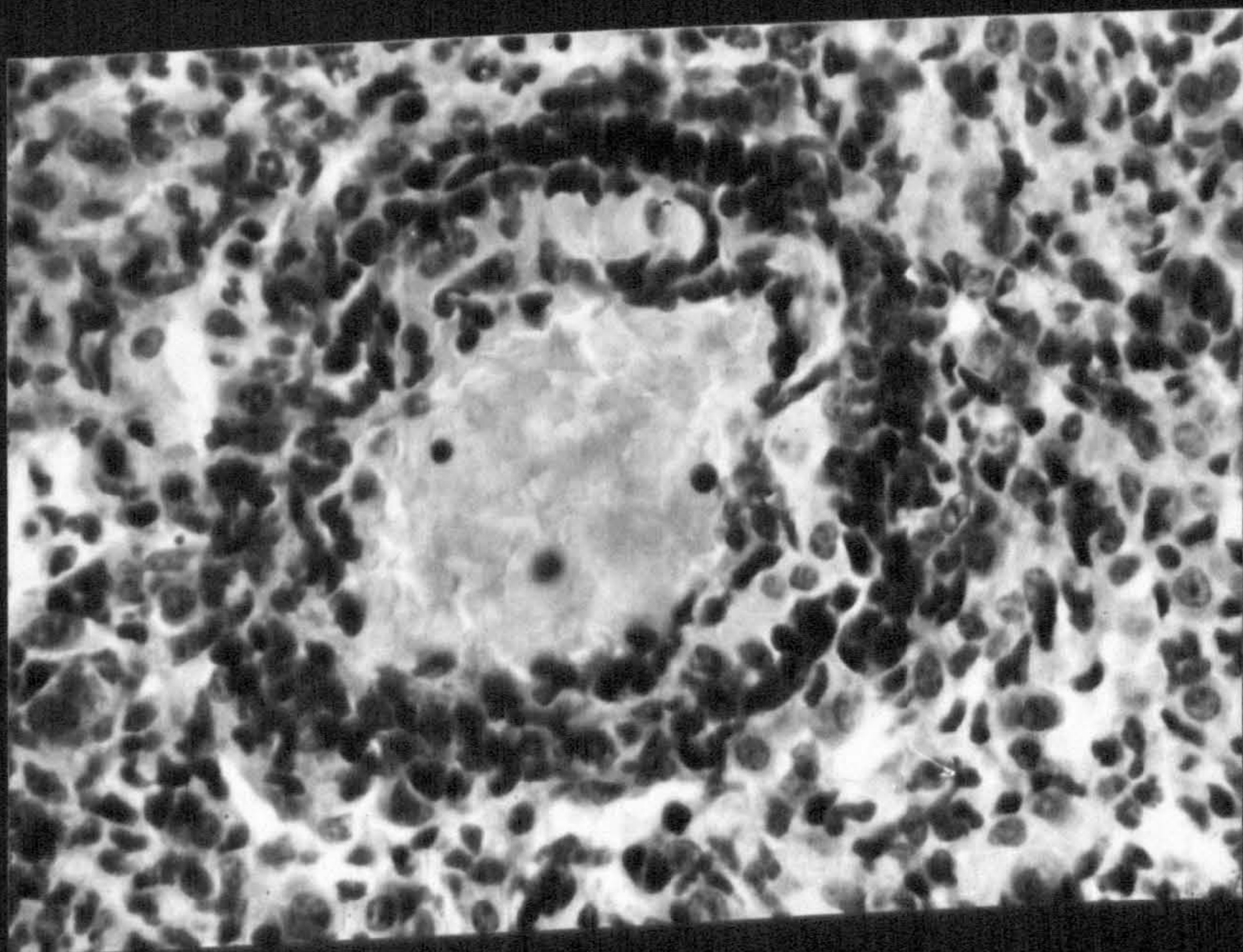
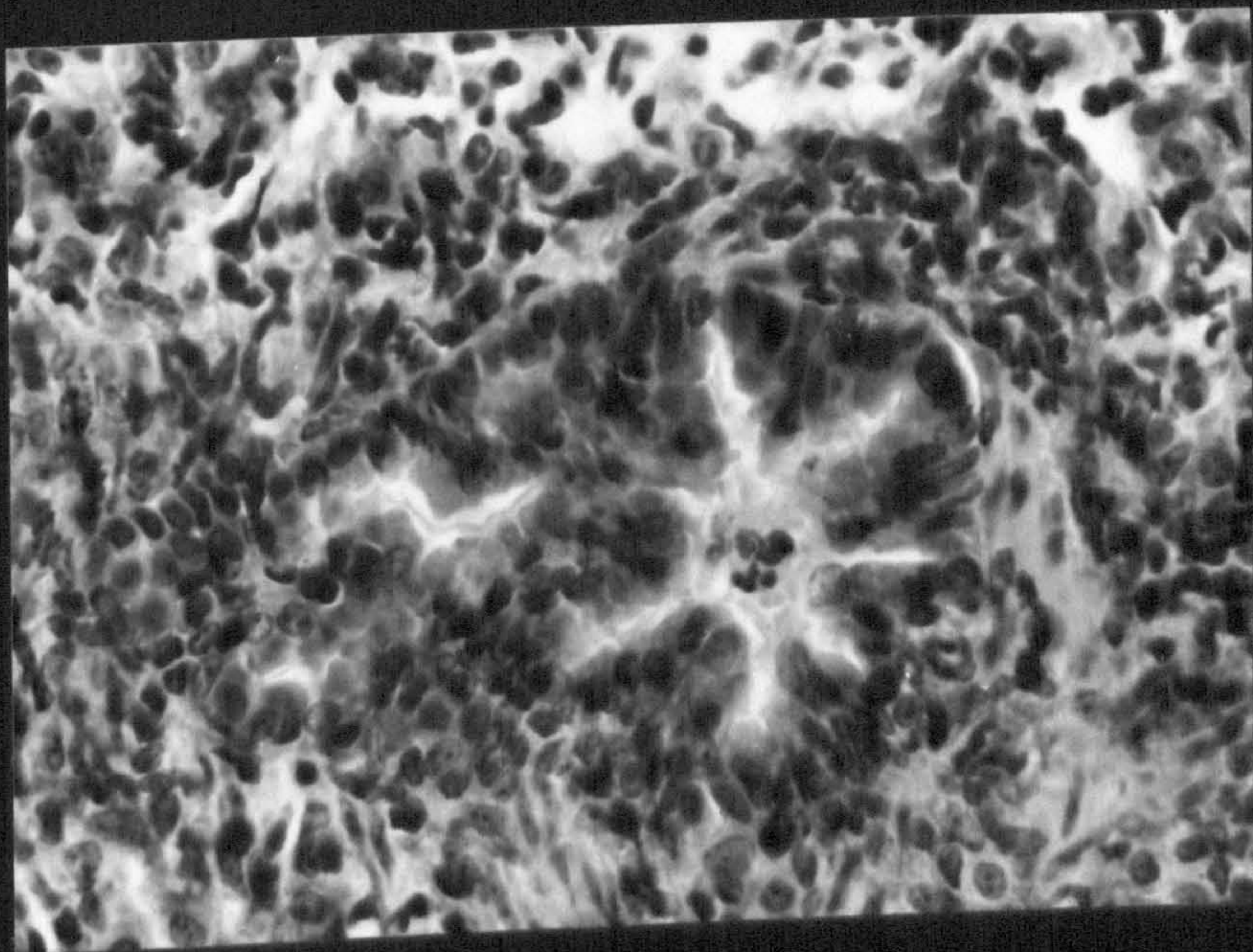
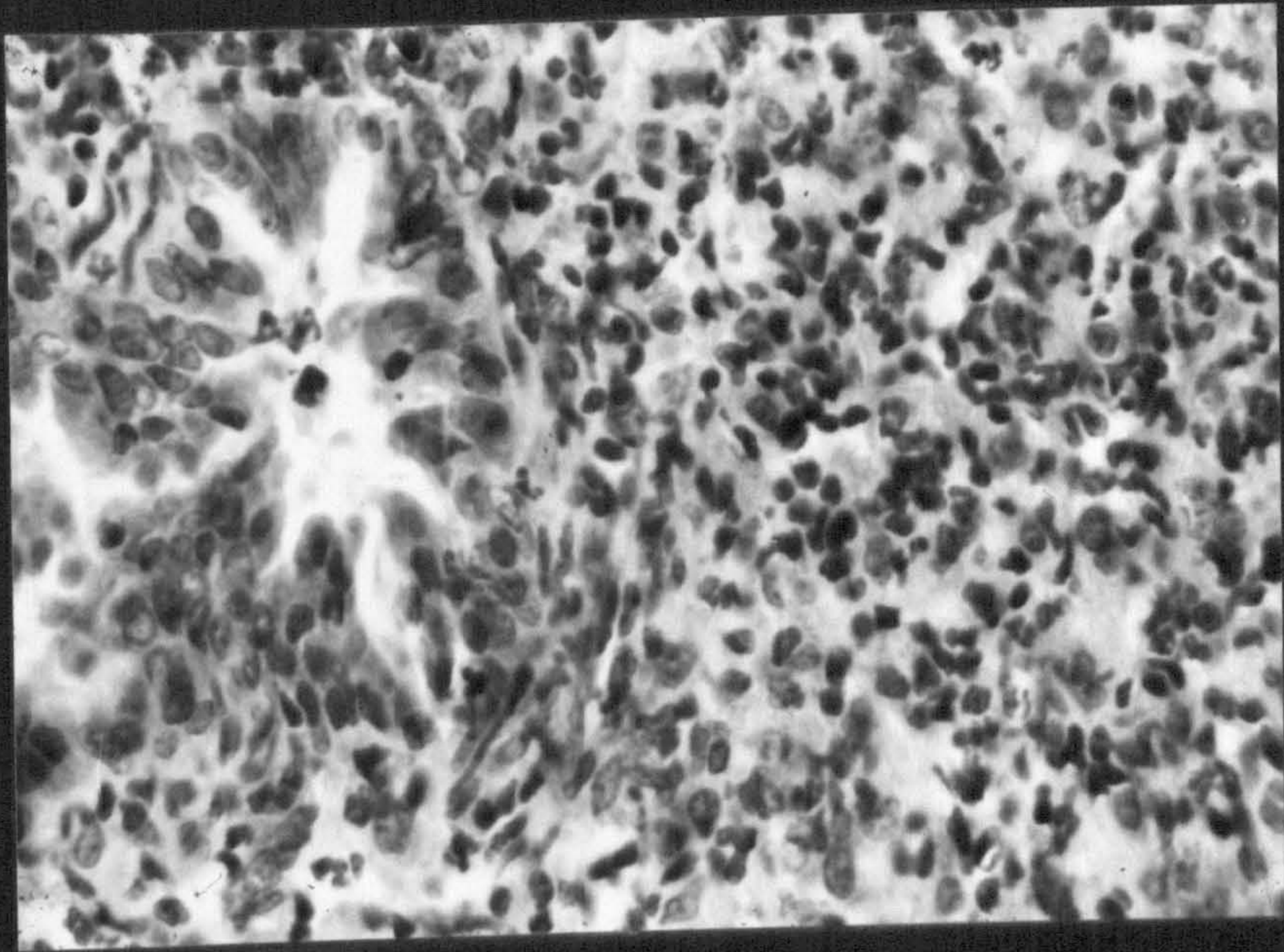
H.E. X875

Plate 1.7 Bronchiole with hyperplastic epithelium

H.E. X875

Plate 1.8 Necrosis of small artery within an area of consolidation. A narrow perivascular lymphoid cuff has formed, and the wall of the artery is infiltrated by mononuclear cells

H.E. X875



It is evident from Table 1.1 that the severity of the lesions observed was greatest in the lungs of mice infected with the largest numbers of organisms. Inoculation of fewer than  $10^{4.3}$  cfu P. haemolytica type A1 did not produce lesions.

No bacteria were observed in the lungs of mice 3 days after infection. This suggested that perhaps they had been cleared from the lung by this time, and Experiment 2 was designed to investigate this possibility.

Experiment 2 - Viable counts of *P. haemolytica* in the  
lungs of mice after infection

It was important to know whether bacteria were cleared from the lung after challenge, or if they multiplied to establish a true infection. This experiment was designed to discover which of these two alternatives occurred.

Design of experiment

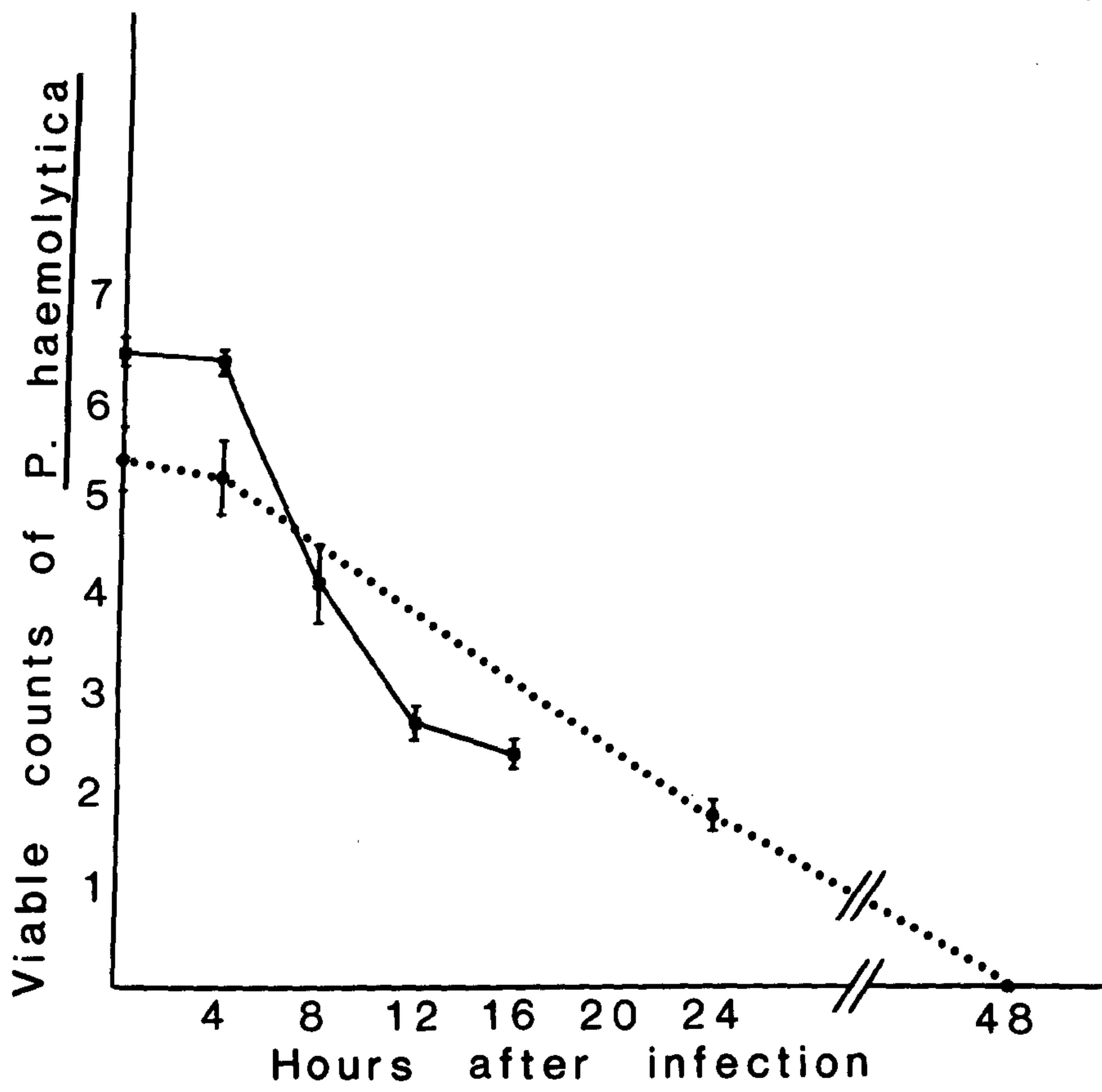
Fifty mice were each inoculated IN with  $10^7$  cfu *P. haemolytica* type A1, as described previously. Groups of 10 mice were killed at the time of challenge and 4 hours, 8 hours, 12 hours and 16 hours after challenge. Viable counts were performed on the lungs of individual mice. In the second part of this experiment, 50 mice were infected with  $10^7$  cfu *P. haemolytica* type A1 and killed in groups of 10 at the time of challenge and 4 hours, 24 hours, 48 hours and 72 hours later. Viable counts were performed on the lungs.

Viable counts were expressed as  $\log_{10}$ , and the mean and standard error (SE) calculated for each group.

## Results

The results are depicted graphically in Fig. 1.1. It appears that no multiplication of P. haemolytica type A1 occurs in the lungs of infected mice, but that the organisms are removed or killed in situ and 48 hours after challenge the lungs are substantially free from P. haemolytica. From this data it is impossible to tell whether the bacteria are killed within the lung, or if they are transported from the lung. Lesions are present within the lungs 3 days after infection, yet no viable bacteria are present in the lungs at this time (not shown in Fig. 1.1).

Figure 1.1    Viable counts of P. haemolytica type A1  
in the lungs of mice after IN infection



Experiment 3 - Comparison of halothane and thiopentone sodium as anaesthetics for intranasal infection

Experiment 3 was performed to discover if the anaesthetic given by inhalation was responsible for the lesions.

Mice are exposed to halothane by inhalation, and it is conceivable that this anaesthetic may be partly or wholly responsible for the lesions seen in the lungs 3 days after infection. To eliminate this possibility, mice were anaesthetised with either halothane, or thiopentone sodium given IP, before IN infection with P. haemolytica type A1.

Design of experiment

Twenty-eight mice were randomly allocated to two groups of 9 mice and two groups of 5 mice each (Table 1.2). The 9 mice in Group 1 were anaesthetised with halothane, and infected IN with  $10^{6.8}$  cfu P. haemolytica type A1 as described previously. The 9 mice in Group 2 were anaesthetised by IP injection of 0.2 ml of 10% thiopentone sodium (Intraval sodium: May and Baker Ltd., Dagenham,



Table 1.2

<u>Group No</u>	<u>Challenge with</u>		<u>No mice</u> <u>per group</u>	<u>Severity of lesions in lung</u> <u>3 days after challenge</u>						<u>Score</u>
	<u>P.</u>	<u>haemolytica</u>		<u>Anaesthetic</u>	3+	2+	1+	+	-	
1	+		9	halothane	2*	1	1	2	3	10.0
2	+		9	thiopentone sodium	2	3	0	1	3	12.5
3	-		5	halothane	0	0	0	0	5	0.0
4	-		5	thiopentone sodium	0	0	0	0	5	0.0

\* Number of mice in each group with lesions of this severity

Essex), and infected IN with  $10^{6.8}$  cfu P. haemolytica type A1. Groups 3 and 4 each consisted of 5 mice, which were anaesthetised with halothane and thiopentone sodium respectively, prior to inoculation with 0.05 ml sterile PBS.

Three days after infection, all mice were killed, and samples removed from their lungs for histological examination. Lungs from individual mice were assessed in this experiment, and awarded a score to represent the extent and severity of lesions. Mice scoring 3+ were awarded 3 points, those scoring 2+ were awarded 2 points, those scoring 1+, 1 point, and mice scoring  $\frac{+}{-}$  were given 0.5. The total for each group was calculated, to enable comparisons to be made between the different groups.

### Results

The results of Experiment 3 are shown in Table 1.2. The challenge inoculum in this experiment was slightly lower than usual ( $10^{6.8}$  cfu P. haemolytica type A1 per mouse), and probably as a result of this, three mice in both Group 1 and Group 2 showed no lesions in their lungs. Lesions in groups 1 and 2 varied from very severe (3+) to negative (-), and the spread was similar in the two groups.

Control mice given sterile PBS IN showed no lesions.

It can be concluded from this experiment that halothane is not responsible for the lesions 3 days after challenge, since mice given the same challenge, but anaesthetised with thiopentone sodium (Group 2), had similar lesions to mice in Group 1, and the mice in Group 3, which were anaesthetised with halothane, but not infected, displayed no lesions.

Experiment 4 - Comparison of IN infection with live and  
heat-killed P. haemolytica

No detectable bacteria are present in the mouse lung when the lung lesions are present, and Experiment 3 demonstrated that the lesions were not a direct result of anaesthesia. These two observations led to the suggestion that the lesions may be the result of the presence of large amounts of bacterial material in the lung, and that dead P. haemolytica may produce similar results. Experiment 4 was designed to test this hypothesis.

Design of experiment

An inoculum of P. haemolytica type A1 was prepared as described previously. One half was heat-inactivated at 60°C for 30 minutes. Two dilutions ( $10^{-1}$  and  $10^{-2}$ ) of both live and heat-killed inocula were made, and viable counts performed to determine the number of organisms present, and to check the sterility of the heat-killed preparation.

Forty-two mice were randomly allocated to 7 groups of 6 mice each. Mice were anaesthetised with halothane,

and inoculated IN with either live or heat-killed P. haemolytica at undiluted,  $10^{-1}$  or  $10^{-2}$  dilutions. The seventh group was inoculated with sterile PBS. The treatments given to each group are detailed in Table 1.3.

Three days after challenge, the mice were killed and samples of lung examined histologically. Scores were awarded as in Experiment 3.

### Results

The results are shown in Table 1.3. The largest live inoculum was that given to mice of Group 6 ( $10^{6.2}$  cfu P. haemolytica type A1/mouse), and lesions of varying severity were observed in five of the six mice in this group. Mice in Group 3 were given equivalent numbers of heat-killed bacteria and lesions were also present in five of the six mice. The remaining mice in these two groups showed no convincing lesions. Smaller inocula, either live or dead, produced few positive results. The control mice (Group 7), which were given sterile PBS, showed no lesions in the lung.

Table 1.3

<u>Group No</u>	<u>No of P. haemolytica</u> type A1 per mouse	<u>Live or</u>		<u>Severity of lesions in the</u>					<u>Score</u>
		<u>heat-killed</u>	<u>inoculum</u>	<u>Lung 3 days after challenge</u>		<u>+</u>		<u>-</u>	
1	$10^{4.2}$	heat-killed	inoculum	2+	1+	+	-	-	2.5
2	$10^{5.2}$	heat-killed	inoculum	0	2*	1	0	3	1.0
3	$10^{6.2}$	heat-killed	inoculum	0	1	0	0	5	5.5
4	$10^{4.2}$	live	inoculum	0	0	1	2	4	1.0
5	$10^{5.2}$	live	inoculum	1	0	0	0	5	2.0
6	$10^{6.2}$	live	inoculum	2	3	0	0	1	7.0
7	Sterile PBS	-	inoculum	0	0	0	0	6	0.0

\* Number of mice in each group with lesions of this severity

It can be concluded from this experiment that similar lesions can be produced in the lungs of mice 3 days after IN challenge with an inoculum of  $10^{6.2}$  cfu of either live or heat-killed P. haemolytica type A1, and that these lesions are not the result of ongoing infection, but are probably due merely to the presence of bacterial material in the lung.

Experiment 5 - Intraperitoneal challenge procedure;  
preparation of inocula for IP challenge of mice and  
determination of LD<sub>50</sub>

Design of experiment

A 300 ml volume of NB was inoculated from a fresh blood agar culture of P. haemolytica type A1. The broth was incubated for 10 hours at 37°C, and the bacteria obtained by centrifugation at 1000 g for 20 minutes at 4°C. The bacteria were washed twice in 0.15 M saline, and resuspended in 10 ml saline. A series of 10-fold dilutions was made in saline from this suspension, and a viable count performed. The undiluted suspension and dilutions 10<sup>-1</sup>-10<sup>-5</sup> were incorporated into a 5% suspension of hog gastric mucin, pH 7.2 (ICN Pharmaceuticals Inc., Life Sciences Group, Plainview, NY), at a ratio of 1 part bacteria to 4 parts gastric mucin. Groups of 5 mice were inoculated IP with 0.5 ml of each dilution and the number of deaths recorded 48 hours later. Generally, no further deaths occurred after this time. The LD<sub>50</sub> was calculated by the method of Reed and Muench (1938).



## Results

The LD<sub>50</sub> of this strain of P. haemolytica type A1 was 10<sup>5.3</sup> cfu when given IP in gastric mucin to mice. In subsequent experiments with this method of challenge, the inoculum was always greater by at least 10<sup>0.5</sup> cfu than the LD<sub>50</sub>.

Experiment 6 - Growth of P. haemolytica type A1 in the  
livers and spleens of normal mice

This experiment was designed to determine if the organisms multiplied within the mouse and established a true infection.

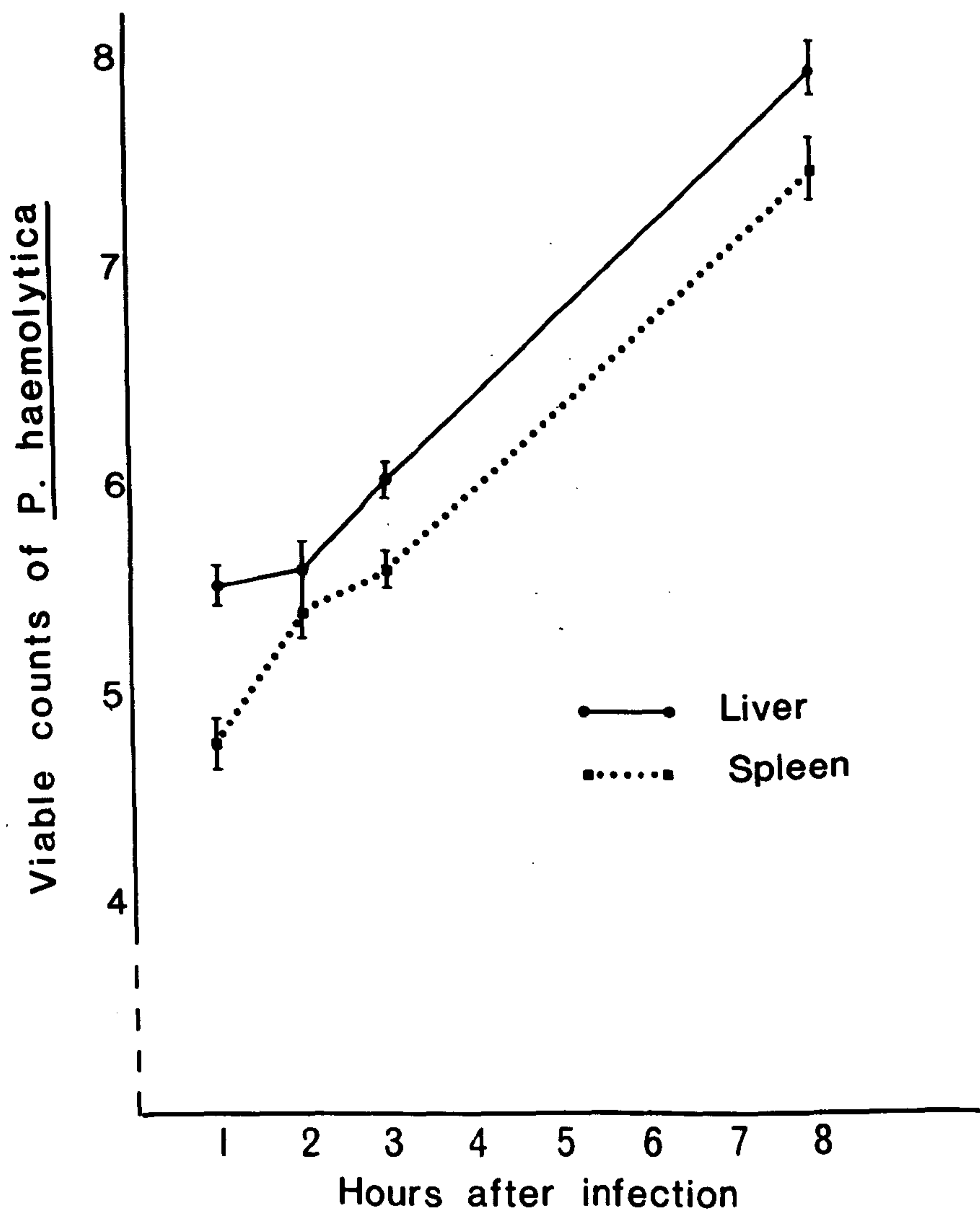
Design of experiment

An inoculum of P. haemolytica type A1 was prepared as described in the preceding experiment. A group of 20 mice was inoculated IP with  $10^{7.1}$  cfu P. haemolytica type A1 in gastric mucin. At time intervals of 1, 2, 3 and 8 hours after challenge groups of 5 mice were killed by cervical dislocation, and their livers and spleens removed aseptically. These organs were individually macerated with 9 ml peptone water in a homogeniser and serial 10-fold dilutions in peptone water plated out for viable counting. The mean counts  $\pm$  SE for each group were calculated and expressed as  $\log_{10}$  cfu.

Results

The viable counts of P. haemolytica type A1 in the livers and spleens of mice are shown in Fig. 1.2. Each

Figure 1.2 Viable counts of P. haemolytica type A1  
in the livers and spleens of mice following IP infection



point plots the mean  $\pm$  SE of the counts for 5 livers or spleens, expressed at  $\log_{10}$  cfu. Counts from individual livers and spleens are given in Appendix 1. From 2 hours after infection, the organisms multiplied in both organs, with similar growth rates in each. This showed that "true" infection was taking place, with multiplication of the organisms within the host.

Experiment 7 - Vaccination of mice by IP and SC routes  
prior to challenge with P. haemolytica type A1

This experiment was designed to determine if the model of IP infection with P. haemolytica could be used to demonstrate protection afforded by vaccination with an experimental P. haemolytica vaccine, and to ascertain the most suitable route for vaccination.

Design of experiment

Fifty mice were randomly allocated to 5 groups of 10 mice each. Mice in Group 1 were unvaccinated controls. Those in Group 2 were inoculated SC with 0.1 ml of P. haemolytica type A1 SSE adsorbed onto Alhydrogel and emulsified in mineral oil, and mice in Group 4 were inoculated with 0.1 ml of this vaccine IP. Mice in Group 3 and Group 5 were given 0.1 ml of BA adsorbed onto Alhydrogel and emulsified in mineral oil, by the SC and IP routes respectively. Details of these vaccine preparations are given in General Materials and Methods.

Three weeks after vaccination, inocula of P. haemolytica type A1 in gastric mucin were prepared as described

for previous IP challenges. Five of the mice in each group were challenged IP with  $10^{8.6}$  cfu and the remaining 5 with  $10^{7.6}$  cfu. The number of deaths was recorded 48 hours later.

### Results

Deaths 48 hours after challenge are shown in Table 1.4. All 10 control mice died. Mice vaccinated SC with P. haemolytica vaccine were protected from challenge with  $10^{7.6}$  cfu, but four of the five mice challenged with  $10^{8.6}$  cfu died. Two of the five mice in Group 3 (vaccinated SC with BA vaccine) survived challenge with the smaller number of organisms, but all those given the higher challenge died.

In contrast, mice in Group 4 (vaccinated with P. haemolytica vaccine IP) survived both challenge doses, and mice in Group 5 (vaccinated IP with BA vaccine) all survived the lower challenge and four of the five mice challenged with  $10^{8.6}$  cfu, survived.

IP inoculation of the P. haemolytica vaccine afforded complete protection from both challenge doses, whereas only

Table 1.4

The effect of vaccination by IP and SC routes upon IP challenge with P. haemolytica type A1

Group No	Vaccine	Route of administration	Number of deaths 48 hours after challenge in groups of five mice	
			$10^{8.6}$ cfu	$10^{7.6}$ cfu
1	-	-	5	5
2	<u>P. haemolytica</u> *	SC	4	0
3	BA*	SC	5	3
4	<u>P. haemolytica</u>	IP	0	0
5	BA	IP	1	0

\* Both antigens adsorbed onto aluminium hydroxide and emulsified in mineral oil.



one mouse vaccinated SC with the P. haemolytica vaccine survived. However, the administration of the BA vaccine IP was shown to have a protective effect comparable to IF vaccination with the P. haemolytica vaccine. It is reasonable to conclude that the protection demonstrated with IP administration of the P. haemolytica vaccine is, at least in part, due to some non-specific reaction in the peritoneum. For this reason, the SC route of vaccination was chosen in preference to the IP route in subsequent experiments.

Experiment 8 - The effect of vaccination of mice with  
*P. haemolytica* type A1 vaccine upon the growth of  
organisms in the liver following IP challenge with  
*P. haemolytica* type A1 3 weeks later

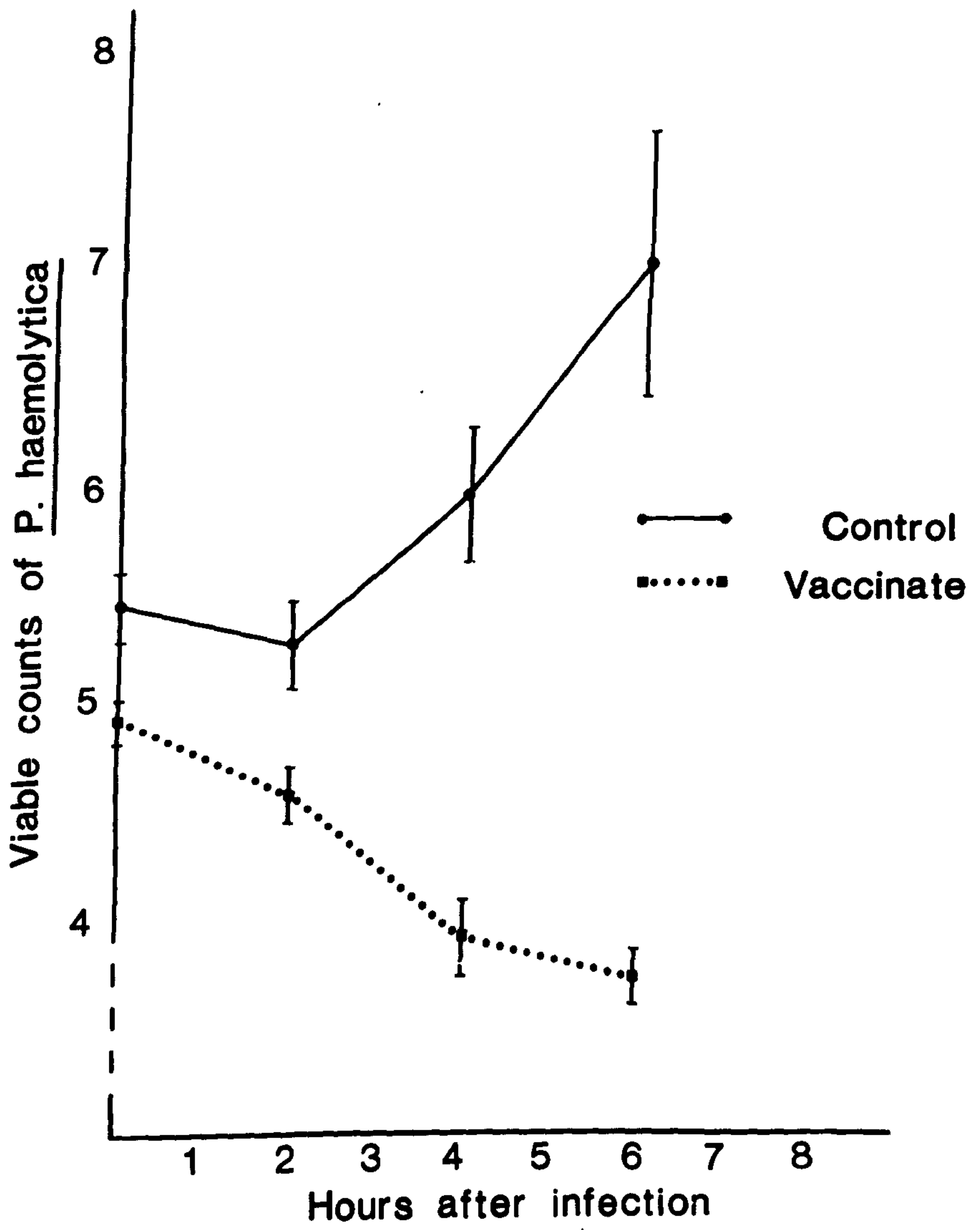
Design of experiment

Twenty-five mice were each inoculated SC with 0.1 ml *P. haemolytica* type A1 vaccine. Three weeks later, these mice and a group of 25 uninoculated controls were challenged IP with  $10^{6.6}$  cfu *P. haemolytica* type A1 in gastric mucin. Five vaccinated and 5 control mice were killed at the time of challenge, and 2, 4, and 6 hours thereafter. Their livers were removed aseptically, and viable counts determined.

Results

Viable counts of *P. haemolytica* type A1 in the livers of vaccinated and control mice are shown in Fig. 1.3. Each point represents the mean  $\pm$  SE of counts from 5 mice, expressed as  $\log_{10}$  cfu. Counts from individual livers are given in Appendix 1. Viable counts increased exponentially in the livers of untreated mice, whereas there was a reduction in the number of viable bacteria in the livers of vaccinated mice. Five control mice

Figure 1.3    Viable counts of P. haemolytica type A1  
in the livers of control and vaccinated  
mice following IP infection



which were not sacrificed all died with 19 hours. In contrast, 5 infected vaccinates appeared healthy at that time, and P. haemolytica could not be recovered from their livers.

### Discussion

The purpose of the work described in this chapter was the development of a method of P. haemolytica challenge in mice which would be suitable for the study of experimental vaccine preparations.

At first, the model of IN infection seemed promising. Lesions developed in the lung, the organ primarily involved in pneumonic pasteurellosis, and these were not caused by the anaesthetic used during challenge, so were probably the result of an effect of the bacteria. However, there was no net multiplication of P. haemolytica in the lung, which was cleared by 48 hours after infection. Rushton (1978) recovered P. haemolytica in varying numbers from the lungs of some mice 3 days after challenge with P. haemolytica by this method. In that study, viable counts were performed upon pools of lungs from 5-10 mice, and not from individual mice, as in this experiment. It is not evident from these results whether P. haemolytica multiplies within the lung and is removed from it at a faster rate, or whether the bacteria are cleared without any multiplication. Bacteria may be killed within the lung, or removed from it. The former possibility is

the more likely. Green and Kass (1964) showed that when mice were exposed to an aerosol of radio-labelled Staphylococcus aureus or Proteus mirabilis, and the rate of disappearance of viable bacteria compared with the rate of their mechanical removal, bacterial viability declined by 80-90% in 4 hours, whereas radioactivity declined by only 14-20%. The disparity between these rates indicated that mechanical removal comprised a relatively small fraction of the total clearing process. The decline in viability of P. haemolytica in the mouse lung is not as rapid, but it is probable that methods of removal are similar.

The fact that P. haemolytica does not multiply in the mouse lung is a serious disadvantage, but more important is the fact that the bacteria do not have to be living to cause lung lesions. The lesions therefore are not necessarily related solely to infection with viable P. haemolytica. Rushton (1978) claimed of this model that "the mouse pneumonia, besides being quickly, easily and cheaply induced, provides an opportunity for studying P. haemolytica in the lung without the development of extensive changes and subsequent death". Despite these

obvious advantages of low cost, and rapidity of induction of lesions, it was considered that the experiments described in this study showed that the lesions were due to a toxic effect of the bacteria, and that the model was therefore unsuitable for the study of P. haemolytica infection, particularly the study of immunity. Attention was turned to the model of infection of mice with P. haemolytica by the IP route, which was developed by Smith (1958).

In this model, P. haemolytica multiplies within the host and causes death within 48 hours. In his original work, Smith (1958) recorded death or survival of mice, and did not quantify the number of organisms present. However significant the differences between death or survival may be, data of this kind reveal nothing of the mechanism of bacterial inactivation by the host. Obviously, the survival of 100% of a group of vaccinated mice, in the face of 100% mortality in controls, represents a convincing demonstration of protection, but the interpretation of, for example, 50% survival of vaccinated mice, or increased time to death, is more difficult. This becomes an even greater problem if the virulence of the infecting agent has been enhanced by some form of immuno-



suppression of the host.

Serial enumeration of the populations of bacteria in both vaccinated and control mice circumvents some of these criticisms. The criterion used to estimate the efficacy of vaccines is a significant shift in the bacterial growth rate, which may be sufficient to prevent disease. Such studies are complementary to mortality/survival data.

Detailed growth curves obtained using both actively and passively immunised animals have revealed the nature of the immune response to a number of microbial parasites, including *Salmonella* species (Collins, 1974). Extensive studies of a similar nature have been performed with *P. multocida* infection of mice (Woolcock and Collins, 1976). Two injections of heat-killed vaccine, incorporated in CFA, protected absolutely against parenteral challenge with *P. multocida*, and growth of *P. multocida* in the livers and spleens of vaccinated mice was 100-fold lower than in controls. Hyperimmune mouse serum, administered IP prior to SC challenge, was shown to be highly protective. In view of these experiments, enumeration of viable *P. haemolytica* in challenged mice was considered to be of greater value than mortality/

survival data alone.

An aim of the present study was development of a model which could be used for screening potential vaccine preparations. By 5-6 hours after infection, viable P. haemolytica in the livers of control mice had increased by at least 10-fold, and those in the livers of successfully vaccinated mice had decreased sufficiently for differences between the two groups to be convincingly demonstrated. In subsequent experiments, it was considered sufficient to kill mice from each group only at the time of challenge, and at one time interval thereafter. This meant that fewer mice would be required to screen each vaccine preparation, an advantage for investigation of vaccine preparations on a large scale. The determination of LD<sub>50</sub> values for each vaccine would require far greater numbers of animals.

The P. haemolytica and BA vaccines administered IP gave similar degrees of protection (Experiment 7). Non-specific stimulation of peritoneal macrophages can occur following the introduction of a variety of substances, such as serum and mineral oil, into the peritoneum (Stuart,

Habeshaw and Davidson, 1978). It is possible that such stimulation took place in these experiments and for that reason, the IP route of vaccination was avoided in later experiments. It is interesting to note that Smith (1959A) demonstrated a non-specific protective effect if diluted mouse serum, saline or veronal-saline buffer were inoculated IP, 5-6 hours prior to challenge with P. haemolytica in gastric mucin. This effect was evident only at lower challenge doses of P. haemolytica, when fewer deaths occurred in treated mice than in controls.

Smith (1959A) demonstrated protection of mice from IP challenge with P. haemolytica by IP inoculation of living P. haemolytica or a formol-saline P. haemolytica vaccine. Protection afforded by IP vaccination with either of these preparations was superior to that given by a single SC vaccination with the formol-saline vaccine flocculated by sodium bicarbonate and potassium aluminium sulphate, then emulsified in mineral oil. Although the strain of P. haemolytica used in these experiments was isolated from a case of lamb septicaemia, and the strain used in the present study was from a case of sheep pneumonia, these results are in agreement. Smith (1959A) also found that IV administration of the formol-saline vaccine gave a

similar degree of protection to that achieved when it was given IP. The vaccine used in the present study was an oil emulsion, and unsuitable for IV inoculation.

The major disadvantages of the SC immunisation and IP challenge of mice model is that it bears no resemblance to the field disease of pneumonic pasteurellosis in sheep, and that the mouse is not a natural host of P. haemolytica, necessitating the use of gastric mucin to enhance its virulence. The model of pneumonic pasteurellosis in sheep (Sharp, Gilmour, Thompson and Rushton, 1978) also has the disadvantage that the host must be compromised, in this case by PI3 virus infection, before infection with P. haemolytica can be established. However, the lesions produced in lambs by this method do resemble those of the field disease. The P. haemolytica type A1 vaccine used in this study has also been shown to protect SPF lambs against challenge with P. haemolytica type A1 superimposed on PI3 virus infection (Gilmour, Martin, Sharp, Thompson and Wells, 1979; Chapter 2 of this thesis), which suggests that there may be some correlation between results obtained in the two challenge systems. This model of P. haemolytica infection in mice has in its favour that an infection can be established, and its

progress followed in the face of various vaccination procedures. Further experiments using this model are described in later chapters.

Mechanisms of immunity to *P. haemolytica* in sheepIntroduction

Assessment of the efficacy of *P. haemolytica* vaccines has previously been based upon serological criteria. The IHA test has been widely used for the detection of antibodies to *P. haemolytica* in sera from vaccinated lambs (Cameron and Smit, 1970). There is a lack of evidence to suggest that measurement of serum antibody titres to *P. haemolytica* gives any indication of the ability of vaccines to prevent disease. Correlation has been demonstrated between serum IHA titres of lambs vaccinated against *P. haemolytica* type A1 and the protective capacity of these sera given passively to mice (Gilmour, personal communication). The resistance of individual lambs to challenge with *P. haemolytica* type A1 did not correlate with either of these parameters. Consequently, the role of humoral immunity in protection against *P. haemolytica* is not clear.

Sterility of the lungs is normally maintained by the combined antibacterial activity of the mucociliary, phagocytic and immune systems. Inhaled bacteria which impact in the upper regions of the respiratory airways

are physically removed by the mucociliary transport systems (Kilburn, 1967; Green, 1968), and bacteria invading the lung are intercepted minutes after their entry by alveolar macrophages (AM) (Goldstein, Lippert and Warshauer, 1974). The sequence of events involved in bacterial inactivation in the lung has been extensively studied in mice, rats and guinea pigs (Goldstein, Green and Seamans, 1970; Goldstein, Lippert and Warshauer, 1974; Green and Kass, 1964; Jakab and Green 1973A and 1973B). Little is known about the local defence mechanisms which operate in the sheep lung during pasteurellosis.

Two experiments are described in this chapter. The first was an investigation into phagocytosis of P. haemolytica by sheep lung wash cells in vitro, in the presence and absence of serum from immunised lambs. In the second experiment, lambs were passively immunised with hyperimmune serum prior to challenge with P. haemolytica. These two experiments were performed in the hope that some insight would be gained into the mechanisms which may be important in immunity to P. haemolytica in sheep.

## Materials and Methods

### Lambs

All lambs were hysterectomy-derived, colostrum-deprived, and were reared under SPF conditions (Hart, Mackay, McVittie and Mellor, 1971).

### Vaccine

The vaccine used throughout was P. haemolytica type A1 SSE, adsorbed onto Alhydrogel and emulsified in mineral oil, as described in General Materials and Methods.

### Bacteria

P. haemolytica type A1 as described in General Materials and Methods was used in both experiments.

### Preparation of antiserum pool for Experiment 2

The pool of antiserum to P. haemolytica type A1 used in Experiment 2 was prepared from blood collected from 4 colostrum-deprived lambs. These lambs were reared for the first two weeks of life under gnotobiotic conditions, then transferred to a room which had been thoroughly cleaned and disinfected. The lambs had no direct or



indirect contact with conventional animals and were thus protected from acquiring unintentional P. haemolytica infection. P. haemolytica type A1 vaccine was injected intramuscularly on 3 occasions, the 2nd and 3rd injections being given 1 month and 2 months respectively after the first. Blood samples were taken at weekly intervals, and the serum titre of IHA antibody to P. haemolytica type A1 determined. On the basis of these results blood for preparation of the serum pool was collected in the 2 weeks prior to and the 4 weeks after the final injection of the vaccine. After separation from the clot, the serum was clarified by filtration and sterilised by passing it through a 22  $\mu$ m membrane filter. The antiserum pool had an IHA titre to P. haemolytica type A1 of 1024, and lacked antibodies to PI3 virus, as detectable by haemagglutination inhibition assay as adapted to microtitre plates (Smith, 1975).

A negative control pool of serum obtained from 4 to 6 week old SPF lambs was prepared in a similar manner. No antibodies to P. haemolytica type A1 or PI3 were detected in this serum pool.

Experiment 1 - Growth of *P. haemolytica* in vitro in the presence of lung wash cells and serum

The aim of this experiment was to demonstrate phagocytosis of *P. haemolytica* by lung wash cells from SPF lambs in vitro, and to determine the effect of vaccination upon this parameter.

Design of experiment

Eight SPF lambs were allocated to 2 groups of 4 lambs each. Lambs in Group 1 were vaccinated SC in the brisket with 2 ml *P. haemolytica* type A1 vaccine at 3 weeks of age. Lambs in Group 2 were untreated.

Serum samples were collected from all 8 lambs at the time of vaccination and at intervals of 1, 2, 3, and 5 weeks post-vaccination. An additional sample was taken prior to slaughter. Antibodies of IgG class to *P. haemolytica* in these samples were measured by means of the ELISA technique as detailed in General Materials and Methods.

Seven to nine weeks after vaccination, lambs were killed in pairs (1 vaccinate and 1 control) by IV injection

of 5 ml pentobarbitone sodium (Euthatal (200 mg/ml): May & Baker Ltd., Dagenham, England). The chest was opened and the trachea clamped before removal of the lungs to avoid contamination of the lungs by blood. Lungs were lavaged by pouring sterile HBSS containing 0.1% gelatin (gelatin-Hanks) into the trachea, now with the clamp removed, through a sterile filter funnel covered with gauze. The lungs were kneaded gently, and the fluid tipped out into sterile centrifuge jars. The cells were sedimented by centrifugation at 200 g for 20 minutes at 4°C. The supernate was removed, and the cells washed twice more in gelatin-Hanks. The cells were counted in an Improved Neubauer Haemocytometer, and viability estimated by exclusion of trypan blue from the cytoplasm. The majority of the cells had morphology of AM, although other cell types, such as lymphocytes were present. Culture mixtures were set up in sterile plastic tissue-culture grade tubes with screw caps (Sterilin Ltd., Teddington, Middlesex, England) as detailed in Table 2.1. The total volume of each culture was 2.5 ml, and each was set up in triplicate. To each tube was added 10 µl of P. haemolytica suspension, giving a final concentration of  $10^{5.0}$  -  $10^{5.9}$  cfu/ml. This inoculum was prepared from a 50 ml NB culture, incubated for 6 hours at 37°C with agitation. The bacteria were

Table 2.1

Composition of cultures in Experiment 1

<u>Culture</u>	<u>Source of Serum (16%)</u>	<u>Source of lung wash cells</u> <u>(10<sup>5.3</sup>/ml)</u>
A	V	V
B	C	V
C	V	-
D	C	C
E	V	C
F	C	-

V = vaccinate

C = control

washed twice in saline by centrifugation and resuspended in saline at an estimated concentration of  $10^7$ - $10^8$  cfu/ml. These experiments were performed on the same day as the lambs were killed.

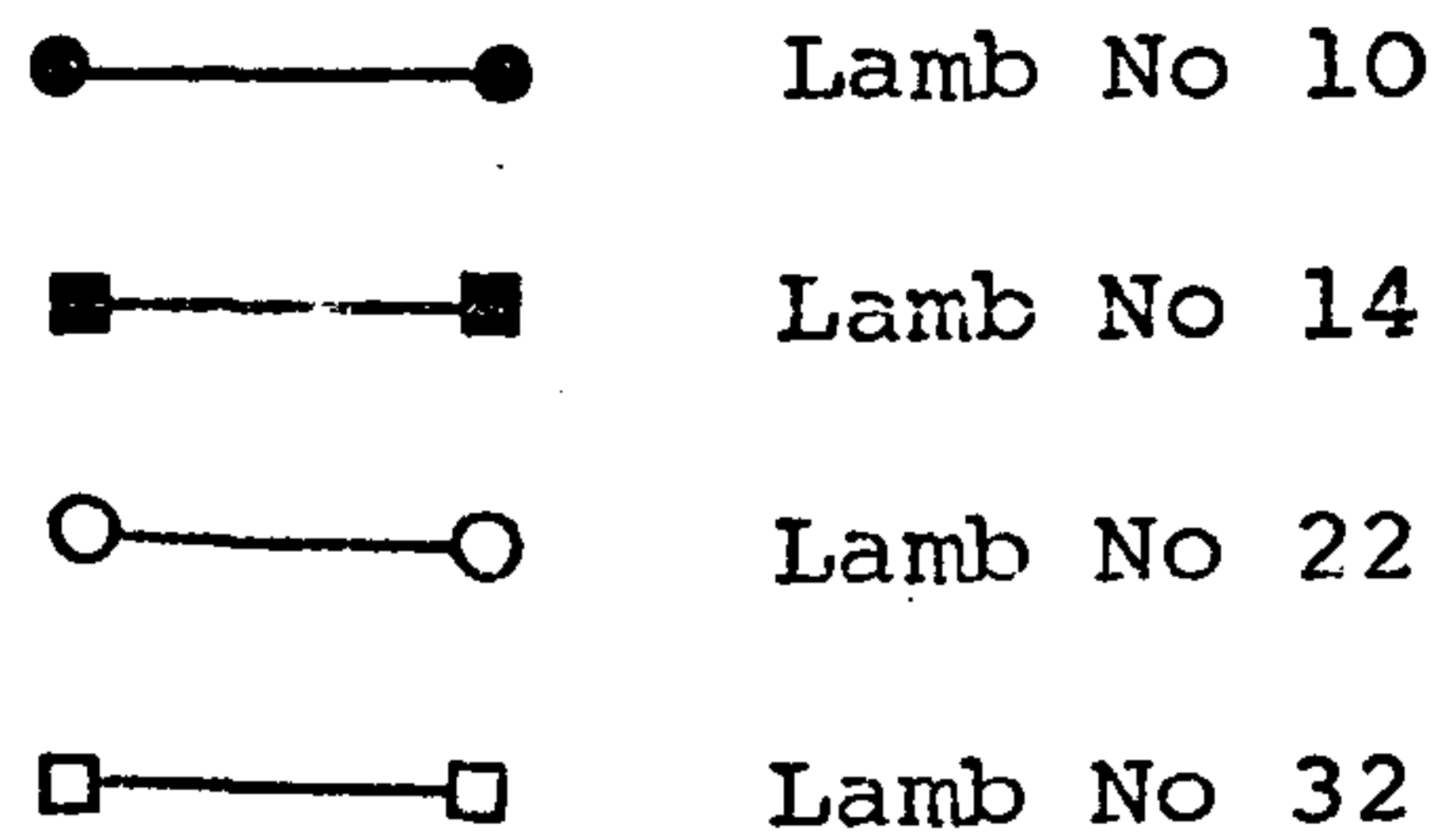
Cultures were incubated at  $37^{\circ}\text{C}$  with rotation. At the start of the experiment, and 30, 60 and 120 minutes later, 0.5 ml aliquots were removed and added to 1.5 ml ice-cold gelatin-Hanks, to stop any reaction, and centrifuged at 110 g for 4 minutes. Preliminary experiments showed that this centrifugation sedimented the lung wash cells, but did not alter the viable count of bacteria in the supernate. Ten-fold dilutions of the supernate were made, and viable counts performed by the method of Miles, Misra and Irwin (1938).

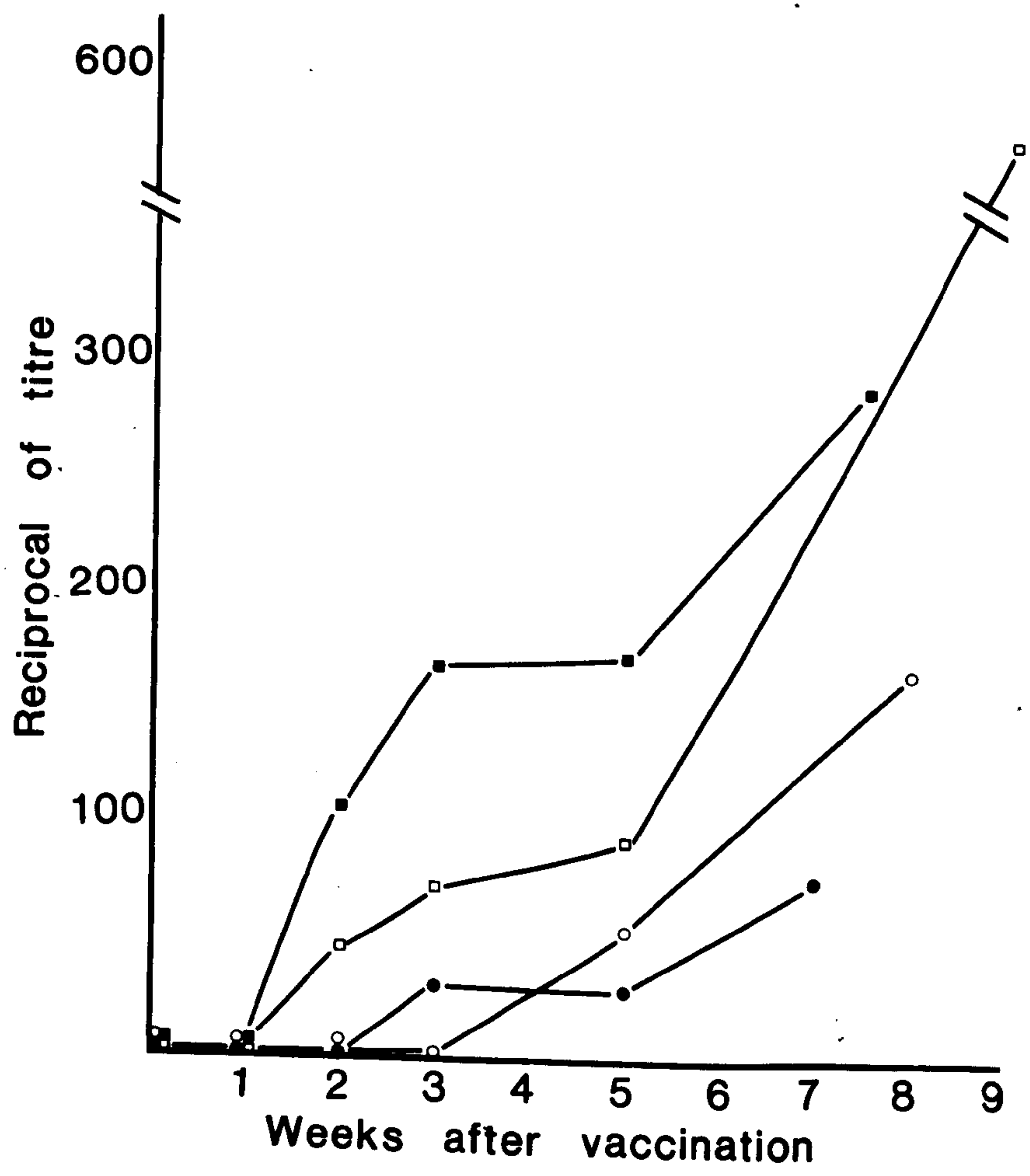
## Results

### Antibody to *P. haemolytica* as measured by the ELISA technique

The sera of the lambs in Group 2 (unvaccinated) were negative for *P. haemolytica* antibodies, as measured by this test, for the duration of the experiment. The titres of the vaccinated lambs are shown in Fig. 2.1. Specific

Figure 2.1 Titres of IgG antibodies to P. haemolytica type A1 in sera of vaccinated lambs, measured by the ELISA technique





circulating IgG was detected as early as 2 weeks post-vaccination (lamb 14 - 1/104, lamb 32 - 1/47). The titres in all 4 lambs increased by varying degrees up to week 5, and rose sharply thereafter.

Growth of *P. haemolytica* in vitro in the presence of lung wash cells and serum

Viable counts in the supernates of cultures were converted to  $\log_{10}$ , and means calculated for each culture. An analysis of variance was performed on the results, which are depicted graphically in Figs. 2.2-2.5.

The cultures in which serum from control lambs was included (Cultures A, C and E) supported the growth of *P. haemolytica* in the supernate, and over the 2 hours of the experiment the numbers of organisms increased by approximately  $10^{1.5}$  cfu/ml. In cultures which contained serum from vaccinated lambs (Cultures B, D and F), the increase in numbers of *P. haemolytica* in the supernate was less than 10-fold. The difference between these 2 groups was highly significant ( $P < 0.001$ ). This effect of serum was the same regardless of whether the lung wash cells in the system were from vaccinated or control lambs,



Figure 2.2 Viable counts of P. haemolytica type A1  
in the supernates of cultures A and B  
at various time intervals after infec-  
tion. SED = 0.150

○—○ culture B  
●—● culture A

Figure 2.3 Viable counts of P. haemolytica type A1  
in the supernates of cultures D and E  
at various time intervals after infec-  
tion. SED = 0.150

○—○ culture D  
●—● culture E

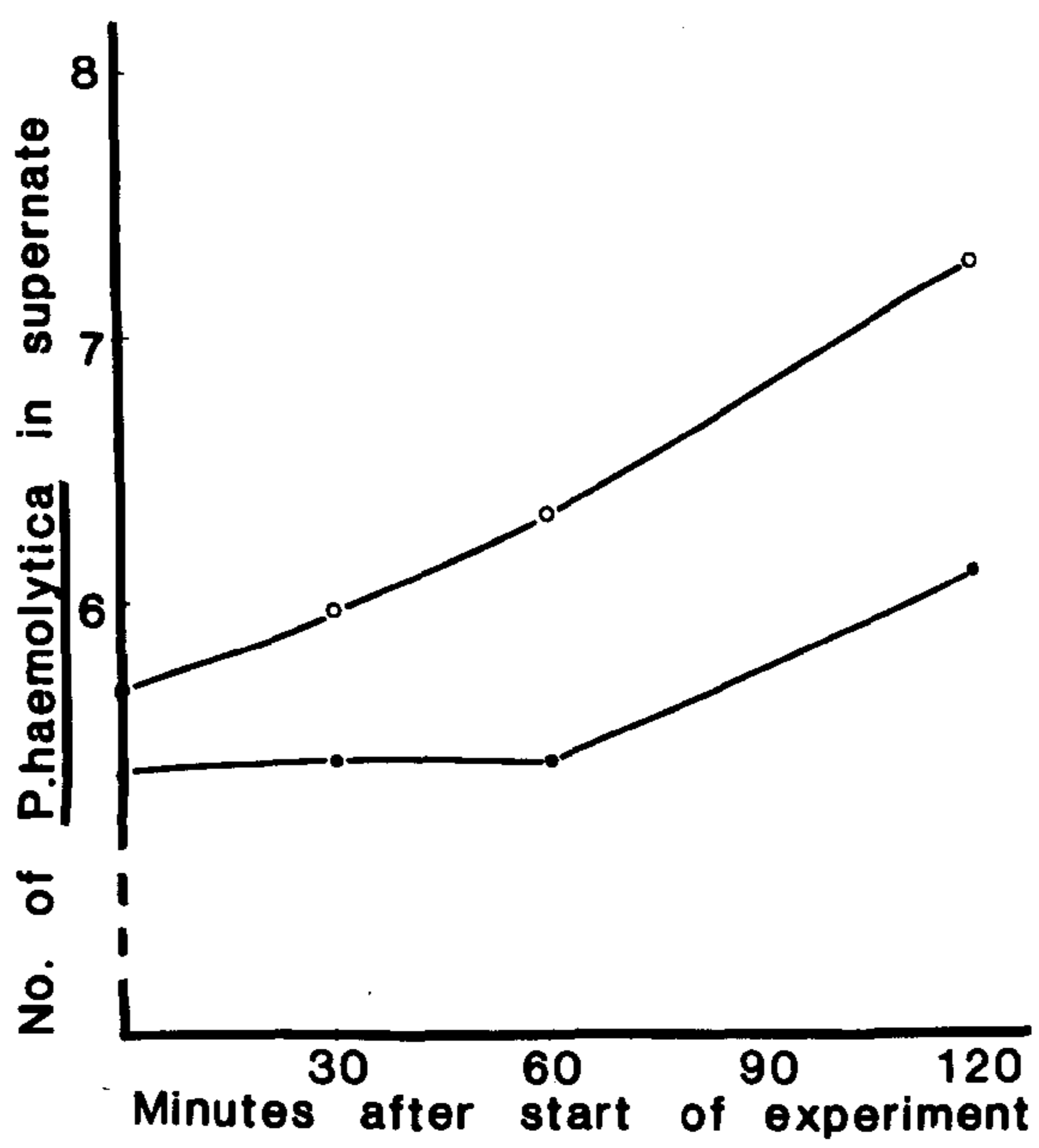
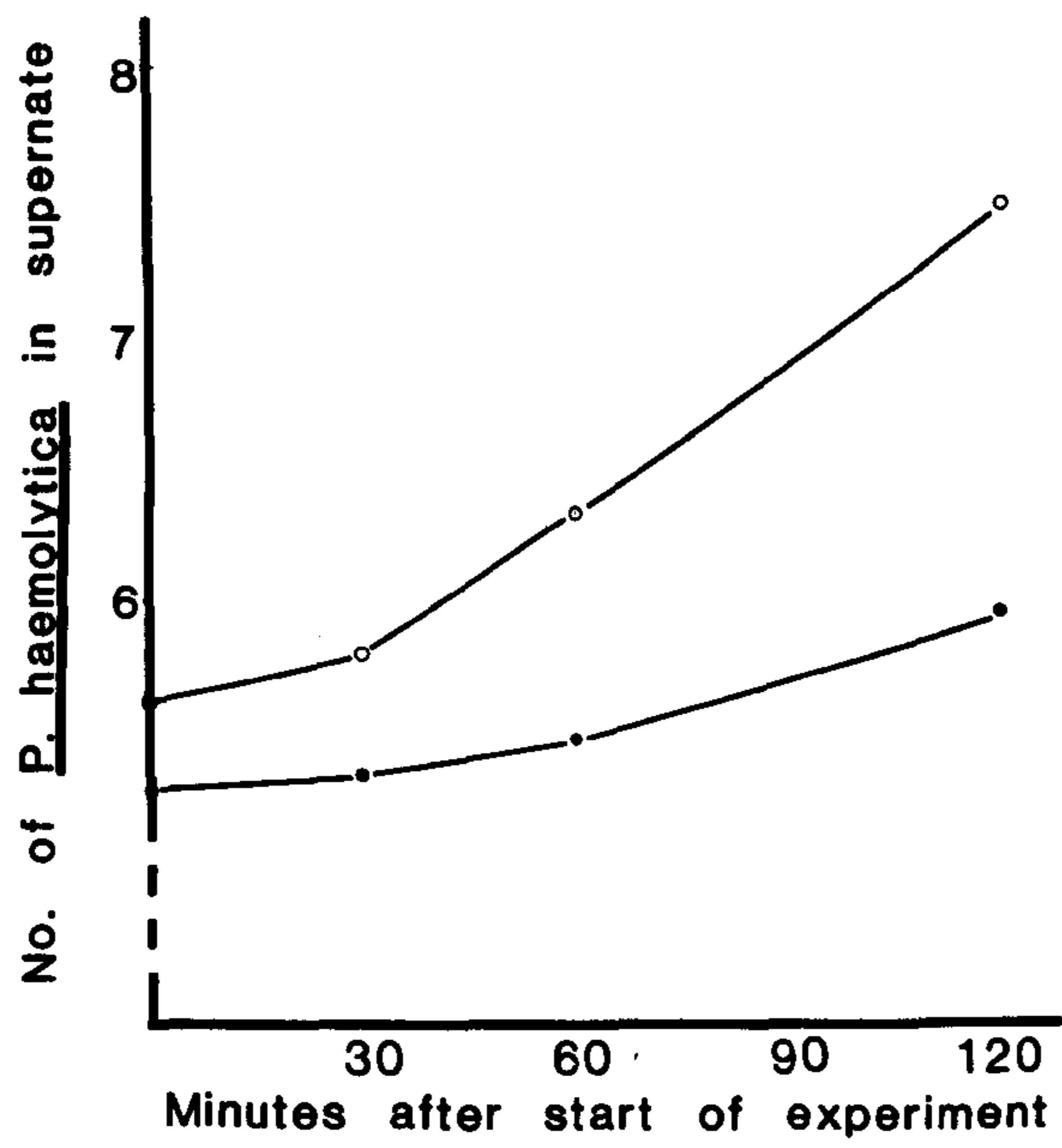
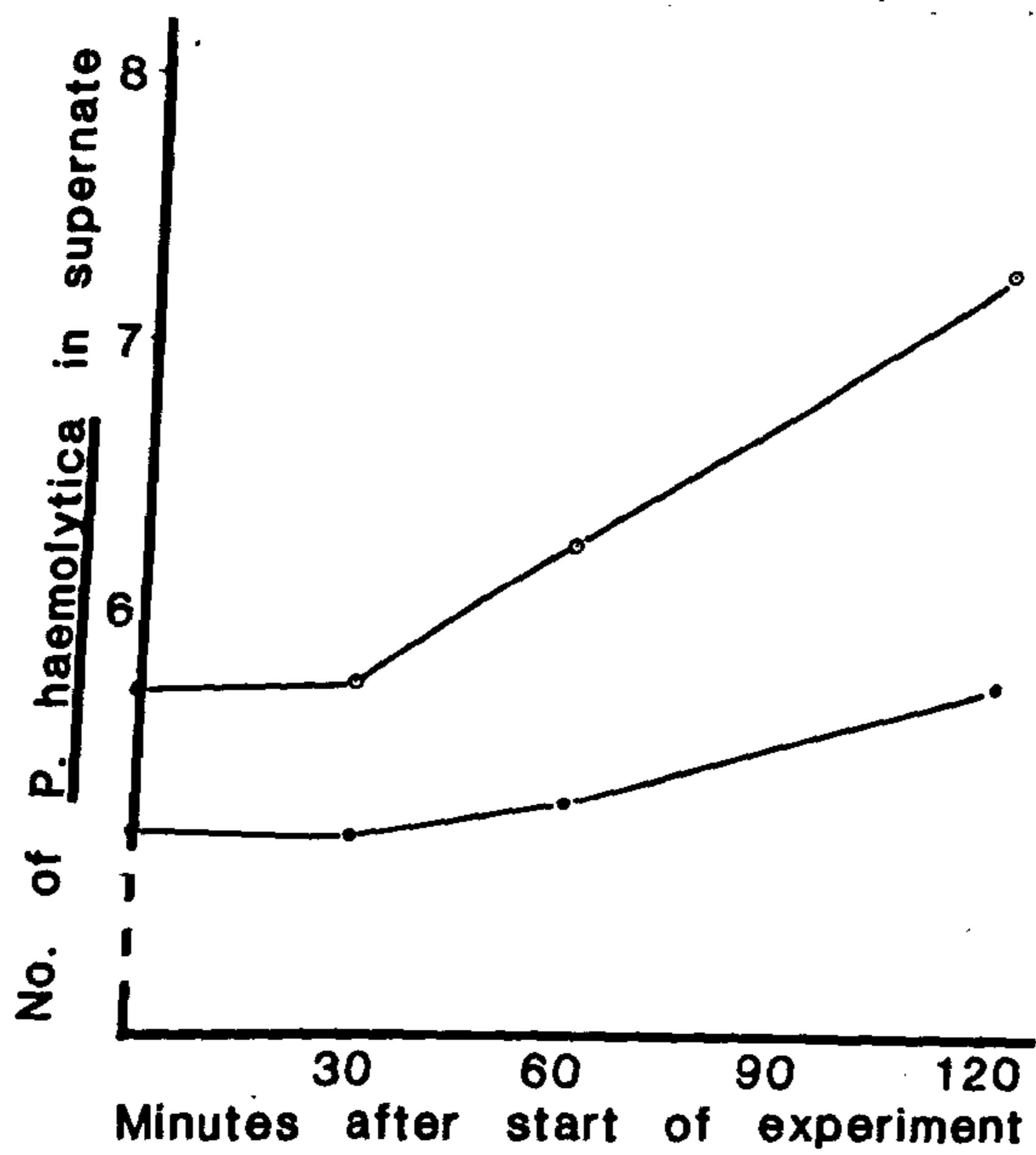
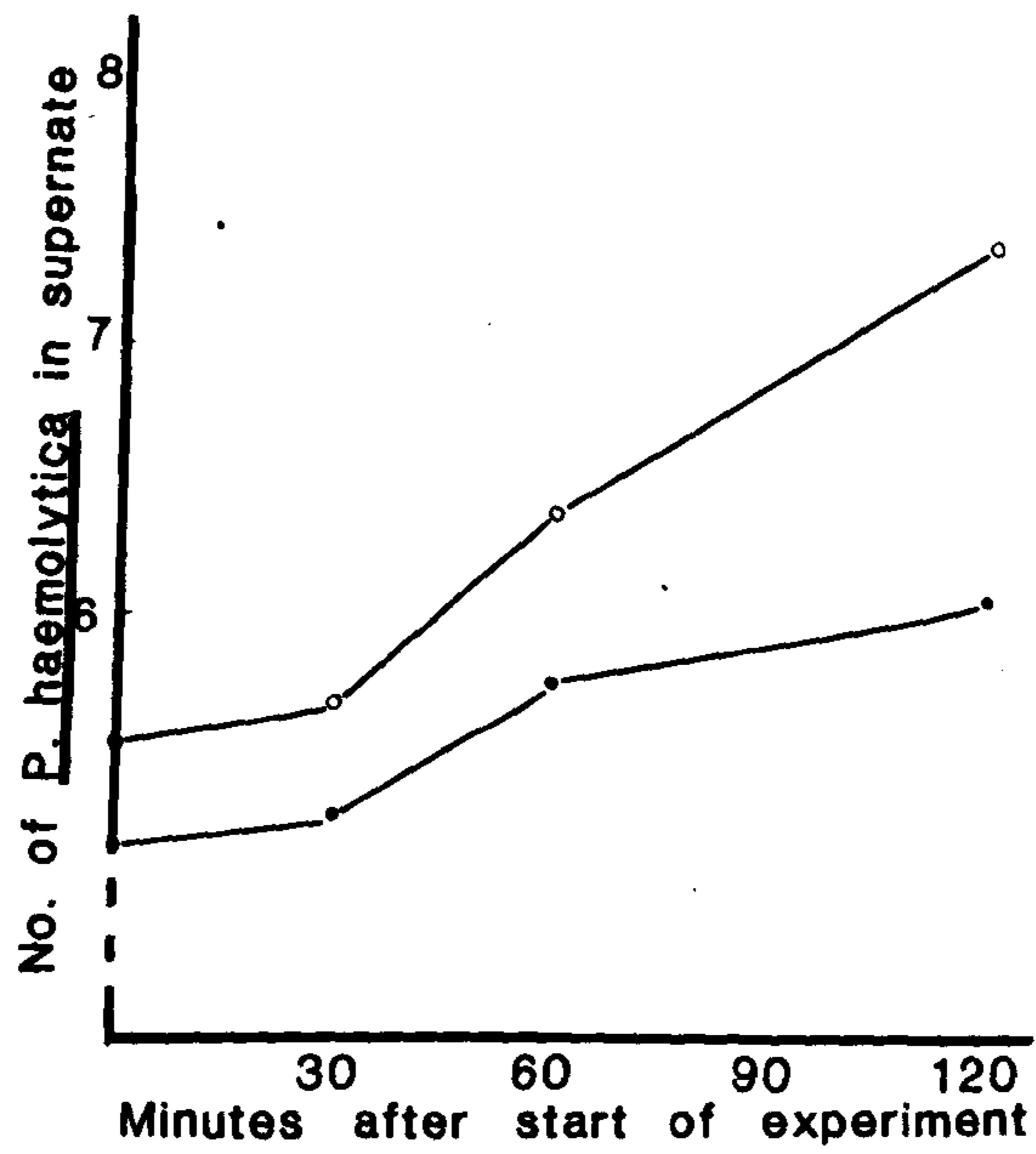


Figure 2.4 Viable counts of P. haemolytica type A1 in the supernates of cultures C and F at various time intervals after infection. SED = 0.150

○—○ culture F  
●—● culture C

Figure 2.5 Mean viable counts of P. haemolytica type A1 in the supernates of cultures containing serum from vaccinates (cultures A, C and E) and cultures containing serum from controls (cultures B, D and F) at various time intervals after infection. SED = 0.087

○—○ cultures B, D and F  
●—● cultures A, C and E



or indeed if there were no lung wash cells present (Cultures C and F). This is illustrated in Fig. 2.5, in which the means at each time interval have been plotted for all cultures containing serum from vaccinates (Cultures A, C and E) and all those containing serum from controls (Cultures B, D and F). The curves in this graph are similar to those in Figs. 2.2-2.4, which illustrate the curves from each combination of cells and sera. No phagocytosed bacteria could be seen within lung wash cells, which had been exposed to P. haemolytica type A1, smeared onto a slide, and stained with Giemsa.

It can be concluded from this experiment that 7-9 weeks after vaccination, sera from lambs vaccinated against P. haemolytica have a bacteriostatic effect upon the growth of P. haemolytica. The presence of lung wash cells, whether from vaccinated or control lambs, had no influence upon this effect. It is debatable as to whether phagocytosis of P. haemolytica in these cultures occurred, but if it did, the level was too low to detect in this system.

Experiment 2 - The roles of antibody and cell-mediated immunity (CMI) in combined infection of SPF lambs with PI3 virus and P. haemolytica type A1

Many aspects of this experiment were carried out by collaborators at the Moredun Research Institute and the principal role of the author was in experimental design and interpretation of the results. The lymphocyte transformation tests were performed by Mr. C. Burrells, clinical examinations of lambs by Dr. J. M. Sharp, and pathology by Dr. B. Rushton. The serology was carried out by Mr. D. A. Thompson. The method of challenge of SPF lambs with P. haemolytica, superimposed upon PI3 virus infection is described in detail in "General Materials and Methods - Techniques employed by collaborators".

Design of experiment

Twenty-seven hysterectomy-derived, colostrum-deprived lambs were reared under SPF conditions. These lambs were allocated to 2 groups of 8 lambs, a third group of 7 lambs, and a fourth group of 4 lambs. The treatments given to each group are summarised in Table 2.2. At 2 weeks of

Table 2.2

Summary of Treatments given to Lambs in Experiment 2

<u>Group</u>	<u>Number of Lambs</u>	<u>Treatment at age (days)</u>			
		<u>14</u>	<u>35</u>	<u>41</u>	<u>42</u>
1	8	Vaccinated with <u>P. haemolytica</u>	Infected with PI3	n.t.	Infected with <u>P. haemolytica</u>
2	8	n.t.	Infected with PI3	Antiserum IP	Infected with <u>P. haemolytica</u>
3	7	n.t.	Infected with PI3	Negative Serum IP	Infected with <u>P. haemolytica</u>
4	4	n.t.	n.t.	n.t.	n.t.

n.t. = not treated

age, the 8 lambs in Group 1 were vaccinated SC with 2 ml P. haemolytica type A1 vaccine over the sternum. Lambs in Groups 2 and 3 were not treated at this time. Three weeks later, the lambs in Groups 1, 2, and 3 were challenged intrabronchially and intratracheally with  $10^{7.5}$  TCID<sub>50</sub> PI3 virus (see General Materials and Methods). Six days after infection with PI3 virus, the lambs in Group 2 each received 200 ml of the antiserum to P. haemolytica type A1 raised in colostrum-deprived lambs, and the lambs in Group 3 received 200 ml of the control serum. The serum was administered by IP injection in 2 doses of 100 ml, and the second dose given 6 hours after the first. Eighteen hours after the second injection, the lambs in Groups 1, 2 and 3 were exposed in groups of 4 for 15 minutes to an aerosol of P. haemolytica type A1 produced by a Wright's nebuliser ( $10^{3.0}$  cfu per litre of air). Lambs in Group 4 were neither vaccinated, nor infected with either PI3 virus or P. haemolytica, and served as controls.

Blood samples were taken from 4 lambs in each group prior to vaccination, and thereafter at intervals during the 4 week period until exposure to P. haemolytica;



lymphocyte cultures were set up from these bloods to determine specific proliferative responses in the presence of P. haemolytica type A1 antigen (see General Materials and Methods). Further cultures were also prepared from the same lambs immediately prior to infection with P. haemolytica type A1, 3 days and 5 days after infection. The lambs in Group 4, which were not infected, were sampled on these latter 3 occasions.

Serum samples were collected from lambs at weekly intervals throughout the experiment, and additional samples were taken prior to challenge. These were tested for antibody to P. haemolytica by the IHA test (see General Materials and Methods).

## Results

### Serology

Lambs vaccinated with the P. haemolytica vaccine responded by production of antibodies detected by the IHA test (Table 2.3). Maximum titres (geometric mean = 45.2) were observed on the day that lambs were challenged by exposure to aerosol of P. haemolytica.

Table 2.3

IHA titres of sera from lambs in Experiment 2

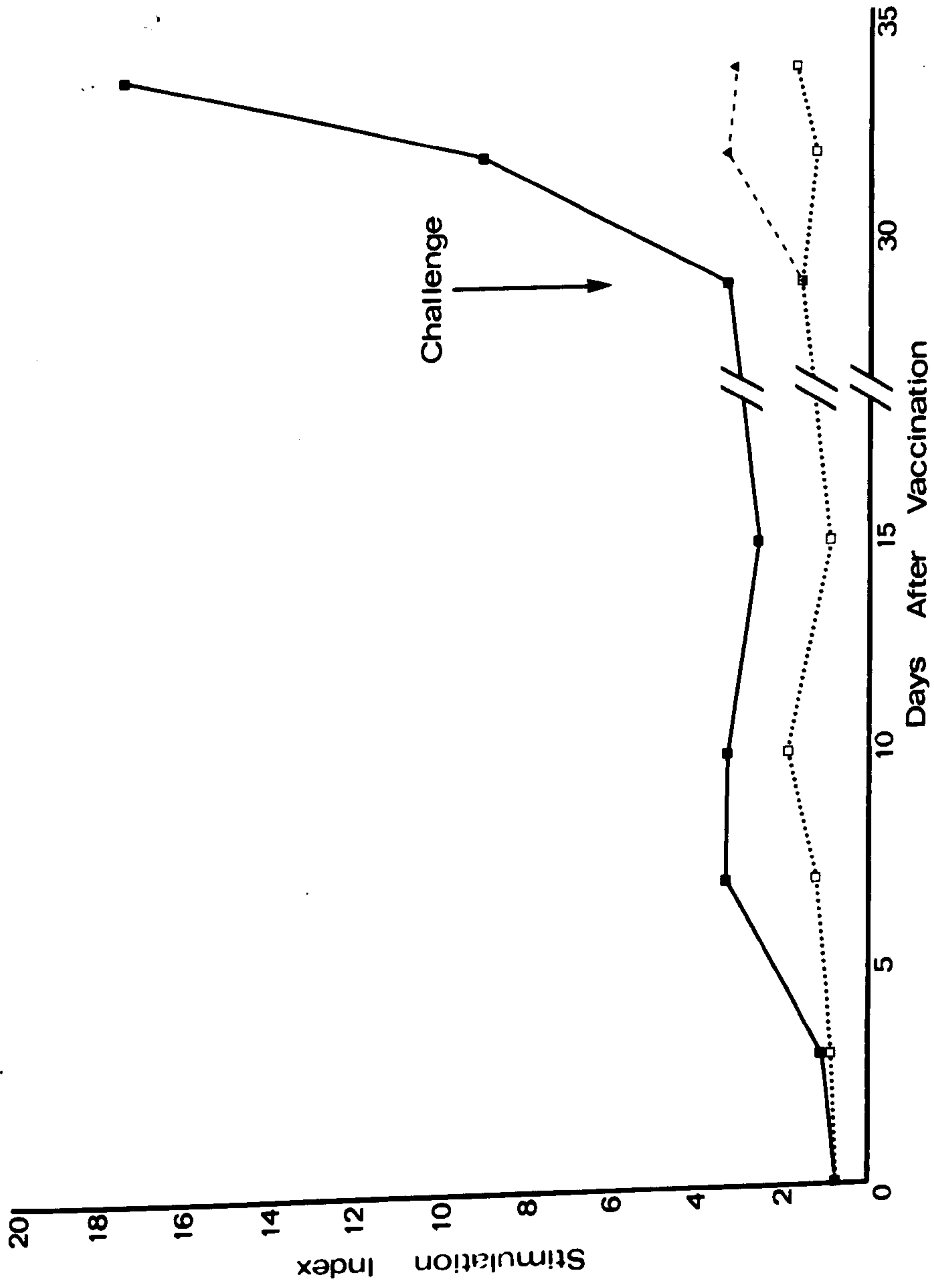
<u>Group</u>	<u>Lamb No</u>	<u>IHA titre in sera at age</u>					
		14d	21d	28d	35d	41d	42d
1	9	-	8	32	64	ND	128
	13	-	4*	8	16	ND	64
	17	-	8	4	32	ND	128
	18	-	4	4	8	ND	8
	21	-	-	8	16	ND	16
	23	-	-	4	16	ND	32
	31	-	4	4	64	ND	64
	34	-	-	4	32	ND	64
2	1	-	ND	-	-	-	8
	2	-	ND	-	-	4	64
	3	-	ND	-	-	-	32
	4	-	ND	4	-	4	32
	6	-	ND	4	-	4	4
	7	-	ND	-	-	-	64
	8	-	ND	-	-	4	-
	90	-	ND	4	-	-	64
3	11	-	ND	4	4	ND	4
	15	-	ND	-	-	ND	-
	20	-	ND	4	4	ND	16
	26	-	ND	-	-	ND	4
	29	-	ND	-	-	ND	4
	33	-	ND	-	4	ND	8
	37	-	ND	-	ND	ND	4

\* Titres of  $\leq 4$  are considered to be negative

In 6 of the lambs in Group 2 treated with antiserum (IHA titre 1024), the treatment was reflected by the appearance of raised IHA titres (geometric mean = 35.9). In 2 other treated lambs, no rise in serum antibody could be detected.

#### Lymphocyte transformation responses

The responses of lymphocytes from vaccinated and unvaccinated lambs to P. haemolytica are shown in Fig. 2.6. Lymphocytes from the unvaccinated control lambs (Group 4) did not respond to stimulation with P. haemolytica type A1 until after challenge when the mean S.I. increased to 3.3 and 3.4 on the 3rd and 5th days respectively after infection. No response to P. haemolytica type A1 was observed in lymphocytes cultured from 4 of the lambs in Group 1 prior to vaccination or 3 days after vaccination. However, by the 7th day after vaccination, the mean S.I. was 3.4, and remained at a similar level during the next 4 weeks, until challenge with the aerosol of P. haemolytica type A1. Within one day of challenge, the mean S.I. had begun to rise, to reach a value of 17.5, 5 days after challenge.



## Clinical responses, microbiology and pathology

The clinical responses, microbiology and pathology of these lambs following challenge are summarised in Table 2.4.

### Clinical responses

Lambs in Groups 1, 2 and 3, which were infected with PI3 virus developed the clinical signs of pyrexia, dyspnoea, anorexia and dullness described previously in association with this infection (Wells, Sharp, Rushton, Gilmour and Thompson, 1978). After exposure to the aerosol of P. haemolytica, lambs in the vaccinated group showed no further signs of clinical illness except a slight pyrexia. This was observed in only two lambs on the day after infection with P. haemolytica. In contrast, 4 of the 7 lambs in Group 3, treated with negative control serum were dull and 7 of the 8 lambs in Group 2 treated with antiserum to P. haemolytica showed pyrexia and dullness during the 5 days following infection with P. haemolytica. Dyspnoea was observed only in the unvaccinated control and antiserum treated lambs. The two lambs which did not appear to have absorbed antibody

Table 2.4

Summary of clinical responses, microbiology and serology from lambs in Experiment 2

Group No	Lamb No	Lesion score	Total score (lesion + clinical)	IHA titre (1 month)	Viable counts in lung (log <sub>10</sub> per g of tissue) Lesion	Normal	
1	9	-	-	128	-	-	
	13	5	6	64	6.4	-	
	17	-	-	128	5.8	-	
	18	5	6	8	-	-	
	21	-	-	16	-	-	
	23	-	-	32	-	-	
	31	-	-	64	-	-	
	34	-	-	64	-	-	
	2	1	5	10	8	7.8	-
		2	5	9	64	6.6	-
3		5	12	32	7.6	3.7	
4		5	7	32	6.6	-	
6		10	23	4	8.8	5.4	
7		5	8	64	6.0	3.7	
8		10	26	-	9.0	6.5	
90		-	-	64	-	-	
3		11	10	21	4	8.2	2.5
		15	5	9	-	7.5	3.7
	20	5	5	16	6.4	-	
	26	-	-	4	-	-	
	29	5	8	4	5.9	-	
	33	5	6	8	7.6	-	
	37	-	1	4	-	-	

in the group treated with antiserum exhibited more marked clinical signs than the other lambs in this group and both these lambs died on the fifth day after infection with P. haemolytica.

### Pathology

Macroscopic lesions of pneumonia were observed in the lungs of 2 of the 8 vaccinated lambs in Group 1, 7 of the 8 lambs in Group 2 and 5 of the 7 lambs in Group 3. The extent of lesions present in vaccinated lambs, as estimated by planimetric measurement of the drawings showing the pneumonic lesions, was significantly less ( $P < 0.01$ ) than in Group 2 lambs treated with the antiserum and Group 3 lambs receiving negative control serum. There was no significant difference between the extent of pneumonic lesions observed in lambs in the antiserum-treated group, and those lambs in Group 3.

Microscopically, the pneumonic lesions observed in lambs from all groups were similar to those associated with P. haemolytica infection (Rushton, Sharp, Gilmour and Thompson, Journal of Comparative Pathology, in press).

Considering both the results of clinical observation and extent of macroscopic lesions, there was no significant difference observed between Groups 2 and 3. In contrast, the severity of disease assessed in this way in the vaccinated group was significantly less ( $P < 0.01$ ) than in either the Group 2 or Group 3.

### Microbiology

P. haemolytica type A1 was isolated from pneumonic areas of lung in 2 of the 8 vaccinated lambs. By comparison, the bacteria were isolated from pneumonic lung tissue from 7 of 8 lambs in Group 2 treated with antiserum and 5 of 7 lambs in Group 3. Mean bacterial counts per gram of pneumonic tissue from these lambs in Groups 2 and 3 were  $10^{7.4}$  (Range  $10^{6.0}$ - $10^{9.0}$ ) and  $10^{7.1}$  (Range  $10^{5.9}$ - $10^{8.2}$ ) respectively compared with  $10^{6.4}$  and  $10^{5.8}$  in the two vaccinates. P. haemolytica type A1 was isolated from apparently normal lung tissue from 4 of the 8 lambs in Group 2 (Range  $10^{3.7}$ - $10^{6.5}$ ) and 2 of the 7 lambs in Group 3 ( $10^{3.5}$ ,  $10^{3.7}$ ).



### Discussion

The phagocytic assay used in Experiment 1 has been successful in demonstrating phagocytosis of Staphylococcus albus by human granulocytes and mouse peritoneal macrophages (van Furth, van Zwet and Leigh, 1978). In Experiment 1, it is doubtful whether phagocytosis by sheep lung wash cells was demonstrated. However, sera from immunised lambs had a bacteriostatic effect upon P. haemolytica in this system, both in the presence and absence of AM. The presence of antibodies of the IgG class to P. haemolytica in these sera was demonstrated by the ELISA technique (Fig. 2.1). As this experiment was performed in vitro, it is difficult to assess whether this effect is of importance in the intact animal. The apparent failure of lung wash cells to phagocytose P. haemolytica may be a reflection upon their inability to phagocytose and inactivate P. haemolytica in vivo, or, alternatively, the conditions of the experiment may not have been optimal for the demonstration of phagocytosis. More recent experiments have indicated that P. haemolytica has some toxic effect upon sheep AM when they are incubated together in vitro under conditions similar to those of Experiment 1, which may suggest that

sheep AM are unable to phagocytose P. haemolytica (Sutherland, personal communication). Benson, Thompson and Valli (1978) found a similar cytotoxic effect on exposure of bovine AM to live P. haemolytica which were not readily phagocytosed by the AM. In contrast, heat-killed P. haemolytica were phagocytosed to a greater extent, and produced only mild cytotoxic changes.

The SSE vaccine of P. haemolytica type A1 has previously been shown to protect against the effects of challenge infection with this type (Gilmour, Martin, Sharp, Thompson and Wells, 1979). Previously, lambs were challenged 2 months after vaccination, and in some cases, 2 doses of vaccine were given. In Experiment 2, protection was demonstrated one month after a single vaccination (Group 1). Control lambs exhibited clinical signs of respiratory disease, and at post-mortem pneumonic lesions were more extensive than in the vaccinates. A vaccine of this type may be of value in the prophylaxis of pneumonic pasteurellosis and more extensive studies have been carried out with a view to its development (Gilmour, Martin, Sharp, Thompson and Wells, 1979).

In 6 of the 8 lambs which received antiserum to P. haemolytica (Group 2) serum antibody titres were similar to those recorded in the vaccinated group (Group 1) at the time of challenge (Table 2.3). However, no protective effect could be attributed to the antiserum, which supports the contention that serum antibody is not the only factor involved in protection of lambs against respiratory disease associated with P. haemolytica infection. Previous experiments have shown that serum antibodies are transferred into the secretions of the respiratory tract of sheep (Smith, Wells, Burrells and Dawson, 1976; Wells, Dawson, Smith and Smith, 1977). Consequently, it is unlikely that any difference between the 2 groups is due to a difference in the concentration of antibody in the secretions bathing the respiratory mucosa.

The antiserum used for passive protection in this experiment was raised by hyperimmunisation of lambs, and it is feasible that the antibodies produced were not protective. The fact that IHA titre does not necessarily correlate with protection from challenge makes this a more likely possibility. This point might be clarified by repetition of the experiment using sera from lambs which

were recovering from P. haemolytica infection.

The data from the lymphocyte transformation studies showed a specific proliferative response in lymphocytes from vaccinates cultured in the presence of P. haemolytica type A1 SSE. Such in vitro responses to antigens are considered to be a measure of cell-mediated immunity (CMI) in vivo. It was shown that in patients suffering from acute infectious mononucleosis, loss of the cutaneous response to tuberculin antigen, which is considered to be a measure of CMI, correlated with depression of lymphocyte transformation responses in vitro (Mangi, Niederman, Kelleher, Dwyer, Evans and Kantor, 1974). Fleer, van der Hart, Blok-Schut and Schellekens (1976) demonstrated good correlation between responses to PPD in skin tests and lymphocyte transformation responses with PPD in healthy individuals. The proportion of lymphocytes capable of this response appears to increase markedly within a few days of aerosol challenge by P. haemolytica type A1. This may be representative of a secondary cellular immune response of rapid onset, and this could play an important part in resistance to infection.

The results of Experiment 1 indicate that serum from immunised lambs affects the growth of P. haemolytica in vitro so humoral immunity cannot be entirely discounted. Antibody may play a valuable ancillary role in bacterial clearance in vivo. In support of this, it may be significant that no antibodies were detected in sera from the 2 lambs in Group 2 which died. Humoral immune responses are also of major importance in protection against the related organism, P. multocida (Woolcock and Collins, 1976).

It can be concluded from these results that monitoring of circulating antibody may not provide a reliable index of the efficacy of vaccines. It would be unwise to disregard serological data entirely, since there may be some relationship between the humoral immune response and CMI induced by vaccination. If CMI responses are involved in protection against P. haemolytica then clearly some method of evaluating these responses would be of value in monitoring potential vaccines.

Non-specific resistance to *P. haemolytica*Introduction

Several Pasteurella vaccines are available commercially, yet despite their widespread use, many outbreaks of pasteurellosis occur (Veterinary Investigation Diagnosis Analysis II Report, 1977). However, it has been suggested on occasion by veterinarians that the Pasteurella vaccine, Carovax (The Wellcome Foundation Ltd., Beckenham, England), administered to sheep during an outbreak of pasteurellosis, may reduce mortality within two days. No data are available to support these observations; if they are true it is possible that by the time an outbreak is recognised, that most of the susceptible animals will have succumbed to the infection, and the number of deaths started to decrease, regardless of vaccine treatment. Alternatively, the vaccine may induce some resistance to infection. Protection of such rapid onset is not likely to be the result of a conventional immune response.

This chapter describes an investigation of the possible existence of such an effect, using the model of IP infection of mice with *P. haemolytica* developed in Chapter 1.

## Materials and Methods

### Mice

C57 black mice, as described in General Materials and Methods, were used in all experiments.

### Vaccine

The vaccine used in this study was Carovax (The Wellcome Foundation Ltd., Beckenham, England), which is a formolised alum-treated culture of P. multocida types II, III and IV, and P. haemolytica types A2, T3 and T10.

### Bacteria and challenge of mice

P. haemolytica type A1, as described in General Materials and Methods, was used for challenge infection of mice by the IP route. This method of challenge infection was similar to that described in Chapter 1, except that 5 hours NB cultures were used for preparation of the challenge inocula.

The strain of E. coli, B188, is also described in General Materials and Methods. Three hundred ml volumes of NB, inoculated from fresh blood plate cultures of E. coli, incubated at 37°C for 18 hours, were used for preparation of

challenge inocula. The bacteria were washed twice in 0.15 M saline, and individual mice were inoculated IP with 0.5 ml of appropriate saline dilutions of washed organisms.

#### Statistical treatment of results

In Experiments 2, 3, 4 and 6, viable organism counts in the livers of mice were expressed as  $\log_{10}$  cfu, and an analysis of variance performed, from which group means and SED were determined.



Experiment 1 - The effect of Carovax given to mice 48 hours, 24 hours and 6 hours prior to challenge upon LD<sub>50</sub> of

P. haemolytica

Design of experiment

One hundred mice were randomly allocated to four groups of 25 mice each (Table 3.1). Mice in Group 1 were uninoculated controls. Mice in Groups 2, 3 and 4 were each injected SC with 0.1 ml Carovax 48 hours, 24 hours and 6 hours respectively, before IP challenge with P. haemolytica in gastric mucin. Five dose levels of challenge organisms were given to 5 mice in each group ( $10^{4.8}$ - $10^{8.8}$  cfu), and deaths were recorded 48 hours later.

Results

The results are shown in Table 3.1. Treatment with the vaccine at all three time intervals prior to challenge caused an increase in LD<sub>50</sub>. The largest increase was in Group 3, when the time interval between vaccine treatment and challenge was 24 hours. The LD<sub>50</sub> for this group was 100-fold greater than that of the controls, indicating that vaccine treatment gave considerable protection.

Table 3.1

Deaths in mice given Carovax at different time intervals prior to challenge with

*P. haemolytica*

Group	Time interval between vaccine and challenge *	Deaths at challenge dose (cfu/mouse)					LD <sub>50</sub>
		10 <sup>8.8</sup>	10 <sup>7.8</sup>	10 <sup>6.8</sup>	10 <sup>5.8</sup>	10 <sup>4.8</sup>	
1	no vaccine given	5	5	5	5	0	10 <sup>5.3</sup>
2	48	5	3	3	0	0	10 <sup>7.0</sup>
3	24	5	5	0	0	0	10 <sup>7.3</sup>
4	6	4	4	5	1	0	10 <sup>6.4</sup>

\* = hours

Experiment 2 - Effect of vaccine treatment of mice at different time intervals prior to challenge with P. haemolytica on growth of bacteria in the liver

This experiment was designed to determine whether conventional commercial vaccine treatment at various time intervals prior to challenge with P. haemolytica retarded the growth of organisms in the liver.

Design of experiment

Groups of 10 mice were injected SC with 0.1 ml of the vaccine on one occasion 14 days, 7 days, 48 hours, 24 hours, 12 hours and 1 hour before challenge with P. haemolytica type A1. The dose regime recommended for Carovax in sheep is two SC injections, given at an interval of 4 weeks, the second given at least 2 weeks before the disease is expected to appear. Therefore, a further group of 10 mice were each injected SC on 2 occasions, at an interval of 4 weeks, with 0.1 ml vaccine.

Each of these groups was challenged IP with P. haemolytica type A1 in gastric mucin at the appropriate time interval after vaccine treatment, along with groups of

10 untreated control mice. Due to the amount of work involved, it was not possible to challenge all groups on the same day, and unavoidably the challenge dose varied slightly from day to day (mean  $\pm$  SE =  $10^{7.05} \pm 10^{0.9}$  cfu/mouse). At the time of challenge and 6 hours later, 5 mice from each experimental group were killed by cervical dislocation.

### Results

Table 3.2 shows the viable counts of P. haemolytica type A1 recovered from control and vaccinated mice immediately after challenge, and 6 hours later. The bacteria multiplied in the livers of control mice, and mice in which the time interval between vaccine treatment and challenge was 1 hour, 48 hours, 7 days, 14 days and the group which had been given 2 doses of vaccine. In contrast, when the time interval between vaccine treatment and challenge was 12 hours or 24 hours, viable counts in the livers decreased after challenge. Viable counts in the livers of these mice and control mice 6 hours after challenge were significantly different ( $P < 0.001$ ). The time interval between vaccine treatment and challenge which had the most marked

Table 3.2

Post-challenge viable counts of *P. haemolytica* type A1 in the livers of control mice and of mice inoculated SC with 0.1 ml vaccine at different time intervals before challenge with

*P. haemolytica* type A1

Hours after challenge	Time interval between treatment with vaccine and challenge		6 weeks		2 weeks								
	C	V	C	V	C	V							
0	5.50*	5.55	5.50	5.51	5.02	5.07	5.10	5.25	5.30	5.64	5.73	5.80	5.70
6	7.53	7.92	7.53	4.89**	7.70	3.84**	7.66	6.68	7.63	8.04	8.15	7.09	6.66

C = control

V = vaccinate

\* = mean ( $\log_{10}$  cfu)

SED = 0.270

\*\* significantly different from control ( $P < 0.001$ )

effect was 24 hours, so this was adopted as a standard time interval in further investigations.

Experiment 3 - Effect of dilution of Carovax on the outcome of challenge with P. haemolytica type A1

This experiment was undertaken to determine the effect of dilution of the vaccine with the standard time interval between inoculation and challenge of 24 hours.

Design of experiment

Groups of 10 mice were each inoculated SC with 0.1 ml of a series of 2-fold dilutions of Carovax in PBS, ranging from 1/2-1/32 dilutions of the original preparation. One control group of 10 mice was inoculated SC with 0.1 ml PBS, and another was untreated. The mice were challenged 24 hours later IP with P. haemolytica type A1 in gastric mucin ( $10^{7.0}$  cfu/mouse) and viable organism counts were performed on the livers of these mice at the time of challenge and 6 hours later.

Results

The results of this experiment are shown in Table 3.3. P. haemolytica type A1 did not multiply in the livers of mice inoculated with undiluted vaccine, and after dilution up to and including 1/4, by 6 hours after infection. Viable counts in the livers of these mice 6 hours after challenge

Table 3.3

Post-challenge viable counts of *P. haemolytica* type A1 in the livers of control mice and of mice inoculated SC with 0.1 ml of dilutions of vaccine, and PBS, 24 hours before challenge

with *P. haemolytica* type A1

Hours after Challenge	Dilution of vaccine given 24 hours before challenge							
	Control	PBS	Undiluted	1/2	1/4	1/8	1/16	1/32
0	6.06*	6.04	6.11	6.04	5.88	5.98	5.96	6.00
6	7.95	7.93	4.78***	5.72***	5.96***	7.53	7.34	7.39

\* mean ( $\log_{10}$  cfu)

SED = 0.263

\*\*\* significantly different from control ( $P < 0.001$ )



were significantly different from controls ( $P < 0.001$ ). These results indicate that the factor responsible for bacterial multiplication was present in a concentration too low to be effective when the vaccine was diluted further than  $.1/4$ .

Experiment 4 - Effect of treatment with individual components of the vaccine on the outcome of challenge with P. haemolytica type A1

Experiment 4 was designed to discover which components of Carovax were responsible for the protective effect observed 24 hours after injection of the vaccine.

Design of experiment

The constituents of the vaccine, without the formalised organisms, were kindly provided by Dr. L. K. Nagy of the Wellcome Foundation Ltd. The vaccine contains the preservative, thiomersal, and either this or the aluminium hydroxide adjuvant could have been responsible for the protective effects observed in mice injected with the vaccine. Placebo vaccines were prepared from the same nutrient broth normally used to grow the vaccine strains of P. multocida and P. haemolytica, aluminium hydroxide and thiomersal. One placebo contained broth and aluminium hydroxide, the other contained in addition thiomersal, all at the same concentrations as they appear in Carovax.

Forty mice were allocated to 4 groups of 10 mice each (Table 3.4). One control group was left untreated and

Table 3.4

Growth of *P. haemolytica* in livers of mice inoculated SC with vaccine or its constituent components 24 hours before IP challenge with *P. haemolytica* type A1 in gastric mucin

Hours after challenge	Vaccine constituents		Vaccine constituents with thiomersal
	Control	no thiomersal	
0	6.15*	6.31	6.09
6	8.29	6.06***	8.12

\* Mean ( $\log_{10}$  cfu)

SED = 0.264

\*\*\* significantly different from control ( $P < 0.001$ )

another received 0.1 ml complete vaccine SC. A third group was injected SC with 0.1 ml of the vaccine constituents without preservative, and the fourth group was given 0.1 ml of the vaccine constituents with preservative. All groups were challenged 24 hours later with P. haemolytica type A1 in gastric mucin ( $10^{7.5}$  cfu/mouse). Five mice were killed at the time of challenge, and the remainder were killed 6 hours later. Viable counts were performed on their livers.

### Results

The results are shown in Table 3.4. Treatment with the complete vaccine resulted in a reduction of viable bacterial counts in the livers 6 hours after challenge, which was significantly different from the controls ( $P < 0.001$ ). The broth and adjuvant preparation did not alter the course of infection with P. haemolytica, regardless of whether the preservative was included. This suggests that the factor responsible for reduced bacterial growth is not a broth constituent or the adjuvant contained in the vaccine, but is probably one of the bacterial components of the vaccine.

Experiment 5 - Determination of LD<sub>50</sub> of E. coli B188 in untreated mice and in mice treated with vaccine 24 hours before challenge

This experiment was devised to determine whether the vaccine would protect mice from challenge with an organism not contained in the vaccine. Challenge was by IP infection with E. coli.

Design of experiment

Twenty-five mice were each injected SC with 0.1 ml Carovax. These mice and 25 controls were allocated to groups of 5 mice each. E. coli B188 was prepared as described in the Materials and Methods section of this chapter, and 10-fold dilutions were made in 0.15 M saline. Five controls and 5 vaccine-treated mice were infected IP with each dilution of this inoculum. The number of organisms in each challenge dose is shown in Table 3.5. Deaths were recorded 48 hours later, and, where possible, the LD<sub>50</sub> was calculated by the method of Reed and Muench (1938).

Results

The results are shown in Table 3.5. The LD<sub>50</sub> of

Table 3.5

Deaths of control and vaccine-treated\* mice  
48 hours after IP challenge with E. coli B188

<u>No of E. coli B188</u> given IP per mouse (cfu)	Deaths in groups of 5 mice	
	Control	Vaccine-treated
$10^{7.4}$	5	0
$10^{6.4}$	4	0
$10^{5.4}$	2	0
$10^{4.4}$	0	0
$10^{3.4}$	0	0
LD <sub>50</sub> (cfu)	$10^{5.7}$	$> 10^{7.4}$

\* Carovax

E. coli B188 when given IP in saline was  $10^{5.7}$  cfu. Mice which had been inoculated SC with 0.1 ml of Carovax 24 hours before challenge, did not succumb to the challenge, even with the highest challenge dose of  $10^{7.4}$  cfu. Protected mice showed no signs of illness. This indicated that treatment with Carovax 24 hours previously could protect mice from challenge with E. coli, a heterologous organism, not present in the vaccine.

Experiment 6 - Effect of treatment with Carovax 24 hours before challenge with E. coli B188 upon the growth of the organism in the livers of mice

This experiment was designed to determine if treatment with Carovax 24 hours before challenge with E. coli B188 affected the growth curve of E. coli in the livers of mice after challenge.

Design of experiment

Ten mice were inoculated SC with 0.1 ml Carovax. Twenty-four hours later, these mice and 10 uninoculated controls were each challenged IP with  $10^{7.6}$  cfu E. coli B188 in 0.5 ml saline, as described in Materials and Methods. At the time of challenge, and 6 hours later, 5 mice from each group were killed by cervical dislocation, their livers removed aseptically, and viable counts performed on them in a manner similar to that described for P. haemolytica (Chapter 1, Experiment 6).

Results

Viable counts from livers of these mice at the time of challenge and 6 hours later are shown in Table 3.6. The number of E. coli B188 in the livers of vaccine-treated



Table 3.6

Viabile counts of *E. coli* B188 in livers of control mice  
and of mice given 0.1 ml vaccine+SC 24 hours before  
challenge IP with  $10^{7.6}$  cfu *E. coli* B188

Hours after challenge	Control	Vaccinate
0	5.48*	5.36
6	7.14	6.07**

\* Mean ( $\log_{10}$  cfu)

SED = 0.284

+ Carovax

\*\* significantly different from control (P<0.01)

mice 6 hours after challenge was significantly different from controls ( $P < 0.01$ ). This indicates that vaccine treatment retards the growth of this organism in mice after challenge.

### Discussion

Mice injected SC with Carovax, a Pasteurella vaccine, 1 hour, 48 hours, 7 days or 14 days before challenge, or given 2 inoculations 6 weeks and 4 weeks before challenge, were not protected against challenge infection with P. haemolytica, using the criterion of growth of organisms in the liver after challenge. A protective response, manifested by significant reduction in bacterial growth, was observed when challenge infection was 12 hours or 24 hours after administration of the vaccine. It is unlikely that this response was due to specific immune mechanisms, since it is of such rapid onset and is operative over such a short period of time after administration of the vaccine. Mice had not been exposed to P. haemolytica prior to treatment with Carovax, eliminating the possibility of a secondary immune response of some kind. Also, no response was observed in the group of mice vaccinated on two occasions prior to challenge.

At dilutions greater than 1/4, the effect was abolished, suggesting that the active component of the vaccine was no longer present in sufficient amount to influence the outcome of challenge. In Experiment 4, the reduction of viable bacterial counts of P. haemolytica in the livers of

vaccinated mice 6 hours after infection was not so marked as in Experiments 2 and 3. The challenge inoculum in this experiment ( $10^{7.5}$  cfu/mouse) was greater than in Experiments 2 and 3 ( $10^{6.3}$  and  $10^{7.0}$  cfu/mouse respectively), which may explain this discrepancy. Nevertheless, viable counts in the vaccinated group of Experiment 4 were still significantly lower than the controls, 6 hours after infection. The strain of P. haemolytica used for challenge of mice was of type A1, which was not a type included in the vaccine, yet a protective effect was observed 24 hours after vaccine treatment. There is no evidence in the literature to suggest that there is cross-protection between types of P. haemolytica; this indicates that the effect may be of a non-specific nature. Further evidence was provided by the protection afforded against challenge 24 hours later with E. coli, which is not a constituent of the vaccine. Indeed, the  $LD_{50}$  of E. coli in treated mice ( $10^{7.4}$  cfu) is slightly higher than that of P. haemolytica in similarly treated animals ( $10^{7.3}$  cfu). The observed effect is therefore of short duration and is non-specific with respect to the challenge organism. There are many reports of phenomena similar to this brought about by the administration of endotoxin to experimental animals.

Among the many effects which bacterial endotoxins exert on the body (Elin and Wolff, 1976) is the stimulation of non-specific resistance to bacterial and viral infections, similar to that described here. The inoculation of mice with bacterial cell walls caused rapid changes in susceptibility to challenge with E. coli (Rowley, 1956). During the first hour after injection of endotoxin, the animals were more susceptible to infection, but after 24 hours a transient state of resistance developed. Non-specific resistance of mice to challenge with Staphylococcus aureus or Salmonella dublin, after administration of E. coli endotoxin, was described by Hill, Hibbitt and Shears (1974A and 1974B).

The vaccine, Carovax, investigated in this study was prepared from gram-negative organisms, and it is probable that lipopolysaccharide material was present in the vaccine, and caused the effect. The mode of action of bacterial endotoxins is uncertain, and controversial. The problem is further complicated by the fact that the level of non-specific resistance induced depends upon the source of endotoxin, the dose given, the strain of mouse studied, and the strain of micro-organism used for challenge. Mice made resistant to the detrimental effects of endotoxin by

repeated low doses, killed S. dublin and Staph. aureus more rapidly in the tissues than did normal mice (Hill, Hibbitt and Shears, 1974B). However, these authors could not attribute this effect to increased phagocytosis. Wells (personal communication) compared clearance of <sup>125</sup>I-labelled polyvinyl pyrrolidone (PVP) from the blood of control mice and mice given Carovax 24 hours previously, and detected no difference in clearance rates in the two groups. PVP is retained by macrophages and the rate at which it is cleared is thought to be an index of macrophage function (Morgan and Soothill, 1975). The clearance rate is reduced by administration of colloidal carbon or hydrocortisone, and increased by oestrogen. Administration of Carovax had no effect, suggesting that macrophage function may be unaltered by this treatment. In contrast, Benacerraff and Sebestyen (1957) showed that, within a few hours of an IV or IP injection of endotoxin into mice and rabbits, the phagocytic cells of the liver and spleen became less effective in clearing injected colloids from the blood. This was followed by a period of increased phagocytic activity, which was considered to be caused by increased production of cells of the phagocytic series. A previous tolerising dose abolished the injury caused by a larger dose, and caused an almost immediate stimulation of

phagocytic cells subsequent to a second large dose.

Using serum from guinea pigs genetically deficient in the C4 component of complement, and thus unable to support activation of the classical pathway, Frank, May and Kane (1973) demonstrated that the components C3 to C9 were consumed in the presence of endotoxin, and that endotoxin was able to activate the alternate pathway. Evidence has also been presented which suggests that endotoxin is capable of activating the classical pathway of complement (Fine, 1974).

It is not evident from the present experiment whether P. haemolytica is inactivated within the peritoneal cavity more efficiently in vaccine-treated mice, and fewer organisms reach the liver, or whether accelerated killing occurs in the liver. In sampling of the liver, bacteria both on the surface and within the organ are measured. Viable counts in vaccine-treated and control mice at the time of challenge are similar, which may suggest that comparable numbers of organisms reach the liver, where they are more rapidly killed in vaccine-treated mice. The vaccine was given by the SC route, so there is no

possibility of non-specific stimulation of peritoneal macrophages due to the presence of foreign material in the peritoneal cavity (Stuart, Habeshaw and Davidson, 1978).

The experiments described in this chapter lend some support to the view that Carovax may have some beneficial effect in animals infected with P. haemolytica but the occurrence of a similar effect in sheep has not been demonstrated.



Chapter 4Further investigations into vaccination and challenge of mice with P. haemolytica

It is essential in the development of a P. haemolytica vaccine to gain some knowledge as to which antigens stimulate a protective immune response, and also to study the existence of any cross-protection between antigens of different types. It is only by gaining such an understanding that a logical choice of vaccine strains can be made, taking into account the relative frequency of isolation of particular strains from field outbreaks of disease. It is not adequate to vaccinate sheep, and examine the serological responses to the various antigens, since the value of such data is questionable (see Chapter 2).

Conventionally reared sheep are unsuitable for study of responses to vaccination with P. haemolytica, since most sheep have P. haemolytica living as commensals in the tonsils or respiratory tract (Gilmour, Thompson and Fraser, 1974) and have antibody titres to P. haemolytica (Gilmour, personal communication). At present, the most successful model by which responses to P. haemolytica vaccines can be studied is by vaccination of SPF lambs, followed by challenge with PI3 virus and P. haemolytica (Sharp, Gilmour, Thompson and Rushton, 1978). The cost of screening

numerous vaccines composed of various combinations of antigens by this method would be prohibitively expensive. Also, the supply of SPF lambs has seasonal limitations.

A method for preliminary screening of experimental vaccine preparations, before final evaluation in SPF lambs, would have many advantages. The use of the SC immunisation and IP challenge system in mice, described in Chapter 1, is investigated in this chapter for the screening of vaccines. The advantages of this system are that mice are comparatively inexpensive, available throughout the year, and that the data obtained gives an indication of the resistance to challenge, rather than antibody responses.

The protection afforded by vaccines containing antigens from more than one type of P. haemolytica, and the effect of vaccinating with one type of P. haemolytica followed by challenge with another type, were investigated. Several of the vaccines tested in mice were also used in experiments using SPF lambs, so it was possible to compare the results obtained with the two models of infection.

## Materials and Methods

### Vaccines

#### Trivalent (Types A1, A2 and A6) P. haemolytica vaccine

A vaccine containing three types of P. haemolytica was prepared. This vaccine contained SSE of P. haemolytica types A1 and A6, and HKO of P. haemolytica type A2. These antigens were individually absorbed onto Alhydrogel at optimal dilution, as described previously. The flocculated antigen-Alhydrogel complex was sedimented by centrifugation (500 g, 10 minutes), and resuspended in part of its own supernate to one third of the original volume. Equal volumes of all three antigens were thoroughly mixed together and emulsified with mineral oil, as described for monovalent P. haemolytica vaccines. Assuming that all the antigen was absorbed onto Alhydrogel, each 0.1 ml dose of vaccine contained the following quantities of each antigen:-

0.019 mg of P. haemolytica type A1 SSE

0.076 mg of P. haemolytica type A2 HKO

0.021 mg of P. haemolytica type A6 SSE.

#### P. haemolytica type A9 vaccines

Vaccines containing various combinations of P. haemol-

ytica type A9 SSE and HKO were also made. These were prepared by adsorbing P. haemolytica type A9 SSE and HKO onto Alhydrogel at their optimal concentrations, in the manner used for the preparation of monovalent vaccines. The flocculated antigen-Alhydrogel complexes were sedimented by centrifugation, and aliquots resuspended in appropriate volumes of their own supernates. Mixtures of SSE and HKO, at the required concentrations were emulsified with mineral oil, as described previously. The compositions of these vaccines are detailed in Table 4.1.

#### Challenge of mice with P. haemolytica

The preparation of inocula for challenge of mice with P. haemolytica, and the method of challenge and enumeration of bacteria in the livers of infected mice were those described in Chapter 1 with the following exceptions. The numbers of bacteria present in bacterial suspensions for challenge of mice were estimated by measuring their absorbance in a colorimeter, as outlined in General Materials and Methods. In all the experiments in this chapter, NB cultures which had been incubated at 37°C for 18 hours were used for challenge of mice.

Table 4.1

Composition of *P. haemolytica* type A9 vaccines

Vaccine	Weight of <i>P. haemolytica</i> type A9 antigen in 0.1 ml vaccine (mg)	
	HKO	SSE
1	0.114	-
2	0.114	0.057
3	0.114	0.284
4	0.114	0.568
5	-	0.057
6	-	0.284
7	-	0.568

Statistical treatment of results

Viabile counts from the livers of mice were expressed as  $\log_{10}$  cfu, and, where possible, analyses of variance were performed. In most of the tables of results, the SED is quoted, but in a few cases, the variances were too heterogeneous for this to be meaningful, so the individual SE is quoted instead. In Experiment 2, some of the results were unsuitable for this statistical treatment, and the Mann-Whitney ranking test (Snedecor and Cochran, 1967) was employed.

Experiment 1 - Determination of LD<sub>50</sub> values of  
P. haemolytica strains for C57 black mice

LD<sub>50</sub> values were obtained for the various strains of P. haemolytica by the method described in Chapter 1. With the exception of P. haemolytica types A7 and A9, in which 10 mice were challenged at each dose level, groups of 5 mice were challenged at each dilution of the inoculum.

Results

The results are shown in Table 4.2. Challenge inocula in further experiments were at least  $10^{0.5}$  cfu/mouse greater than the LD<sub>50</sub> value.

The LD<sub>50</sub> values varied considerably amongst the types of P. haemolytica. For types A2, A6 and T3, it was not possible to calculate exact values since the lowest challenge doses killed more than 50% of the mice. A proportion of mice challenged with the lowest doses of each of these three strains survived challenge, and it was assumed that the LD<sub>50</sub> value would be  $10^{1.0}$ - $10^{2.0}$  cfu lower than the lowest challenge dose.

Table 4.2(i)

LD<sub>50</sub> values for various strains of P. haemolytica when given IP in gastric mucin to

C57 black mice

Type of <u>P. haemolytica</u>	Inoculum/ mouse (log <sub>10</sub> cfu)	* No. deaths by 24 hours	* No. deaths by 48 hours	Total deaths	LD <sub>50</sub>
A2	8.4	5	-	5	< 4.40
	7.4	2	3	5	
	6.4	-	5	5	
	5.4	-	4	4	
	4.4	-	4	4	
A5	7.4	4	1	5	4.23
	6.4	1	2	3	
	5.4	4	1	5	
	4.4	1	2	3	
	3.4	-	2	2	
A6	8.08	5	-	5	< 4.08
	7.08	4	1	5	
	6.08	4	3	5	
	5.08	2	3	5	
	4.08	-	3	3	

NO\* = Number of



Table 4.2 (ii)

Type of <u>P. haemolytica</u>	Inoculum/ mouse (log <sub>10</sub> cfu)	No.* deaths by 24 hours	No.* deaths by 48 hours	Total deaths	LD <sub>50</sub>
A7	7.5	ND	10	10	6.11
	6.5	ND	7	7	
	5.5	ND	1	1	
	4.5	ND	-	-	
	3.5	ND	-	-	
A8	8.30	4	1	5	6.30
	7.30	5	-	5	
	6.30	-	2	2	
	5.30	-	-	-	
	4.30	-	1	1	
A9	8.0	ND	10	10	5.38
	7.0	ND	10	10	
	6.0	ND	10	10	
	5.0	ND	2	2	
	4.0	ND	-	-	

ND = not done  
No.\* = Number of

Table 4.2 (iii)

Type of <u>P. haemolytica</u>	Inoculum/ mouse (log <sub>10</sub> cfu)	No.* deaths by 24 hours	No.* deaths by 48 hours	Total deaths	LD <sub>50</sub>
All	7.9	2	3	5	6.42
	6.9	0	5	5	
	5.9	-	-	-	
	4.9	-	-	-	
	3.9	-	-	-	
A12	8.3	5	-	5	5.92
	7.3	5	-	5	
	6.3	1	3	4	
	5.3	-	-	0	
	4.3	-	-	0	
T3	7.48	5	-	5	< 3.48
	6.48	5	-	5	
	5.48	4	1	5	
	4.48	1	3	4	
	3.48	-	3	3	

No.\* = Number of

Table 4.2 (iv)

Type of <u>P. haemolytica</u>	Inoculum/ mouse (log <sub>10</sub> cfu)	* No. deaths by 24 hours	* No. deaths by 48 hours	Total deaths	LD <sub>50</sub>
T4	7.04	5	-	5	6.54
	6.04	-	-	0	
	5.04	-	-	0	
	4.04	-	-	0	
	3.04	-	-	0	
T10	8.54	1	4	5	6.04
	7.54	-	5	5	
	6.54	3	2	5	
	5.54	-	-	0	
	4.54	-	-	1	

No.\* = Number of

Experiment 2 - Vaccination of mice with a trivalent  
(types A1, A2 and A6) P. haemolytica vaccine and challenge  
with types A1, A2, A6 and A9

It was necessary to discover if all the types of P. haemolytica must be included in a vaccine for use in the field, or if vaccination with one or two types would be sufficient to protect from challenge with other types. The vaccine used in this experiment contained SSE of P. haemolytica types A1 and A6, and HKO of P. haemolytica type A2. Vaccinated mice were challenged with P. haemolytica types A1, A2, A6 and A9.

Design of experiment

Forty mice were each vaccinated twice SC with 0.1 ml of the trivalent P. haemolytica vaccine, with a two week interval between injections. Two weeks after the second injection, these mice and 40 control unvaccinated mice were randomly allocated to four groups each consisting of 10 vaccinates and 10 controls. Each group was challenged with one of four types of P. haemolytica namely, A1 ( $10^{7.2}$  cfu/mouse), A2 ( $10^{6.6}$  cfu/mouse), A6 ( $10^{5.0}$  cfu/mouse), or A9 ( $10^{6.6}$  cfu/mouse). Five controls and five vaccinates in each group were sacrificed at the time of challenge,

and the remaining mice were sacrificed 6 hours later.

Viable counts were performed on liver suspensions.

### Results

The results of this experiment are summarised in Table 4.3. Viable counts in the livers of control mice challenged with each of the four types of P. haemolytica used in this experiment increased in the 6 hours following challenge. Rates of growth similar to those in the livers of control mice were observed in vaccinated mice challenged with P. haemolytica types A2 and A9, and viable counts in the livers of vaccinated mice in these two groups 6 hours after infection were not significantly different from those of their respective controls. In contrast, over the same time interval, viable counts in the livers of vaccinated mice challenged with P. haemolytica type A1 and type A6 dropped dramatically. Six hours after challenge, no organisms were detected in the liver homogenates of any vaccinates challenged with P. haemolytica type A1 or A6, indicating that their content of viable organisms was less than  $10^{2.7}$  cfu/ml, the lower limit of the counting technique employed.

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These results show that this vaccine could afford protection against challenge with P. haemolytica types A1 and A6, but not against challenge with types A2 and A9. This suggests that HKO of P. haemolytica type A2 may not afford protection, even against homologous challenge with the same type. Experiment 3 was designed to investigate the possibility of successfully immunising mice against P. haemolytica type A2.

Experiment 3 - Effect of various vaccination procedures upon challenge of mice with P. haemolytica type A2

In this experiment attempts were made to protect mice from challenge with P. haemolytica type A2, using both living and killed preparations.

Design of experiment

This experiment was performed in several parts. It was thought that the lack of response to P. haemolytica type A2 HKO in Experiment 2 may have been due to either antigenic competition between antigens in the trivalent vaccine, or that insufficient antigen was present in the vaccine. Alternatively, P. haemolytica type A2 HKO may not be a protective antigen. To investigate these possibilities further, two monovalent P. haemolytica type A2 vaccines were made, one of which contained 0.227 mg HKO/0.1 ml dose of vaccine, and the other 0.682 mg HKO/0.1 ml dose of vaccine. The antigens were adsorbed onto Alhydrogel and emulsified in mineral oil as previously described.

a. Vaccination with varying amounts of HKO

Two groups of 10 mice each were vaccinated SC on two occasions with 0.1 ml of these P. haemolytica type A2 HKO



vaccines, the second injection being given 2 weeks after the first. Two weeks after the second injection of vaccine, these mice and a group of control mice were each challenged IP with  $10^{6.7}$  cfu P. haemolytica type A2 in gastric mucin. Five mice from each group were killed at the time of challenge, and five were killed 6 hours later. Their livers were removed for enumeration of viable bacteria.

b. Live immunisation

An attempt was made to protect mice from challenge with P. haemolytica type A2 by giving live P. haemolytica type A2 in saline prior to challenge. The inocula used were 18 hours NB cultures, washed twice in saline and resuspended in saline. One group of 20 mice received 0.2 ml of P. haemolytica type A2 suspension, containing  $10^{7.0}$  cfu, IP, and another group of 20 mice received the same inoculum SC. Eighteen days later, this treatment was repeated for 10 mice in each group. Two weeks later, all mice and 10 controls were challenged IP with P. haemolytica type A2 in gastric mucin ( $10^{6.9}$  cfu/mouse). Five mice per group were killed at the time of challenge, and the remainder were killed 6 hours later. Their livers

were removed and viable counts performed.

c. Live immunisation with *P. haemolytica* type A2 in gastric mucin

In the final part of this experiment, mice were infected with a sub-lethal dose of *P. haemolytica* type A2 in gastric mucin, prior to challenge with a lethal dose. Ten mice were infected IP with 0.5 ml *P. haemolytica* type A2 in gastric mucin ( $10^{5.3}$  cfu/mouse). All mice survived this challenge. Three weeks later, these mice and 10 controls were challenged IP with *P. haemolytica* type A2 in gastric mucin ( $10^{7.0}$  cfu/mouse). Five mice per group were killed at the time of challenge, and the rest were killed 6 hours later; viable counts were performed on their livers.

### Results

Viable counts of *P. haemolytica* type A2 in the livers of mice given the various vaccination treatments, and their respective controls, after challenge with *P. haemolytica* type A2, are shown in Tables 4.4-4.6. The viable counts in the livers of mice vaccinated with *P. haemolytica* type A2 HKO vaccines, or given live *P. haemolytica* type A2 either

Key to Tables 4.4 - 4.23

- \*\*\* = Viable counts in livers of vaccinates significantly lower than controls 6 hours after challenge ( $P < 0.001$ )
- \*\* = Viable counts in livers of vaccinates significantly lower than controls 6 hours after challenge ( $P < 0.01$ )
- \* = Viable counts in livers of vaccinates significantly lower than controls 6 hours after challenge ( $P < 0.05$ )
- ND = Not done.

Table 4.4

Viabile counts of *P. haemolytica* type A2 in livers of control mice and mice vaccinated SC with *P. haemolytica* type A2 HKO vaccines (Experiment 3a)

Hours after challenge	Control	VACCINE		SED
		A2 HKO (0.227 mg/dose)	A2 HKO (0.682 mg/dose)	
0	4.69 ± 0.039	4.55 ± 0.311	3.91 ± 0.312	
6	7.57	7.13	7.30	0.267

Table 4.5

Viabile counts of *P. haemolytica* type A2 in the livers of control mice and mice previously

given *P. haemolytica* type A2 by IP or SC route (Experiment 3b)

Hours after challenge	Control	Route of infection with <i>P. haemolytica</i> type A2 in saline				SED
		IP (once)	IP (twice)	SC (once)	SC (twice)	
0	5.22	5.19	5.13	5.13	5.05	0.210
6	7.62	7.98	7.17	7.51	7.71	0.213

Table 4.6

Viabile counts of *P. haemolytica* type A2 in the livers of control mice and mice previously

given a sublethal dose of *P. haemolytica* type A2 IP in gastric mucin (Experiment 3c)

Hours after challenge	Control	Previously infected	SED
0	4.52	4.46	0.164
6	7.33	7.33	0.208

in saline or gastric mucin, prior to challenge, were not significantly different from the controls, either at the time of challenge or 6 hours later. This indicates that the administration of a sub-lethal dose of live P. haemolytica type A2 is insufficient to protect against challenge with P. haemolytica type A2 in gastric mucin. The lack of response to P. haemolytica type A2 in the trivalent vaccine used in Experiment 1 was not due to either antigenic competition, or insufficient antigen in the vaccine, since the mice in Experiment 2 did not respond to either of the monovalent P. haemolytica type A2 HKO vaccines.

Experiments 4-16 - Cross-protection between different types of P. haemolytica, investigated by the model of IP challenge of mice with P. haemolytica

Experiments 4-16 were designed to determine whether SSE from the various types of P. haemolytica, when adsorbed onto Alhydrogel and emulsified in mineral oil, could be used as vaccines to protect mice from challenge with P. haemolytica of homologous and heterologous types. The designs of these experiments are similar, and are summarised in Table 4.7. Groups of 10 mice were vaccinated SC on two occasions with 0.1 ml of a monovalent P. haemolytica SSE vaccine. Two weeks after injection these mice and 10 controls were challenged IP with P. haemolytica of a specified type, in gastric mucin. Inocula were prepared as described in Chapter 1, except that the cultures were incubated for 18 hours. Viable counts were performed upon the livers of challenged mice.

Results

The results of these experiments are shown in Tables 4.8-4.19, and are summarised in Tables 4.20 and 4.21.

Table 4.7

Summary of designs of Experiments 4-16

Experiment Number	Types of <u>P. haemolytica</u> in monovalent vaccines	Types of <u>P. haemolytica</u> used for challenge of mice	Number of organisms in challenge ( $\log_{10}$ cfu/mouse)
4	A1, A2, A5, A6, A7	A1	6.26
5	A1, A2, A5, A6, A7	A2	6.39
6	A1, A2, A5, A6, A7	A5	6.40
7	A1, A2, A5, A6, A7	A6	5.65
8	A2, A5, A6, A7	A7	7.40
9	A1, A2, A5, A6, A7, A8	A8	7.43
10	A7, A9	A9	7.00
11	A1, A2, A5, A8	A9	7.48
12	A1, A6, A8, A11	A11	7.04
13	A1, A6, A8, A12	A12	5.57
14	T3, T4, T10	T3	6.54
15	T3, T4, T10	T4	7.60
16	T3, T4, T10	T10	6.48



Table 4.8

Experiment 4 - Viable counts of P. haemolytica type A1 in the livers of control mice and mice

vaccinated with P. haemolytica types A1, A2, A5, A6 and A7

Hours after challenge	Control	A1 vaccine	A2 vaccine	A5 vaccine	A6 vaccine	A7 vaccine	SED
0	5.84	5.93	5.91	5.74	5.46	5.92	0.076
6	7.24	5.65 <sup>***</sup>	7.39	7.63	8.10	7.46	0.304

Table 4.9

Experiment 5 - Viable counts of P. haemolytica type A2 in the livers of control mice and mice

vaccinated with P. haemolytica types A1, A2, A5, A6 and A7

Hours after challenge	Control	A1 vaccine	A2 vaccine	A5 vaccine	A6 vaccine	A7 vaccine	SED
0	4.97	5.19	5.11	5.07	5.40	5.32	0.151
6	6.98 ±0.122	7.06 ±0.049	7.42 ±0.057	7.13 ±0.115	7.09 ±0.167	6.78 ±0.447	

Table 4.10

Experiment 6 - Viable counts of P. haemolytica type A5 in the livers of control mice and mice

vaccinated with P. haemolytica types A1, A2, A5, A6 and A7

Hours after challenge	Control	A1 vaccine	A2 vaccine	A5 vaccine	A6 vaccine	A7 vaccine	SED
0	5.32	5.33	5.52	5.49	5.30	5.43	0.153
6	7.45	7.81	7.74	7.57	7.14	7.59	0.297

Table 4.11

Experiment 7 - Viable counts of P. haemolytica type A6 in the livers of control mice and mice

vaccinated with P. haemolytica types A1, A2, A5, A6 and A7

Hours after challenge	Control	A1 vaccine	A2 vaccine	A5 vaccine	A6 vaccine	A7 vaccine	SED
0	4.80 ±0.024	5.16 ±0.118	5.09 ±0.139	4.99 ±0.250	4.76 ±0.245	4.82 ±0.208	-
6	7.79	7.68	7.35	7.52	3.64 <sup>**</sup>	7.10	0.320

Table 4.12

Experiment 8 - Viable counts of *P. haemolytica* type A7 in the livers of control mice and mice vaccinated with *P. haemolytica* types A2, A5, A6 and A7

Hours after challenge	Control	A2 vaccine	A5 vaccine	A6 vaccine	A7 vaccine	SED
0	5.94	5.83	6.08	6.10	6.06	0.085
6	7.47	7.85	7.53	7.65	7.45	0.202

Table 4.13

Experiment 9 - Viable counts of *P. haemolytica* type A8 in the livers of control mice and mice vaccinated with *P. haemolytica* type A1, A2, A5, A6, A7 and A8

Hours after challenge	Control	A1 vaccine	A2 vaccine	A5 vaccine	A6 vaccine	A7 vaccine	A8 vaccine	SED
0	5.83	5.95	5.85	ND	5.90	5.79	5.85	0.101
6	6.64	5.56*	5.62	5.50*	5.77	5.70	4.29*	0.450

Table 4.14

Experiments 10 and 11 - Viable counts of *P. haemolytica* type A9 in livers of control mice and mice vaccinated with *P. haemolytica* types A1, A2, A5, A7, A8 and A9

Hours after challenge	Control	A7 vaccine	A9 vaccine	SED
0	5.95	5.93	5.81	0.120
6	8.13	8.09	7.76	0.388

Hours after challenge	Control	A1 vaccine	A2 vaccine	A5 vaccine	A8 vaccine	SED
0	6.09	5.80	6.14	6.05	6.05	0.204
6	8.01	7.74	7.56	8.07	8.01	0.329

Table 4.15

Experiment 12 - Viable counts of *P. haemolytica* type A11 in livers of control mice and mice vaccinated with *P. haemolytica* types A1, A6, A8 and A11

Hours after challenge	Control	A1 vaccine	A6 vaccine	A8 vaccine	All vaccine	SED
0	6.11	6.15	6.12	6.09	6.07	0.076
6	7.75 ±0.032	7.44 ±0.059	7.11 ±0.044	7.51 ±0.115	5.37* ±0.970	-

Table 4.16

Experiment 13 - Viable counts of *P. haemolytica* type A12 in livers of control mice and mice vaccinated with *P. haemolytica* types A1, A6, A8 and A12

Hours after challenge	Control	A1 vaccine	A6 vaccine	A8 vaccine	A12 vaccine	SED
0	5.44	5.26	5.38	5.15	5.36	0.176
6	6.82	5.97	5.63	6.55	6.03	0.490

Table 4.17

Experiment 14 - Viable counts of *P. haemolytica* type T3 in livers of control mice and mice vaccinated with *P. haemolytica* types T3, T4 and T10

Hours after challenge	Control	T3 vaccine	T4 vaccine	T10 vaccine	SED
0	5.34	5.33	5.39	5.28	0.163
6	8.16	8.00	7.86	7.62	0.490

Table 4.18

Experiment 15 - Viable counts of *P. haemolytica* type T4 in livers of control mice and mice vaccinated with *P. haemolytica* types T3, T4 and T10

Hours after challenge	Control	T3 vaccine	T4 vaccine	T10 vaccine	SED
0	6.33	6.26	6.29	6.26	0.065
6	8.62	8.24	7.80	8.48	0.385

Table 4.19

Experiment 16 - Viable counts of *P. haemolytica* type T10 in livers of control mice and mice vaccinated with *P. haemolytica* types T3, T4 and T10

Hours after challenge	Control	T3 vaccine	T4 vaccine	T10 vaccine	SED
0	5.31	5.37	5.49	5.30	0.101
6	7.73 ±0.040	7.15 ** ±0.130	6.83 *** ±0.044	6.66 * ±0.347	-

Table 4.20

Summary of results of experiments 4-13

Type of *P. haemolytica* used for challenge of mice

	A1	A2	A5	A6	A7	A8	A9	A11	A12
A1	***	-	-	-	ND	*	-	-	-
A2	-	-	-	-	-	-	-	ND	ND
A5	-	-	-	-	-	*	-	ND	ND
A6	-	-	-	***	-	-	-	-	-
A7	-	-	-	-	-	-	-	ND	ND
A8	ND	ND	ND	ND	ND	***	-	-	-
A9	ND	ND	ND	ND	ND	ND	-	ND	ND
A11	ND	ND	ND	ND	ND	ND	ND	*	-
A12	ND	ND	ND	ND	ND	ND	ND	ND	ND

Type of *P. haemolytica* in  
monovalent vaccines



Table 4.21

Summary of results of Experiments 14-16

Type of *P. haemolytica* used for challenge

	T3	T4	T10
Type of <i>P. haemolytica</i> in monovalent vaccines	-	-	**
	-	-	***
	-	-	*

SSE of P. haemolytica types A1, A6, A8, A11 and T10 protected mice against challenge with the homologous type. In addition, both P. haemolytica type A1 and type A5 vaccines afforded some protection against challenge with P. haemolytica type A8, and the P. haemolytica type T3 and T4 vaccines protected against challenge with P. haemolytica type T10.

SSE of P. haemolytica types A2, A5, A7, A9, A12, T3 and T4, did not protect mice from challenge with homologous types of P. haemolytica.

Experiment 17 - Vaccination of mice with *P. haemolytica*  
type A9 SSE and HKO, and challenge with *P. haemolytica*  
type A9

Experiment 17 was designed to investigate the possibility of protecting mice against challenge with *P. haemolytica* type A9, using vaccines composed of combinations of *P. haemolytica* type A9 SSE and HKO. Preliminary experiments had shown that combinations of SSE and HKO of *P. haemolytica* type A2 in a vaccine afforded some protection against homologous challenge (Gilmour, personal communication) and this experiment was designed to determine whether similar results could be obtained with *P. haemolytica* type A9.

Design of experiment

The vaccines used in this experiment contained various combinations of *P. haemolytica* type A9 SSE and HKO, and their compositions are detailed in the Materials and Methods section of this chapter. Groups of 10 mice were each vaccinated SC on two occasions with 0.1 ml doses of vaccine. Challenge was with *P. haemolytica* type A9 in gastric mucin, two weeks after the second vaccination ( $10^{7.0}$  cfu/mouse).

Vaccine 2 (see Table 4.1) was retested using a group of 25 control mice and a group of 25 vaccinates. Challenge was with P. haemolytica type A9 ( $10^{6.8}$  cfu/mouse), and five mice from each group were killed at the time of challenge, and the remaining mice killed 6 hours later.

### Results

These results are shown in Tables 4.22 and 4.23. In the first part of the experiment, the viable counts at 6 hours after challenge from each group of vaccinated mice were compared in turn with the controls by means of a t-test. No statistically significant differences were found, partly due to variance heterogeneity. Some mice in these groups appeared to have responded to vaccination whereas others did not.

Three of the five mice vaccinated with vaccine 2 and killed 6 hours after challenge appeared to have responded to the vaccine, whereas only two mice in the groups given vaccines 3 and 4 responded. For this reason, vaccine 2 was chosen for repetition of the vaccination and challenge with larger numbers of mice. The results are given in Table 4.23. There was considerable variation amongst the

Table 4.22

Viable counts of P. haemolytica type A9 in the livers of control mice and mice vaccinated with P. haemolytica type A9 vaccines

Hours after challenge	Control	Vacc-ine 1	Vacc-ine 2	Vacc-ine 3	Vacc-ine 4	Vacc-ine 5	Vacc-ine 6	Vacc-ine 7
0	5.90	6.18	5.95	5.88	6.18	5.81	6.18	5.81
	6.00	5.88	6.15	5.90	6.04	5.81	5.93	5.60
	5.78	6.04	5.90	6.02	5.90	5.88	5.90	6.00
	5.98	5.93	5.81	6.10	6.00	6.02	5.95	6.06
	5.88	5.98	5.78	6.04	6.15	6.00	6.00	6.04
$\bar{x}$	5.91	6.00	5.92	5.99	6.05	5.93	5.99	5.90
SE	0.008	0.013	0.021	0.009	0.013	0.021	0.012	0.038
6	8.06	8.30	8.54	7.95	7.74	4.11	7.88	6.88
	7.60	7.88	7.81	7.48	6.04	4.06	8.18	7.88
	7.78	8.48	4.88	7.88	4.00	7.74	5.70	7.60
	7.90	8.54	4.00	7.85	7.69	7.93	7.78	7.65
	8.06	8.30	4.78	7.65	3.30	7.85	7.65	7.85
$\bar{x}$	7.88	8.30	6.00	7.76	5.75	6.34	7.44	7.57
SE	0.038	0.067	4.118	0.037	4.218	4.235	0.982	0.164

Table 4.23

Viabile counts of *P. haemolytica* type A9 in livers of control mice and mice vaccinated with *P. haemolytica* type A9 ("vaccine 2")

Hours after challenge		Controls	Vaccinates
0		5.74	5.90
		5.74	5.90
		5.81	5.93
		5.93	5.95
		6.00	5.98
	$\bar{x}$	5.84	5.93
	SE	0.052	0.015
6		7.70	3.00
		7.70	4.48
		7.70	5.11
		7.70	5.18
		8.00	5.90
		8.00	5.95
		8.08	6.08
		8.18	6.30
		8.18	6.70
		8.18	7.00
		8.18	7.08
		8.30	7.11
		8.30	7.18
		8.30	7.30
		8.40	7.39
		8.48	7.48
		8.48	7.84
		8.60	7.95
		8.81	8.08
		9.30	8.40
	$\bar{x}$	8.23	6.58***
	SE	0.090	0.302

viable counts in the livers of the vaccinated mice, but nevertheless the viable bacterial counts in the livers of the vaccinated group 6 hours after infection were significantly lower than the controls ( $P < 0.001$ ).

Discussion

This part of the study investigated the value of IP challenge with P. haemolytica and monitoring of bacterial growth in the liver as a means of assessing the protective value of vaccination with types of P. haemolytica other than type A1. The ability of a vaccine containing one type of P. haemolytica to protect against challenge with another type was also investigated.

In these experiments, inocula were prepared from 18 hours NB cultures, and not 5 hours NB cultures which were previously used. The viable counts of 18 hours NB cultures were variable, but the method for estimating the number of organisms in a suspension by colorimetry proved to be sufficiently accurate that the number of viable organisms in 18 hours NB cultures could be confidently predicted, and these cultures gave equally satisfactory results in challenge experiments.

The SSE antigens in the monovalent vaccines were standardised on the basis of dry weight (see General Materials and Methods). Since the nature of the component active in conferring immunity in the preparations is as



yet unknown, the choice of a parameter by which to standardise them is difficult, and to some extent arbitrary. Another possible criterion is activity of SSE extracts in the IHA test, but the value of this is doubtful, as there is only limited evidence to suggest that IHA activity of the extracts is correlated with protection. In the absence of any specific criteria by which to standardise vaccines they were arbitrarily standardised on the basis of the dry matter content using a standard extraction procedure. Although clearly imperfect, such a procedure is likely to be a crude measure of the quantity of the immunogenic material contained in each vaccine.

In general, variation between viable counts from the individual mice in a group was low at the time of challenge, and was greater in the mice sampled 6 hours after challenge. This was a consistent finding, which may reflect the ability of individual mice to cope with the challenge infection, although it should be pointed out that an inbred strain of mice was used in these experiments, and one would have expected their responses to be similar.

There was little cross-protection between the different types of P. haemolytica, which suggests that the antigens

involved in protection may be type-specific, and it is possible that all the types, or at least those important in causing disease, may have to be included in an effective vaccine. Successful polyvalent pneumococcal vaccines have been produced which contain as many as 12 or 13 serotypes (Austrian, 1976). The antigens used to prepare these vaccines consisted of capsular material, and the immunogenicity of individual components of these polyvalent vaccines was comparable to that resulting from their use in monovalent vaccines. In Experiment 2, in which mice were vaccinated with a trivalent P. haemolytica vaccine, protective responses against challenge with P. haemolytica types A1 and A6 were observed, indicating that there is no competition between these two antigens. This is encouraging for the development of a multivalent P. haemolytica vaccine, and suggests that the development of an immune response to one type may not prevent the development of a response to other types contained in the vaccine. There was no cross-protection between P. haemolytica types A1 and A6 (Experiments 4 and 7), indicating that the responses seen in Experiment 2 were separate responses to the two antigens.

Smith (1959A) vaccinated mice IV with live P. haemolytica of both type A and type T strains, followed 9 days

later with a dose administered subcutaneously. Mice were challenged 12 days after the second dose with P. haemolytica of a type T strain in gastric mucin. Vaccination with the homologous strain gave strong protection, and very little protection was seen with most of the heterologous strains. There was some evidence to suggest that six out of eight type T strains were related immunologically, whereas none of the type A strains gave any protection against challenge with the type T strain.

No success was achieved in protecting mice against challenge with P. haemolytica type A2, even when live organisms were given prior to challenge. Smith (1959A) protected mice from challenge with a type T strain of P. haemolytica by giving two doses of living organisms in broth. Similar protection against P. haemolytica type A2 was not observed in the present experiment. P. haemolytica type A2 is the predominant type incriminated in pneumonic pasteurellosis in the United Kingdom (Thompson, Fraser and Gilmour, 1977), and it is imperative that a successful vaccine provide protection against this organism. Trials with P. haemolytica type A2 vaccines in SPF lambs have met with very limited success (Gilmour, Martin, Sharp, Thompson and Wells, 1979) and it is possible that poor antigenicity

may be partly responsible for the widespread disease caused by this type.

The results of Experiment 17, which investigated P. haemolytica type A9 vaccines, are not conclusive. It appears that some mice responded to the vaccine, and others did not; yet the mice used in this experiment were inbred, and theoretically the response should have been uniform. The variation may have arisen from technical error. The entire experiment was performed by one operator, and the variation seen in the vaccinated group is not reflected in the controls, which makes experimental error less probable. Although statistically significant differences could be convincingly demonstrated between the vaccinated and control group in the second part of the experiment, where the sample size was increased, the biological significance of these results is difficult to interpret. Further experiments have been performed using P. haemolytica type A9 SSE vaccines (Gilmour, personal communication). A vaccine containing ten times the concentration of SSE used in the present experiment gave a high degree of protection. This suggests that insufficient antigen may have been incorporated into the vaccines used in the present experiment, and that more uniform results may have been obtained had a concentrated

preparation been used.

The trivalent vaccine, which contained SSE of P. haemolytica types A1 and A6, and HKO of P. haemolytica type A2, was protective against challenge with P. haemolytica types A1 and A6, but not against challenge with types A2 and A9. This finding is in agreement with data obtained from vaccination-challenge experiments in SPF lambs using the same vaccine and the same strains for challenge (Gilmour, Martin, Sharp, Thompson and Wells, 1979). In addition, monovalent vaccines prepared from P. haemolytica types A1 and A6 SSE protected SPF lambs from challenge with the homologous type. There is evidence for a correlation between the protection afforded by these vaccines in mice, and the protection which they gave to SPF lambs challenged by the method of Sharp, Gilmour, Thompson and Rushton (1978). This model of challenge infection in mice may therefore proved useful in assessment of experimental vaccine preparations, but this can only be confirmed when a greater variety of vaccines have been tested in SPF lambs.

Development of IHA and ELISA tests for the detection  
of antibodies to P. haemolytica in sera from  
vaccinated mice

Introduction

The experiments described in Chapter 4 demonstrated very little cross-protection between the different types of P. haemolytica in vaccinated mice. The mechanism of resistance to P. haemolytica in this model has not yet been determined, and the importance of the humoral immune response is not known. There is some evidence to suggest that serum antibody alone does not confer on sheep protection against P. haemolytica (Chapter 2) but antibody may play an important role and information about serological cross-reactivity between strains of P. haemolytica could be valuable. The experiments to be described in this chapter were devised to determine whether cross-reactions occur in the sera of mice vaccinated with the various types of P. haemolytica.

The most commonly used serological test for estimation of serum antibodies to P. haemolytica in sheep is the IHA test (Shreeve, Biberstein and Thompson, 1972), but its repeatability has been questioned, and there is

poor correlation between serum antibody titres and protection against challenge (Gilmour, personal communication). Smith (1959A) devised an agglutination test for measurement of antibodies to P. haemolytica in mouse sera, but apart from this report, there is no other published information on this subject. The IHA test of Shreeve, Biberstein and Thompson (1972) was adapted to measure antibodies against P. haemolytica in this chapter.

The ELISA technique, devised by Engvall and Perlmann (1972) has been adapted to the detection and quantitation of antibodies to a wide variety of infectious agents. The test has been modified to microplates and has been shown to be convenient, reliable and highly sensitive. ELISA tests have been described for the serodiagnosis of rotavirus infection of calves (Scherrer and Bernard, 1977), Babesia divergens infections in cattle (Purnell, Hendry, Bidwell and Turp, 1976), and bovine brucellosis and hog cholera (Saunders and Clinard, 1976). Estimation of antibodies to P. haemolytica in sheep sera using an ELISA test has been described by Burrells, Wells and Dawson (1979).

Because of its great sensitivity, an adaptation of

this test, to measure antibodies in sera from vaccinated mice, and to study the possible existence of cross-reactivity between the various types of P. haemolytica was developed and is described in this chapter.



## Materials and Methods

### Preparation and collection of mouse sera

Antisera to each type of P. haemolytica were raised in C57 black mice. Groups of 10-20 mice were vaccinated SC on two occasions with 0.1 ml of the monovalent P. haemolytica vaccines described in General Materials and Methods. The second injection was given two weeks after the first. Two weeks after the second injection, the mice were anaesthetised with CO<sub>2</sub> and bled out. The blood from each group was pooled, and left to clot. The sera were separated, and stored at -20°C in 0.5 ml aliquots. Serum from unvaccinated mice was obtained in the same manner.

### Indirect haemagglutination assays

IHA tests were carried out by the method of Burrells, Wells and Dawson (1979). To 3 ml of P. haemolytica SSE of the required type was added 0.2 ml washed packed HuRBC. Due to the presence of bacterial haemolysin, a dilution of SSE which had previously been shown to coat the HuRBC without causing lysis, was used. After mixing and incubation for 2 hours at 37°C, the cells were washed

twice in PBS, containing 0.5% normal rabbit serum (Cameron, 1966) before resuspension in this diluent to a concentration of 1%. Test mouse sera were absorbed with an equal volume of 50% HuRBC for 1 hour at room temperature to remove any non-specific agglutinins, and heated at 64.5°C for 30 minutes to inactivate IgM (Reid and Doherty, 1971) and remove complement. Two-fold dilutions (0.025 ml) were made from 1/8-1/2048 in round-bottomed microtitre plates and 0.025 ml HuRBC added to each well. The end-point dilutions were those showing approximately 50% agglutination.

#### Reagents for ELISA test

The buffers and other reagents for the ELISA test are detailed in Appendix 2.

#### Micro-ELISA assays

The technique of the test was basically a microplate modification (Voller and Bidwell, 1975) of the ELISA technique (Engvall and Perlmann, 1972). An appropriate dilution of P. haemolytica SSE in 0.05 M carbonate buffer, pH 9.6, was used to sensitise the wells of polystyrene microtitre plates (type M129A, Dynatech Laboratories Ltd.,

Billinghamurst, Sussex). Individual wells were filled with 300  $\mu$ l of antigen solution, and held at 4°C overnight. Excess antigen was removed by washing the wells three times with PBS containing 0.05% Tween 20 (PBS/Tween). Sera were not inactivated or pre-treated in any way before testing. The required dilutions of sera were made in PBS/Tween containing 0.02% sodium azide and 300  $\mu$ l added to each well of the antigen-coated plate. After incubation for 2 hours at room temperature, the plate was washed again three times with PBS/Tween. Dilutions in PBS/Tween of enzyme-conjugated rabbit anti-mouse IgG were then added (300  $\mu$ l/well) and incubated at room temperature for 3 hours. The plates were again washed with PBS/Tween and a 300  $\mu$ l volume of the enzyme substrate at a concentration of 1g/l in substrate buffer was added to each well. One hour later the reaction was stopped by the addition of 50  $\mu$ l of 3 M NaOH to each well. Absorbance of each sample was read at a wavelength of 400 nm in a colorimeter (Vitatron UPS Photometer) fitted with a micro flow-through cuvette. Results were recorded as peaks on a chart, which corresponded to the absorbance (E). Standardisation of antigens and sera are detailed in the preliminary experiments (Experiments 2 and 3) described in this chapter.

Experiment 1 - Determination of end-point dilutions for mouse sera in IHA tests using homologous and heterologous

*P. haemolytica* SSE extracts

Aliquots of HuRBC were coated with SSE from all twelve types of *P. haemolytica*. Two-fold dilutions of each pool of mouse serum were made, and reacted with each of these coated HuRBC. End-point dilutions were recorded.

Results

End-point dilutions are shown in Table 5.1. Values of  $\leq 4$  were considered negative. Antibody to homologous antigen was evoked with A1, T3, T4, A8 and T10. In addition mice immunised with A2 reacted to T3, with A5 to A9 and with T3 to T10. These results suggest that either a very low response is evoked by the vaccine, or that this IHA test is too insensitive to detect the antibodies present.

The ELISA test is reputed to be up to 160 times as sensitive as the IHA test (Burrells, Wells and Dawson, 1979) and for this reason was developed in an attempt to detect IgG antibodies in these mouse sera.

Table 5.1

End-point dilutions of mouse sera in IHA tests, against homologous and heterologous

P. haemolytica SSE

Type of P. haemolytica against which mice were vaccinated

	Control	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12
A1	-	32	-	-	-	-	-	-	-	-	-	-	-
A2	-	-	8	-	-	-	-	-	-	-	-	-	-
T3	-	-	8	8	-	-	-	-	-	-	-	-	-
T4	-	-	-	8	-	-	-	-	-	-	-	-	-
A5	-	-	-	-	-	-	-	-	-	-	-	-	-
A6	-	-	-	-	-	-	-	-	-	-	-	-	-
A7	-	-	-	-	-	-	-	-	-	-	-	-	-
A8	-	-	-	-	-	-	-	16	-	-	-	-	-
A9	-	-	-	-	-	8	-	-	-	-	-	-	-
T10	-	-	-	16	-	-	-	-	-	-	16	-	-
A11	-	-	-	-	-	-	-	-	-	-	-	-	-
A12	-	-	-	-	-	-	-	-	-	-	-	-	-

Type of P. haemolytica SSE used to coat  
HuRBC

Experiment 2 - Preliminary titration of *P. haemolytica*  
type A1 SSE in the presence of immune mouse serum and  
titrations to determine optimal dilutions of conjugate

A series of checkerboard-type titrations was carried out to determine the most suitable dilutions of antigen and serum to use in this test. Reaction times were those detailed in Materials and Methods, which had been found to be optimal in the measurement of antibodies to *P. haemolytica* in sheep sera (Burrells, Wells and Dawson, 1979).

Titration of mouse anti-*P. haemolytica* type A1 serum pool  
against *P. haemolytica* type A1 SSE

All these ELISA tests were carried out as detailed in Materials and Methods.

An initial checkerboard titration was performed to determine the dilutions of the mouse anti-*P. haemolytica* type A1 serum pool and *P. haemolytica* type A1 SSE which gave a value of E in the test closest to 1.0. Dilutions of the serum and antigen, in their appropriate diluents, were made (1/20-1/5000). Negative controls which lacked either antigen or serum or both were included since the

enzyme substrate contributes some background colouration. In this test, an arbitrary dilution of the conjugate was used (1/200).

### Results

The dilutions of antigen and serum in which the E value most closely approached 1 were, when both were diluted 1/500 (E=0.90) and at dilutions of 1/200 and 1/500 respectively (E=0.87).

### Titration of conjugate

Standardisation of the conjugate was undertaken using both of these combinations of dilutions. Mouse anti-P. haemolytica type A1 sera and control sera at 1/500 and 1/1000 dilutions were added to the wells of a microtitre plate coated with P. haemolytica type A1 SSE at dilutions of 1/200 and 1/500, respectively. Serial dilutions of conjugate (1/50-1/10000) were prepared and added to the wells after the reaction of the antigen and sera.

### Results

Values of E at 400 nm using various dilutions of conjugate are shown in Fig. 5.1. The greatest difference

Figure 5.1 Determination of optimal dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG



1/500 dilution of mouse anti-P. haemolytica type A1 serum pool



1/1000 dilution of mouse anti-P. haemolytica type A1 serum pool

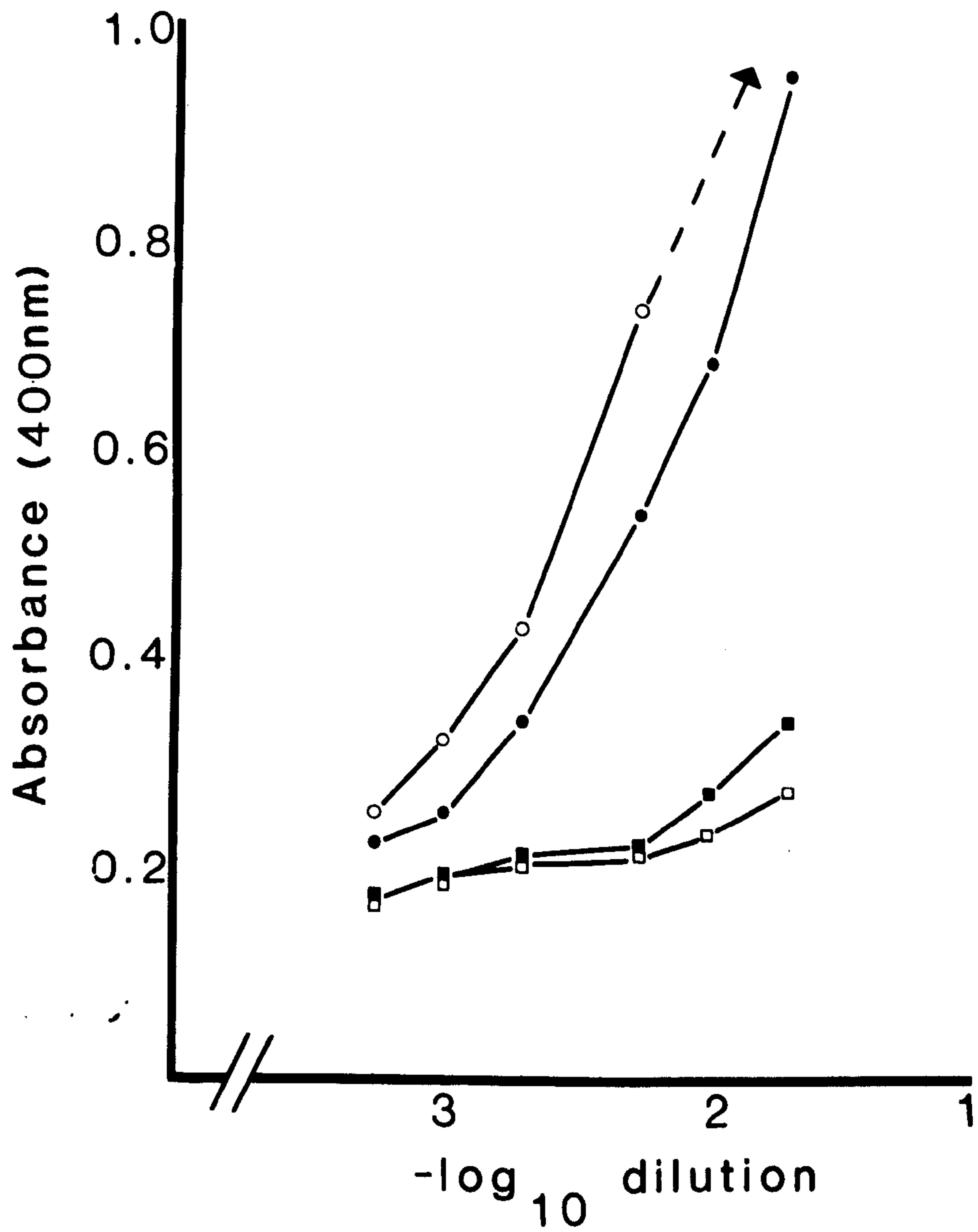


1/500 dilution of control mouse serum



1/1000 dilution of control mouse serum





between absorbance by the positive and negative sera at the same dilution was when the sera and antigen were both diluted to 1/500, and the conjugate to 1/200. The conjugate was used at 1/200 in all further experiments.

Experiment 3 - Titrations of sera and SSE antigens of  
*P. haemolytica* types A1 - A12 using conjugate at 1/200  
dilution to achieve comparable absorbances at 400 nm

A series of dilutions prepared from each pool of mouse sera was titrated initially against homologous SSE antigen at an arbitrary dilution of 1/100. The dilution of serum which gave an absorbance closest to 0.7 was determined. Some of the types did not attain values of E greater than 0.7, so this value was used to standardise the sera in this particular titration. These dilutions were used in a further titration to determine the optimal dilution of antigen for use with these dilutions of sera. Dilutions of each antigen were made (1/50 - 1/2000) and reacted with each of the sera at their pre-determined dilutions.

A final titration was performed, in which the optimal dilutions of SSE were used to coat the wells, and reacted with a series of dilutions of homologous sera (1/50 - 1/2000). This titration served to check the previous two, and from it the dilutions of both antigen and sera which gave absorbances of 0.7 could be determined.

Results

The concentrations of sera and antigens which gave values of E of 0.7 are shown in Table 5.2.

Table 5.2

Dilutions of SSE extracts and antisera which give

E = 0.70 with a dilution of conjugate of 1/200

Type of <u>P. haemolytica</u>	Dilution of sera	Dilution of SSE
A1	1/500	1/200
A2	1/200	1/100
T3	1/50	1/200
T4	1/50	1/50
A5	1/500	1/100
A6	1/100	1/100
A7	1/200	1/50
A8	1/100	1/200
A9	1/100	1/100
T10	1/50	1/200
A11	1/2000	1/50
A12	1/100	1/100

Experiment 4 - Determination of the degree of cross-reaction between the types of *P. haemolytica* in the

ELISA test

Design of experiment

In this experiment, all sera and antigens were used at concentrations determined in Experiment 3 (Table 5.2) which gave a value of  $E=0.70$ . Sera against each type of *P. haemolytica* were reacted with antigen of each type. Controls which lacked either serum or antigen were also included.

Results

The values obtained for absorbance at 400 nm were corrected for background colouration by subtraction of the appropriate control which contained antigen, but no serum. These values are shown in Table 5.3.

An attempt was made to interpret antigenic relatedness between strains in terms of the antibody titre ratio. The calculation involved was described by Archetti and Horsfall (1950) and Gois, Kuksa and Taylor-Robinson (1974).

Table 5.3

Absorbance at 400 nm of reaction between sera and SSE extracts of *P. haemolytica* types A1-A12

Type of *P. haemolytica* SSE used to coat wells of microplate

	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12
A1	0.90	0.81	0.61	0.60	0.91	0.69	0.51	0.64	0.81	0.47	0.30	0.57
A2	0.10	1.01	0.73	0.67	1.01	0.99	0.66	0.70	0.91	0.45	0.59	0.59
T3	0.11	0.26	0.93	0.43	0.17	0.22	0.10	0.19	0.31	0.33	0.13	0.08
T4	0.21	0.43	0.70	1.02	0.38	0.39	0.19	0.29	0.47	0.41	0.13	0.17
A5	0.65	0.81	0.71	0.50	1.06	0.74	0.50	0.57	0.70	0.46	0.26	0.50
A6	0.80	0.91	0.78	0.53	0.96	0.94	0.72	0.66	0.76	0.44	0.18	0.56
A7	0.90	1.11	0.73	0.68	1.01	1.04	1.07	0.75	0.91	0.69	0.42	0.90
A8	0.65	0.76	0.78	0.49	0.71	0.79	0.64	0.68	0.71	0.44	0.21	0.54
A9	0.90	0.91	0.64	0.55	0.91	0.79	0.56	0.69	0.71	0.46	0.25	0.67
T10	0.17	0.28	0.73	0.50	0.28	0.26	0.13	0.15	0.39	0.72	0.09	0.12
A11	0.56	0.76	0.73	0.54	0.86	0.65	0.62	0.60	0.70	0.50	1.53	0.34
A12	1.10	1.11	0.73	0.71	1.01	0.99	0.82	0.80	1.01	0.56	0.36	0.90

Type of *P. haemolytica* against which mouse sera were raised

The geometric mean of the ratios is given by the formula:-

$$r = r_1 \times r_2$$

where

$$r_1 = \frac{\text{heterologous titre (strain 2)}}{\text{homologous titre (strain 1)}}$$

$$r_2 = \frac{\text{heterologous titre (strain 1)}}{\text{homologous titre (strain 2)}}$$

The value  $r$  measures similarities between serological reactions to different strains. When the homologous and heterologous titres are equal,  $r$  is maximum (ie, 1/1). The greater the denominator, when  $r$  is expressed as a ratio, the greater the difference between two strains.

Values of  $1/r$  are given in Table 5.4. To simplify the table, the denominators of the fractions only have been given (ie, the reciprocal of  $r$ ). The larger the value of  $1/r$ , the less closely the two strains are related antigenically.



Table 5.4

Values of 1/r for the different types of *P. haemolytica* as determined by the ELISA test

	A1	A2	T3	T4	A5	A6	A7	A8	A9	T10	A11	A12
A1	1.0											
A2	3.4	1.0										
T3	3.5	2.2	1.0									
T4	2.7	2.0	1.8	1.0								
A5	4.0	1.1	2.9	2.4	1.0							
A6	1.2	2.2	2.3	2.3	1.2	1.0						
A7	1.5	1.4	3.7	2.9	1.5	1.2	1.0					
A8	1.2	1.2	2.1	2.2	1.3	1.1	1.2	1.0				
A9	0.9	1.0	1.8	1.7	1.1	1.1	1.2	1.0	1.0			
T10	2.9	2.4	1.7	1.9	2.4	2.4	2.9	2.7	1.7	1.0		
A11	2.9	2.6	3.9	4.7	2.7	3.5	2.5	2.9	2.5	5.0	1.0	
A12	1.1	1.2	3.8	2.8	1.4	1.2	1.1	1.2	1.0	3.1	3.4	1.0

### Discussion

The ELISA technique is becoming increasingly useful for measurement of antibodies to a diversity of antigens. The particular test used in this chapter was first successfully adapted for use with sheep sera by Burrells, Wells and Dawson (1979). The technique has many advantages; absorption and heating of test sera are avoided, and as the test is read photometrically no subjective influence is placed upon the results. The volume of serum required is extremely small. Sensitivity of the ELISA test is equal to that of radio-immunoassay, and up to 200 times more sensitive than haemagglutination inhibition or complement fixation tests.

Provided that the reaction times for each test are strictly adhered to, the test is reproducible. For example, if the reaction between the enzyme and its substrate, which gives the colouration, is allowed to proceed for an extra few minutes, the value of E may be significantly raised. There is a slight discrepancy between the E value obtained in Experiment 2 for P. haemolytica type A1 where both antigen and serum were diluted 1/500 (E = 0.90), and the value for the same

reaction in the following titration in which the conjugate was standardised ( $E = 0.73$ , Fig. 5.1). The dilutions of sera and antigens which gave  $E = 0.70$  in Experiment 3, gave values of  $E$  closer to 0.90 in Experiment 4. Routine use of this test would require inclusion of reference positive and negative sera, to enable comparisons to be made between tests. As no such comparisons were made here, this was not necessary.

It is evident from Experiment 4 that cross-reactions between the different types of P. haemolytica occur. The sensitivity of the test means that cross-reactions will be detected which would be missed in a less sensitive test such as the IHA test which was shown to be extremely insensitive (Experiment 1). The values of  $r$ , as calculated by the method of Archetti and Horsfall (1950) give a measure of the relative relatedness of types. No attempt was made to assess the significance of these differences as any decisions based on this would be arbitrary. There is some suggestion that there is less cross-reaction between A and T types, than between the A types with each other, or amongst the T types, although there is still considerable cross-reaction between the A and T types.

Type A11 appears to be the least cross-reactive of all the types of P. haemolytica. Type A9 seems to cross-react strongly with a number of other types of P. haemolytica, such as types A1, A2, A5, A6, A7, A8 and A12, and yet no protection against challenge with type A9 was seen in mice vaccinated with these other types (Chapter 4).

In Chapter 4, it was shown that very little cross-protection occurs between the different types of P. haemolytica. The present ELISA test indicates that there are antigens in common between the types of P. haemolytica, to which IgG antibodies are raised in the mouse, which can be detected by this test, but not by the IHA test. Several explanations for this are plausible. The crude SSE may contain antigens capable of adhering to the polystyrene plates used in the ELISA test, which are incapable of attaching to HuRBC used in the IHA test. This would mean that the ELISA test would measure antibodies undetected by the IHA test. It is possible that such antibodies may not be involved in immunity, and that their presence is preventing the detection of the antibodies important in immunity to P. haemolytica infection. An alternative explanation would be that antibodies alone

cannot be responsible for protecting mice in the vaccination-challenge system, and that some other parameter, such as the stimulation of CMI or peritoneal macrophages is involved in resistance to challenge with P. haemolytica. The passive transfer of immune serum to SPF lambs did not protect them from challenge with P. haemolytica (Chapter 2), suggesting that factors other than the humoral response were important in protection against P. haemolytica infection in sheep. Although the model of P. haemolytica infection in mice is in many respects unlike that in SPF lambs, it may be that the humoral response does not play a major role in this model either. This question may be resolved by a study of passive transfer of immune serum in mice prior to challenge.

The ELISA test could be useful for monitoring the humoral immune response in vaccinated mice. The small volume of serum required makes it possible to take serial samples from individual mice, and so the response could be followed over a period of time. The conjugate used here is specific for IgG antibodies, but although it was unavailable at the time of these experiments a conjugate of rabbit anti-mouse IgM could be produced for measurement of antibodies of the IgM class.

CONCLUDING DISCUSSION

This study was undertaken with a view to elucidating the mechanisms which are important in the resistance of sheep to disease associated with P. haemolytica infection. Pasteurellosis of sheep is a sporadic yet widespread disease, and sufficiently important to merit development of an effective vaccine. The microbiology and pathology of the disease are well documented, but surprisingly little is known about the immunological aspects. In the development of an effective vaccine, a knowledge of the specific host defences important in resistance is of value so that appropriate tests may be used to evaluate methods of antigen preparation or alternatively to monitor antigen batches for efficacy. There are twelve types of P. haemolytica (Biberstein, Gills and Knight, 1960; Biberstein and Thompson, 1966), and it is essential to know whether serological cross-reactions and cross-protection occur between these types, and also whether combinations of antigens of several types in a vaccine will be as immunogenic as these antigens in monovalent vaccines.

The most suitable model of infection for study of these problems is that of Sharp, Gilmour, Thompson and Rushton (1978). SPF lambs are infected intratracheally and intra-

bronchially with PI3 virus, and 7 days later P. haemolytica is given by aerosol. Lesions similar to those of the field disease of pneumonic pasteurellosis develop. This model was used in the present study of mechanisms of immunity. The major drawback of the model is expense, and it was necessary to develop an alternative model of P. haemolytica infection, and to show that results of experiments with such a model bore relationship to results obtained with sheep. The work in this thesis mainly describes attempts to develop a challenge system in mice.

This concluding discussion examines the extent to which the original aims of this study have been realised, the questions which remain to be answered, and the type of research which may in the future provide additional useful information.

Mechanisms of immunity to *P. haemolytica* in sheep

*P. haemolytica* is a commensal which resides in the naso-pharynx and tonsils of clinically normal sheep (Gilmour, Thompson and Fraser, 1974) and yet, for reasons unknown, can invade the lower respiratory tract, and often cause fatal pneumonia. Since *P. haemolytica* is widely distributed among flocks, it might be expected that sheep would have some natural immunity to the organism, and indeed the majority of sheep have circulating antibodies to several types of *P. haemolytica* (Gilmour, personal communication). It may be questioned therefore whether circulating antibodies alone protect against infection, and if the level of circulating antibody is a reliable index of immunity to infection. Experiment 2 in Chapter 2, in which passively transferred hyperimmune serum failed to protect SPF lambs from challenge, casts doubt upon the importance of serum antibody alone in protection against *P. haemolytica*. It was also shown in Chapter 2 (Experiment 1) that serum from vaccinated lambs had a bacteriostatic effect upon *P. haemolytica* in vitro. Consequently, the role of serum antibody in defence against *P. haemolytica* infection is unresolved.

Although serum antibody itself is unable to protect



against experimental pasteurellosis, it could still play an important auxiliary role. It has been suggested that P. haemolytica "may be called the animal equivalent of the pneumococci in man" (Biberstein, Gills and Knight, 1960). Phagocytosis and intracellular killing of Streptococcus pneumoniae responsible for pneumococcal pneumonia of man, require the presence of both complement and antibody (Robbins, 1978). For opsonisation to occur, the antibody must be directed against the capsular component of the cell. P. haemolytica is an encapsulated organism, and lipopolysaccharide is a constituent of SSE, although it is not yet known whether antibodies are produced against this component (Donachie, personal communication). The situation may be analogous to that of Strep. pneumoniae, but until the protective antigens of P. haemolytica have been identified, this remains speculative.

The site of multiplication of P. haemolytica is the lung, and the role of local defence mechanisms must be considered. Intramuscular vaccination of SPF lambs with SSE in adjuvant provides protection from challenge. It is unlikely that intramuscular vaccination would stimulate production of antibody in the lung and although the transfer of antibody of both IgG<sub>1</sub> and IgG<sub>2</sub> classes from

serum of sheep to nasal secretions has been demonstrated (Wells, Dawson, Smith and Smith, 1977), it is not certain whether serum derived antibody is present or active in the lower respiratory tract in sheep. Pulmonary host defences of rabbits immunised with *Pseudomonas* antigens have been studied (Reynolds, 1974) and it was shown that either IN or intramuscular immunisation resulted in the appearance of antibody in bronchial secretions. Antibody to *Pseudomonas aeruginosa* was measured by agglutination; agglutinating activity in the bronchial secretions from intranasally immunised rabbits was present in the IgA and IgG fractions, whereas in the bronchial specimens from intramuscularly immunised rabbits, agglutination was confined to the IgG fractions. IgM was not detected in bronchial secretions and complement was present only in trace amounts. Using an in vitro culture system with rabbit AM and <sup>14</sup>C-labelled organisms it was demonstrated that IgG was a more effective opsonin than IgA. Assuming that the situation in sheep is similar to that in rabbits, it would be expected that IgG would pass into the bronchial secretions and be available for opsonisation of *P. haemolytica*.

It was concluded therefore that measurement of serum

antibody against P. haemolytica is not a reliable index of immunity and that it was necessary to look further at other parameters which might give a more accurate indication of immune status.

Although phagocytosis and intracellular killing by AM were not demonstrated (Chapter 2, Experiment 1), this does not rule out the possible importance of AM in defence of the lung against P. haemolytica. The experiment was performed in vitro, and the lack of demonstrable phagocytosis need not reflect upon the situation in the intact animal. Clearance of P. haemolytica from the lungs of calves has been studied (Lopez, Thomson and Savan, 1976), and it has been found that the organism is cleared effectively within 4 hours of exposure. However, exposure to PI3 virus 7 - 11 days prior to exposure to P. haemolytica markedly inhibited clearance of the bacteria. Studies similar to this, performed in sheep, may give insight into the role of clearance of P. haemolytica from the lung, and hence almost certainly the role of the AM in defence against P. haemolytica. The development of a method for obtaining samples of AM from infected lambs by lavage would be useful in such a study.

P. haemolytica appears to have a toxic effect upon both bovine (Benson, Thomson and Valli, 1978) and ovine (Sutherland, personal communication) AM in vitro, and it is important to know whether a similar effect occurs in vivo. Under normal circumstances P. haemolytica does not enter the lung, which is kept free from bacteria by the mucociliary system. P. haemolytica only invades the lower respiratory tract when these defences are compromised, as they might be during a virus infection. If the proliferating P. haemolytica themselves depress the activation of AM it is clear that a very delicate balance must exist between the multiplying organisms and the host defence mechanisms. It is important to know if P. haemolytica can exert a toxic effect upon AM in vivo, and a study of this phenomenon and the mechanisms by which it operates would contribute greatly to knowledge of the pathogenicity of P. haemolytica.

No marked toxic effects upon AM were observed when the test organisms in the system were P. multocida, Serratia marescens, Listeria monocytogenes or Staph. aureus (Benson, Thomson and Valli, 1978). Antiphagocytic activity by other organisms has been reported. Some strains of Bacteroides melaninogenicus and Bacteroides fragilis can inhibit phagocytosis by leucocytes (Ingham, Sisson, Tharagonnet, Seldon and

Codd, 1977) and it is thought that this may be an important factor in post-operative sepsis. This activity is cell-associated in contrast to the toxic effect of P. haemolytica which can be produced using the supernates of cultures. Another interesting phenomenon has been described in experimental pneumococcal pneumonia of rats (Ansfield, Woods and Johanson, 1977). Bacterial clearance from the lung proceeds rapidly in the early stages of infection, and then the anti-pneumococcal defences become depressed as pneumonia develops. This effect is thought to be selective for Strep. pneumoniae; the affected lungs are still able to inactivate organisms such as Staph. aureus, and the inhibition is caused by accumulation of capsular material in the lung. During growth in liquid culture, P. haemolytica sheds capsular material into the supernate, and it is conceivable that a mechanism similar to that described for Strep. pneumoniae may be involved in the pathogenesis of pneumonic pasteurellosis.

It was demonstrated (Chapter 2, Experiment 2) that vaccinated lambs which were protected from P. haemolytica challenge had elevated lymphocyte transformation responses to P. haemolytica SSE, indicating the possible importance of CMI in resistance to P. haemolytica infection. The

lymphocyte transformation responses of lambs increased markedly after aerosol challenge with P. haemolytica, reflecting an increase in the proportion of lymphocytes capable of responding to the P. haemolytica SSE antigen. This increase was greater in vaccinated lambs than in controls, and probably represents development of a secondary response after challenge. There is every indication that lymphocyte transformation responses to P. haemolytica SSE antigens may be a useful means of assessing immunity to P. haemolytica in sheep.

The experiments undertaken in Chapter 2, which examined the role of the immune response in resistance to P. haemolytica went some way to determining the important aspects, but clearly many questions remain unanswered. The cell-mediated immune response probably plays an important part, but its exact role in resistance to P. haemolytica is not known, and further study of this is required.

The ability of sensitised lymphocytes to release lymphokines in the presence of P. haemolytica antigens has not been investigated. These factors amplify the cell-mediated immune response in a variety of ways, including recruitment of more lymphocytes, attraction and retention

of macrophages at the site of infection, and their presence in the lung could be an important part of its defences against P. haemolytica. Macrophage migration inhibition factor (MIF) is released in vitro in the presence of antigens (Valentine, 1974) and it would be of considerable interest to determine whether MIF was also released by sensitised lymphocytes in the presence of P. haemolytica antigens.

### Models of *P. haemolytica* infection in mice

An attempt was made to develop a laboratory animal model for the study of *P. haemolytica* infection, with a view to evaluating the efficacy of experimental vaccine preparations. The model of IN infection of mice with *P. haemolytica* (Rushton, 1978) proved to be unsuitable for this purpose, since the organisms were rapidly cleared from the lung, and similar lesions could be produced by inoculation of dead and living *P. haemolytica* (Chapter 1). This suggests that damage in the lung may be caused by the mere presence of bacterial material exerting a toxic effect. Such an effect is unlikely to be in any way specific and clearly lacks the feature of bacterial multiplication in the tissues which must be an essential part of any bacterial pneumonia.

Enhancement of infections with *P. pneumotropica* and *Staph. aureus* in the lung can be achieved by prior infection with Sendai virus (Jakab and Green, 1972; Jakab, 1974). In view of the fact that dead *P. haemolytica* cause lesions in the lung no attempt was made to explore the effect of prior viral infection on infection with *P. haemolytica*.



The advantages and disadvantages of the model of IP infection of mice with P. haemolytica in gastric mucin have been discussed (Chapter 1). The model is by no means ideal, but in view of the fact that P. haemolytica is not a pathogen of laboratory animals, it may be the best than can be achieved.

The model of IP infection of mice with P. haemolytica in gastric mucin has proved useful for the evaluation of vaccine preparations. It would be imprudent to assume that vaccines which protect mice from IP challenge with P. haemolytica would necessarily be effective in protecting sheep from pneumonic pasteurellosis. However, results to date are encouraging. Several vaccines have been tested in mice (Chapters 1 and 4) and SPF lambs (Gilmour, Martin, Sharp, Thompson and Wells, 1979) in vaccination-challenge experiments. Vaccines which protected mice against P. haemolytica infection also protected SPF lambs. Vaccines which contained antigens of P. haemolytica type A2 gave no protection against this type in either model. Too few vaccines have been compared in this way to state categorically that the behaviour of vaccines in each model is the same, and it is necessary to test more vaccines using both systems. However, it is reasonable to suggest that the model of IP challenge

of mice with P. haemolytica in gastric mucin may be important in the preliminary screening of potential vaccine preparations.

There are many dangers inherent in using a laboratory animal model to test vaccines for use in another species, especially as, in the present model, the organism is non-pathogenic and the host defences must be comprimised before infection can be established. There is always the possibility that mice may not respond to antigens to which sheep are responsive, and the danger that a potentially useful antigen may be discarded on the basis of preliminary screening in mice. The amount of antigen required to elicit an immune response in the mouse bears no relationship to the dose required in another species.

The experiments described in Chapter 4, in which it was demonstrated that in general cross-protection between the different types of P. haemolytica does not occur, have implications as to the type of vaccine required to combat pasteurellosis. If it is confirmed in experiments with SPF lambs that there is very little cross-protection between the types, a vaccine containing antigens from several types of P. haemolytica must be developed. Since the incidence

of types of P. haemolytica varies, all the commonly occurring types would have to be included in a vaccine. It is possible that after repeated use of such a vaccine that the distribution of types of P. haemolytica would change, and types previously rare would appear more frequently. In such an event, their inclusion in the vaccine would have to be considered. The trivalent vaccine (types A1, A2 and A6) was tested in both SPF lambs (Gilmour, Martin, Sharp, Thompson and Wells, 1979) and mice (Chapter 4), and results indicated that the presence of other antigens caused no diminution of the protective responses to P. haemolytica types A1 or A6, suggesting that antigenic competition may not be a problem in the development of polyvalent P. haemolytica vaccines.

All attempts to protect mice from challenge with P. haemolytica type A2 were unsuccessful. Type A2 is most frequently incriminated in outbreaks of pneumonic pasteurellosis (Thompson, Fraser and Gilmour, 1977) and a vaccine which fails to protect against this serotype would be unlikely to be acceptable. Most attempts to protect SPF lambs against P. haemolytica type A2 have been unsuccessful (Gilmour, Martin, Sharp, Thompson and Wells, 1979).

Preliminary experiments have shown that some degree of protection of mice from challenge with P. haemolytica type A2 can be obtained using a vaccine containing P. haemolytica type A2 HKO and concentrated SSE (Gilmour, personal communication). This suggests that a protective antigen is present, albeit in low concentration, and that perhaps the HKO contained in this vaccine is either having some adjuvant effect, or that protective antigen is also contained in this preparation. The solution to successful vaccination against this organism may be found through study of its individual antigenic components and their properties, rather than immunisation with crude extracts such as those used in the present study.

The SSE antigens examined in this study are crude, and contain both protein and polysaccharide components (Mould, personal communication). Further analysis of extracts of P. haemolytica, to determine both the chemical composition and immunogenicity of the components, would clarify the situation. Purified extracts would probably be available in small quantities, and the production of sufficient amounts with which to test immunogenicity might pose a problem. The model of SC immunisation of mice followed by IP infection with P. haemolytica would be very

useful in a study of this type, as only small amounts of antigen combined with adjuvant are required for vaccination.

The model of IP infection of mice with P. haemolytica was also used to investigate whether the Pasteurella vaccine, Carovax, could bestow short-term non-specific immunity shortly after its administration (Chapter 3). The results suggested that the vaccine could exert some influence upon the resistance of mice to challenge with P. haemolytica 12 - 24 hours after the vaccine was given. A thorough investigation of this phenomenon in SPF lambs would involve a large number of animals, as the time course of such an event would not necessarily be the same in sheep and mice. If such a phenomenon does occur in sheep, administration of vaccine during an outbreak would be of some benefit, although it is clearly preferable that a vaccine confers immunological protection as well.

### Serological studies

Experiments in which antibodies in mouse sera were measured by the IHA and ELISA tests were described in Chapter 5. The ELISA test detected antibodies to P. haemolytica in sera which were negative when examined by the IHA test. The IHA test depends upon binding of antigenic components to HuRBC, and it is possible that some components failed to do this, although they did bind to the polystyrene plates of the ELISA test. If this were so, antibodies to such components would go undetected in the IHA test, but would be measured in the ELISA test. An alternative possibility might be that the antibodies produced were of low valency, and incapable of agglutinating antigen-coated HuRBC. Results of the ELISA test would be unaffected by this. The volumes of serum required to perform the ELISA test are small, and for this reason the technique may prove useful for detecting mouse anti-P. haemolytica antibodies from serial samples of serum.

This study has elucidated some of the mechanisms by which sheep resist infection with P. haemolytica. It suggests that serum antibody alone is not responsible for immunity, and that its measurement is not a reliable indication of the immune status of the sheep. Cell-mediated immunity is probably important in resistance to pasteurellosis, and lymphocyte transformation responses in the presence of P. haemolytica SSE antigen reflect the ability of sheep to resist infection with P. haemolytica.

The model of SC immunisation followed by IP challenge of mice with P. haemolytica in gastric mucin (Chapter 1) has to date given similar results with experimental vaccines to the model of infection of SPF lambs with P. haemolytica super-imposed upon PI3 virus infection (Sharp, Gilmour, Thompson and Rushton, 1978). This model of infection has proved useful in assessing experimental vaccines, and, assuming that there is a correlation between results obtained and the behaviour of these vaccines in sheep, experiments have implied that there is little cross-protection between the different types of P. haemolytica, and also that antigenic competition between different antigens in a polyvalent vaccine may not be a problem. This strongly suggests that

a successful P. haemolytica vaccine would have to include antigens from several types of P. haemolytica, but there would be no antigenic competition associated with such a polyvalent vaccine.



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Appendix 1Chapter 1: Experiment 6

Viablc counts of *P. haemolytica* type A1 in livers  
and spleens of mice after infection

Time after infection (hours)		Viablc counts of <i>P. haemolytica</i> type A1	
		Liver	Spleen
1		5.40	4.90
		5.76	4.65
		5.20	4.95
		5.52	4.18
		5.60	4.95
	$\bar{x}$	5.50	4.73
	SE	0.10	0.15
2		5.70	5.18
		5.04	5.65
		5.65	5.57
		5.78	5.20
		5.60	5.15
	$\bar{x}$	5.55	5.35
	SE	0.13	0.11
3		5.88	5.59
		6.18	5.70
		5.88	5.20
		5.88	5.60
		6.20	5.70
	$\bar{x}$	6.00	5.56
	SE	0.08	0.09
8		8.20	8.04
		7.95	7.32
		7.78	7.20
		7.54	7.46
		7.93	7.18
	$\bar{x}$	7.88	7.44
	SE	0.11	0.16

Chapter 1: Experiment 8

Viabile counts of *P. haemolytica* type A1 in livers of vaccinated and control mice after infection

Time after infection (hours)		Controls	Vaccinates
0		5.15	4.53
		5.48	4.93
		5.70	4.81
		MV*	4.95
		MV	5.28
	$\bar{x}$ SE	5.44 0.16	4.90 0.12
2		5.00	3.90
		5.74	5.15
		4.60	4.40
		5.60	5.26
		5.40	4.08
	$\bar{x}$ SE	5.27 0.21	4.56 0.28
4		7.00	3.60
		5.40	3.78
		6.90	5.04
		5.44	3.18
		MV	MV
	$\bar{x}$ SE	6.18 0.44	3.90 0.40
6		7.38	3.60
		7.85	3.90
		7.48	2.70
		5.30	4.20
		MV	4.18
	$\bar{x}$ SE	7.00 0.58	3.72 0.28

\* MV = Missing value



Composition of reagents for ELISA assaysAntigen diluent - Carbonate/bicarbonate buffer

(0.05 mol/l, pH 9.6) 0.02% azide

NaCO <sub>3</sub>	1.59 g	} 1000 ml distilled water
NaHCO <sub>3</sub>	2.93 g	
NaN <sub>3</sub>	0.2 g	

Plate washing solution (PBS/Tween)

0.5 g/l Tween 20 in PBS

(Tween 20: Polyethylene sorbitan monolaurate, Sigma Chemical Company, Poole, Dorset).

Substrate buffer

Diethanolamine	100 ml
Distilled water	840 ml
MgCl <sub>2</sub> (0.5 mmol/l)	0.0475 g
NaN <sub>3</sub>	0.2 g/l

Adjust to pH 9.8 with HCl at 1 mol/l (~ 60 ml/l).

Appendix 2 (ii)Substrate

p-nitrophenyl phosphate disodium (1 g/l) in substrate buffer.

p-nitrophenyl phosphate disodium: Sigma 104 phosphatase substrate tablets, Sigma Chemical Company, Poole, Dorset. Stored at  $-20^{\circ}\text{C}$ . Fresh solutions of this were made for each test.

Reaction arrester

3 mol/l NaOH.

Conjugate for ELISA test to detect IgG antibodies againstP. haemolytica in mouse sera

IgG fraction of rabbit anti-mouse IgG (Miles Laboratories, Slough, England: Cat. No. 65--57-2), labelled with alkaline phosphatase, Sigma type VII (Sigma Chemical Company, Poole, Dorset) by the method of Engvall and Perlmann (1972) and stored at  $4^{\circ}\text{C}$ .

Appendix 2 (iii).Conjugate for ELISA test to detect IgG antibodies against  
P. haemolytica in sheep sera

A mono-specific pig anti-sheep IgG serum dialysed against 0.01 M sodium phosphate buffer, pH7.6, containing 0.03 M NaCl, was passed through a Whatman DE 52 cellulose column equilibrated with the same buffer. The whole IgG fraction was collected and concentrated by pressure dialysis against PBS to 5 mg/ml. This specific anti-sheep IgG was conjugated with alkaline phosphatase, Sigma type VII by the method of Engvall and Perlmann (1972) and stored at 4°C.

Antigens

The antigens used in the ELISA test were standardised preparations of SSE, prepared as described in General Materials and Methods.

Publications arising from this thesis

Evans HB and Wells PW (1979). A mouse model of Pasteurella haemolytica infection, and its use in assessment of the efficacy of P. haemolytica vaccines. *Research in Veterinary Science*, 27, 213-217.

Evans HB and Wells PW. Non-specific resistance of mice to Pasteurella haemolytica. *Research in Veterinary Science*, in press.

Wells PW, Evans HB, Burrells C, Sharp JM, Gilmour NJL, Thompson DA and Rushton B. Inability of passively acquired antibody to protect lambs against experimental pasteurellosis. *Infection and Immunity*, in press.

## A mouse model of *Pasteurella haemolytica* infection and its use in assessment of the efficacy of *P haemolytica* vaccines

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A method for infecting mice with *Pasteurella haemolytica* is described. Mice were inoculated intraperitoneally with *P haemolytica* incorporated in gastric mucin, killed at various time intervals thereafter and viable counts of bacteria were performed on liver suspensions. *P haemolytica* grew at an exponential rate in the livers of normal mice. Mice vaccinated against *P haemolytica* A1 were protected against homologous challenge and viable counts decreased rapidly in their livers. Mice given trivalent *P haemolytica* vaccine (types A1, A2 and A6) were protected against challenge with *P haemolytica* types A1 and A6, but were not protected against challenge with types A2 and A9. These results correlate with findings in sheep.

OVINE PASTEURELLOSIS CAUSES substantial loss every year (Veterinary Investigation Diagnosis Analysis II Report 1977). Although commercial vaccines are available, much effort is being directed towards devising vaccines which are more effective against this disease. The recent development of an experimental model of pneumonic pasteurellosis in specific pathogen-free (SPF) lambs (Sharp *et al* 1978) has provided a more dependable means of assessing the protective capacity of experimental vaccines than was previously possible. However, experimentation with SPF lambs is costly and the number of animals available is limited and seasonal.

A laboratory animal model of infection with *Pasteurella haemolytica* would have the advantages of relatively low cost and ready availability of greater numbers of experimental animals throughout the year. *P haemolytica* is not a pathogen of laboratory animals and this has hindered the development of a model to study immunity to the organism. Smith (1958) devised two methods by which true infections with *P haemolytica* could be established in mice, one by intracerebral inoculation and the other by intraperitoneal inoculation with granular mucin. This paper describes a modification of Smith's original intraperitoneal means of infection, and its use in assessing the efficacy of *P haemolytica* vaccines.

### Materials and methods

#### Mice

Inbred C57 black mice, four to six weeks old, of both sexes, were used throughout.

#### Bacteria

The serotype and strains of *P haemolytica* used for challenge of mice are listed in Table 1, together with their origin and LD<sub>50</sub> for C57 black mice. Bacteria were maintained on blood agar plates incubated overnight at 37°C then stored at 4°C.

#### Determination of LD<sub>50</sub> of *P haemolytica* for C57 black mice

A 300 ml volume of nutrient broth (Oxoid Nutrient Broth No 2) was inoculated from a fresh blood agar culture of *P haemolytica*. The broth was incubated for 10 h at 37°C, and the bacteria obtained by centrifugation at 1000 g for 20 min at 4°C. The bacteria were washed twice in 0.85 per cent saline, and resuspended in 10 ml saline. A series of 10-fold dilutions was made in saline from this suspension and a viable count was performed by the method of Miles *et al* (1938). The undiluted suspension and dilutions 10<sup>-2</sup> to 10<sup>-8</sup> were incorporated into a 5 per cent suspension of hog gastric mucin, pH 7.2 (ICN Pharmaceuticals), at a ratio of one part bacteria to four parts gastric mucin. Groups of five mice were inoculated intraperitoneally with 0.5 ml of each dilution, and the number of deaths per group recorded 48 h after challenge. In general, no further deaths occurred after this time. The LD<sub>50</sub> for each strain of *P haemolytica* was calculated by the method of Reed and Muench (1938).

#### Growth of *P haemolytica* FA1 in the livers and spleens of normal mice

An inoculum of *P haemolytica* strain FA1 (bio-

TABLE 1: Serotype and origin of *Phaemolytica* strains used in mouse infection tests and LD<sub>50</sub> values assessed by death 48 h after intraperitoneal inoculation

<i>P haemolytica</i>			LD <sub>50</sub> in C57 black mice log <sub>10</sub> cfu*
Serotype	Strain	Origin	
A1	FA1	Lung of experimentally infected lamb	5.3
A2	FA2	Lung of experimentally infected lamb	5.9
A6	FA6	Lung of experimentally infected lamb	3.8
A9	—	Referred from Edinburgh Veterinary Investigation Centre	5.1

\* cfu = colony forming units

type A, serotype 1) was prepared as described above. A group of 20 mice was inoculated intraperitoneally with  $10^{7.1}$  colony forming units (cfu) *P haemolytica* FA1 in gastric mucin, using the same method as for determination of the LD<sub>50</sub>. At time intervals of 1, 2, 3 and 8 h after challenge, groups of five mice were killed by cervical dislocation, and their livers and spleens removed aseptically. These organs were individually macerated with 9 ml peptone water (Oxoid) in a Colworth stomacher (Seward). Serial 10-fold dilutions in peptone water were plated out for viable counting by the method of Miles *et al* (1938). The mean viable count and standard error of the mean (SE) for each group was calculated and expressed as log<sub>10</sub> cfu.

#### Vaccines

Using the methods of N. J. L. Gilmour, D. A. Thompson and D. L. Mould (unpublished) capsule extract from *P haemolytica* type A1 was prepared by extraction with sodium salicylate. Bacteria from an overnight broth culture were shaken in M sodium salicylate for 3 h at 37°C. The bacteria were removed by centrifugation and the supernate was dialysed for 48 h against dilute phosphate-saline buffer (0.02 M sodium phosphate, 0.03 M sodium chloride, pH 7.6) at 4°C. The preparation was concentrated by ultrafiltration through a Diaflo XM100A membrane (Amicon).

The extract was adsorbed onto Alhydrogel (Miles Laboratories) at a concentration determined by a titration carried out according to the manufacturer's recommendations. This preparation was emulsified in an equal volume of Bayol F (Esso) containing 10 per cent Arlacel A (Sigma). A trivalent vaccine, consisting of capsule extract from *P haemolytica* type A1, *P haemolytica* type A6 and heat-killed organisms of *P haemolytica* type A2 was similarly combined with adjuvant.

#### Vaccination of mice with *P haemolytica* A1 capsule vaccine and subsequent challenge with *P haemolytica* FA1

Twenty-five mice were each inoculated subcutaneously with 0.1 ml *P haemolytica* A1 capsule vaccine. Three weeks later, these mice and a group of 25 uninoculated control mice were challenged intraperitoneally with  $10^{6.8}$  cfu *P haemolytica* FA1 in gastric mucin. Five vaccinated and five control mice were killed at the time of challenge and 2, 4 and 6 h thereafter. Their livers were removed aseptically and viable bacterial counts were determined.

Ten mice were each given two doses of 0.1 ml *P haemolytica* A1 capsule vaccine subcutaneously, with an interval of two weeks between the two inoculations. Two weeks after the second inoculation, these mice and a group of 10 uninoculated controls were challenged intraperitoneally with  $10^{5.8}$  cfu *P haemolytica* FA1 in gastric mucin. Immediately following challenge and 6 h later, five mice from each group were killed and viable counts were performed on liver suspensions.

#### Vaccination of mice with trivalent *P haemolytica* vaccine, followed by challenge with *P haemolytica* types A1, A2, A6 and A9

Forty mice were each vaccinated twice subcutaneously with 0.1 ml of the trivalent *P haemolytica* vaccine (types A1, A2 and A6), with a two week interval between inoculations. Two weeks after the second inoculation, these vaccinated mice and 40 control unvaccinated mice were randomly allocated to four groups, each consisting of 10 vaccinated mice and 10 controls. Each group was challenged with one of four serotypes of *P haemolytica*, namely, FA1 ( $10^{7.2}$  cfu per mouse), FA2 ( $10^{6.6}$  cfu per mouse), FA6 ( $10^{5.0}$  cfu per mouse) or A9 ( $10^{6.6}$  cfu per mouse). Five control and five vaccinated mice in each group were killed at the time of challenge, and the remaining five vaccinated and five control mice were killed 6 h later. Viable counts were performed on liver suspensions.

#### Statistical treatment of results

Viable counts were expressed as log<sub>10</sub> cfu, and means and SE were calculated. Where possible, Student's *t* tests were performed and, where the data were unsuitable, the Mann-Whitney ranking method was employed.

#### Results

##### LD<sub>50</sub> of *P haemolytica* strains for C57 black mice

The LD<sub>50</sub> values of *P haemolytica* strains FA1,

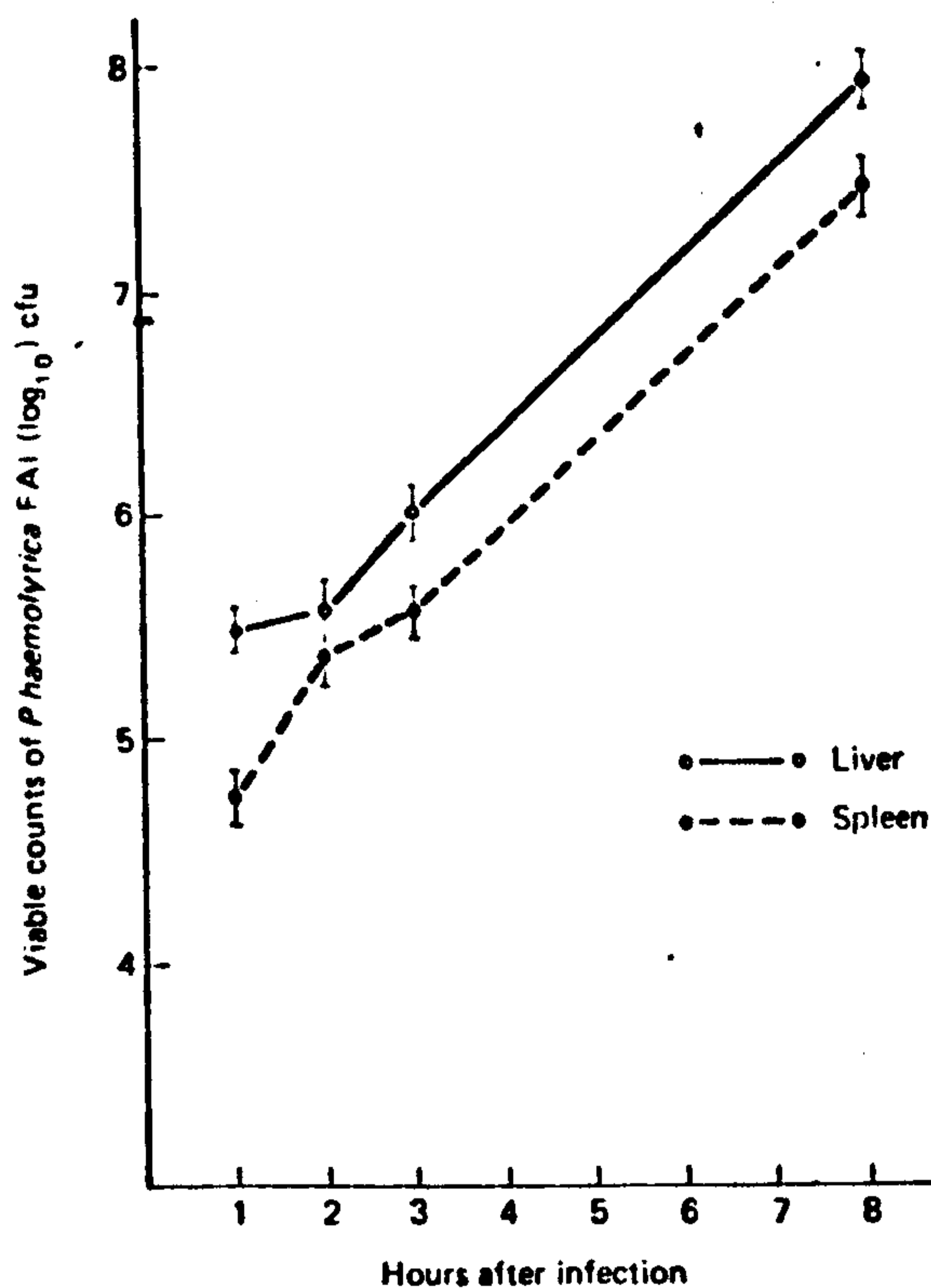


FIG 1: Viable counts (mean  $\pm$  SE) of *P haemolytica* FA1 in livers and spleens of normal mice after intraperitoneal inoculation of  $10^{7.1}$  cfu in gastric mucin

FA2, FA6 and the type A9 strain are listed in Table 1. In general, mice which succumbed to infection were dead or moribund 24 h after inoculation. The challenge doses of *P haemolytica* used in subsequent experiments were always greater by at least  $10^{0.8}$  cfu than the LD<sub>50</sub> for each strain.

*Growth of P haemolytica FA1 in the livers and spleens of normal mice*

The viable counts of *P haemolytica* FA1 in the livers and spleens of normal mice are shown in Fig 1. Each point plots the mean of the counts for five livers or spleens,  $\pm$  standard error, expressed as log<sub>10</sub> cfu. From 2 h after infection, growth of *P haemolytica* FA1 in both organs was exponential, with similar growth rates in each. In most subsequent experiments, mice were sampled only at the time of infection and 6 h later, but in the following experiment, additional mice were killed 2 and 4 h after infection. In order that a greater number of treatments could be investigated simultaneously, viable counts in livers only were examined in later experiments.

*Growth of P haemolytica FA1 in mice vaccinated with P haemolytica A1 capsule vaccine*

Viable counts of *P haemolytica* FA1 in the livers of vaccinated and control mice are shown in Fig 2. Viable counts of *P haemolytica* FA1 increased exponentially in the livers of untreated mice, whereas there was a reduction in the number of viable bacteria in the livers of vaccinated mice. Five infected control mice which were not killed all died within 19 h. In contrast, five infected vaccinated mice appeared healthy at that time and *P haemolytica* FA1 could not be recovered from their livers.

Viable counts in the livers of control and double vaccinated mice at the time of challenge and 6 h after challenge are given in Table 2. The mean viable counts in the livers of vaccinated mice 6 h after infection were significantly lower than those in the controls ( $P = 0.008$ ).

*Effect of vaccination of mice with trivalent (A1, A2, A6) P haemolytica vaccine upon challenge with P haemolytica types A1, A2, A6 and A9*

The results of this experiment are summarised in

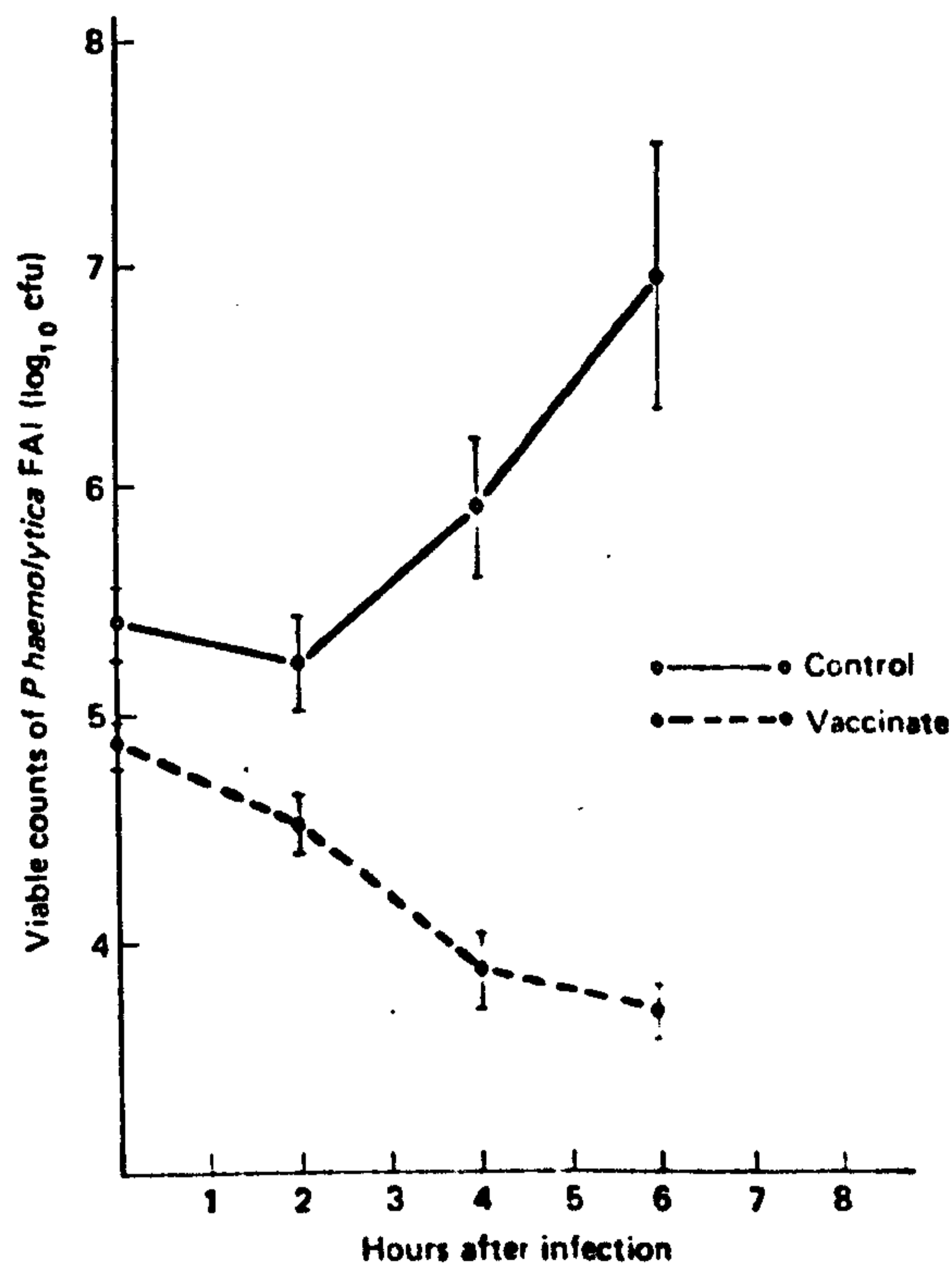


FIG 2: Post-challenge viable counts (mean  $\pm$  SE) in livers of control mice and mice vaccinated three weeks previously with *P haemolytica* A1 capsule vaccine. The challenge inoculum was  $10^{6.8}$  cfu in gastric mucin, given intraperitoneally

TABLE 2: Post challenge  $\log_{10}$  viable counts (geometric mean  $\pm$  SE) of *P haemolytica* FA1 in the livers of control mice and of mice vaccinated with *P haemolytica* A1 capsule four and two weeks before intraperitoneal inoculation of  $10^{10.0}$  cfu in gastric mucin

Hours after challenge	Control	Vaccinated
0	4.72 $\pm$ 0.09	4.71 $\pm$ 0.06
6	6.29 $\pm$ 0.20	4.20 $\pm$ 0.62*

\* Significantly lower than control value ( $P = 0.008$ )

Table 3. Viable counts in the livers of control mice challenged with each of the four serotypes of *P haemolytica* used in this experiment increased in the 6 h following challenge. Rates of growth similar to those in the livers of control mice were observed in vaccinated mice challenged with *P haemolytica* strain FA2 and *P haemolytica* A9, and viable counts in the livers of vaccinated mice 6 h after infection in the two groups were not significantly different from those of their respective controls. In contrast, over the same time interval viable counts in the livers of vaccinated mice challenged with *P haemolytica* FA1 and *P haemolytica* FA6 dropped dramatically. Six hours after challenge no organisms were detected in the liver homogenates of any vaccinated mice challenged with *P haemolytica* FA6, or *P haemolytica* FA1 indicating that their content of viable organisms was less than  $10^{2.7}$  cfu/ml, the lower limit of the counting technique employed.

### Discussion

The low virulence of *P haemolytica* for laboratory animals has hampered the development of a suitable laboratory animal model of infection with this organism, and it is necessary to include in the challenge inoculum substances which effectively increase virulence. Gastric mucin was first used to enhance the virulence of *P haemolytica* for mice by Smith (1958), although its mode of action still remains unknown. Mucin has been shown to be anti-complementary *in vitro* by Lambert and Ritchley (1952), but a comparison of various mucins revealed little correlation between antiproperdin activity and the ability to promote infection with *Escherichia coli* or *Staphylococcus aureus* (DeWitt 1958). The introduction of high molecular weight dextran sulphates at the time of infection with *P haemolytica* increases virulence to the same extent as gastric mucin but related compounds, such as heparin, are less effective (Wessman 1967).

In his original work with intraperitoneal infection of mice with *P haemolytica*, Smith (1958) merely recorded death or survival of challenged mice. In the present experiments, post-challenge numbers of

TABLE 3: Post challenge  $\log_{10}$  viable counts (geometric mean  $\pm$  SE) of *P haemolytica* in the livers of control mice and mice vaccinated four and two weeks before intraperitoneal inoculation with *P haemolytica* types A1, A2, A6 and A9

Hours after challenge	Challenge with <i>P haemolytica</i> FA1		Challenge with <i>P haemolytica</i> FA2		Challenge with <i>P haemolytica</i> FA6		Challenge with <i>P haemolytica</i> A9	
	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated
0	5.77 $\pm$ 0.089	5.44 $\pm$ 0.141	5.38 $\pm$ 0.033	5.59 $\pm$ 0.097	3.34 $\pm$ 0.098	3.30 $\pm$ 0.077	5.54 $\pm$ 0.089	5.30 $\pm$ 0.113
6	7.65 $\pm$ 0.127	< 2.7*	8.05 $\pm$ 0.112	7.65 $\pm$ 0.144***	5.59 $\pm$ 0.220	< 2.7**	7.68 $\pm$ 0.080	6.74 $\pm$ 0.375***

\* Significantly lower than control value ( $P = 0.016$ , Mann-Whitney ranking method)

\*\* Significantly lower than control value ( $P = 0.006$ , Mann-Whitney ranking method)

\*\*\* Not significantly lower than control value ( $P = 0.06$ , Student's *t* test)



viable organisms in the livers of mice were determined. Preliminary studies with experimental vaccines showed insufficient difference in LD<sub>50</sub> between control and vaccinated mice for assessment of the efficacy of vaccine preparations (Evans and Wells unpublished). In the present experiments, post-challenge numbers of viable organisms in the livers of mice were determined. By using a suitable challenge dose, exceeding the LD<sub>50</sub> by at least 10<sup>0.5</sup> cfu, large differences in the abilities of control and successfully vaccinated mice to inactivate *P haemolytica* were detected. This criterion for the assessment of the efficacy of vaccines would appear to be more sensitive than that of LD<sub>50</sub>.

Non-specific stimulation of peritoneal macrophages can occur following the introduction of a variety of substances into the peritoneum, such as serum and mineral oil (Stuart *et al* 1978). The administration intraperitoneally of bovine serum albumin (BSA) adsorbed onto aluminium hydroxide and emulsified with oil was shown to have a protective effect comparable with intraperitoneal vaccination with *Phaemolytica* A1 capsule vaccine (Evans and Wells unpublished). However, no such effect was observed when this BSA preparation was inoculated subcutaneously. For this reason the subcutaneous route of vaccination was chosen in preference to the intraperitoneal one in these experiments.

From the results of initial experiments in which mice were killed and sampled at frequent time intervals after challenge, a standard sampling time of 6 h following infection was chosen for all subsequent work. By that time, growth of the organisms in the livers of control mice had become exponential and numbers of viable organisms in the livers of successfully vaccinated mice had declined (Fig 2). Vaccination times were standardised as four weeks and two weeks before challenge.

Under the conditions described the vaccine containing *P haemolytica* type A1 capsule extract protected mice against challenge infection with *P haemolytica* FA1. This vaccine has also been shown to protect SPF lambs against the effect of infection with *P haemolytica* FA1 (Gilmour *et al* 1979).

The trivalent *P haemolytica* vaccine, which contained capsules of serotypes A1 and A6 and heat killed organisms of serotype A2, was protective

against challenge with types A1 and A6 but not against challenge with types A2 and A9. This finding is in agreement with data obtained from vaccination-challenge experiments in SPF lambs using the same vaccine (Gilmour *et al* 1979). It is notable that in both species protection was associated with capsule antigens (types A1 and A6) but was apparently not stimulated by heat-killed antigens (type A2). The results are evidence of a correlation between the protection afforded by these vaccines in mice and the protection which they give to SPF lambs challenged by the method of Sharp *et al* (1978). The model of infection in mice may therefore prove useful in assessment of experimental vaccine preparations and experiments are being undertaken to determine the degree of cross-protection between different serotypes of *P haemolytica*.

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