EXPERIMENTAL INFECTION WITH BORDETELLA PERTUSSIS IN THE MOUSE BRAIN

A THESIS

submitted by

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Abstract

Two types of infecting strains of Bordetella pertussis have been studied. One type was of high virulence for the mouse brain, a dose of 5×10^3 viable bacteria killing all the mice in 5 to 8 days. The other type was of low virulence for the mouse brain, a dose of 5×10^3 viable bacteria giving rise to a transitory infection, all brains being sterile 5 days after challenge.

It was shown following an infection with a low virulence strain that the brains were less infectable to a second infection with the same low virulence strain but were unchanged to a second infection with a high virulence strain.

Previous infection with either vaccinia virus or influenza virus resulted in brains being uninfectable with either low or high virulence strains of B. pertussis.

The high virulence strains were less resistant than the low virulence strains to the bactericidal action of complement in vitro.

Cortisone, injected subcutaneously, greatly enhanced infections with both low and high virulence strains and vitamin A and streptolysin-O also enhanced infection with low virulence strains.

Virulence did not seem to be dependent on the release of

lysosomal enzymes in the brain.

X-irradiation of the head alone greatly stimulated infection with low virulence strains. It was suggested that irradiation had damaged neuroglial cells which when stimulated to divide by a B. pertussis infection either failed to do so or died.

In the immune mouse X-irradiation of the head alone did not decrease the number of mice surviving a lethal challenge but irradiation to the body alone, the head being protected, resulted in fewer mice surviving lethal challenge.

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1. Introduction

Bordetella pertussis is a Gram negative, non-motile, non-sporing, cocco-bacillus first isolated by Bordet and Gengou in 1906. All strains appear to be obligate parasites inhabiting the respiratory tract and are pathogenic in man. It is the causative organism of bacterial whooping cough and seems seldom to play a harmless parasitic role.

Vaccination against whooping cough was first employed in 1923 in the Faroe Islands (Madsen, 1933) during a severe epidemic. The number of deaths and severe cases was considerably reduced in the vaccinated group of children.

The mouse was introduced as an experimental model for whooping-cough and the intranasal route of challenge was developed by Burnet and Timmins (1937). Ten years later Kendrick et al. (1947) published the results of their study on the intracerebral infection in mice and this route of challenge was adopted for potency testing of B. pertussis vaccines following the Medical Research Council's Reports on Vaccination against Whooping Cough (1951, 1956, 1959). Standfast (1958b) showed that results in the field correlated better with potency tests using the intracerebral challenge route than the intranasal challenge route.

Most of the work on Bordetella pertussis has been

concerned with the mouse high virulence strain 18/323 and little attention has been paid to other high virulence strains or, more especially, to the low virulence strains. In this study it has been found that these latter strains grow in the mouse brain for twenty-four to forty-eight hours before rapidly disappearing, the mouse brain being sterile by the fourth to fifth day. The altered reaction of the mouse to these strains, following administration of certain drugs and X-irradiation has also been studied.

In the immune mouse the bacteria multiplied intracerebrally for four days before rapidly disappearing. Apart from the need for specific protective antibody little is known of the other factors involved in this mechanism of protection.

2. Historical

2.1. The Mouse as an Animal Model for Whooping Cough

Burnet and Timmins (1937) were the first workers to study an infection with Bordetella pertussis in the mouse. Two drops (0.05 ml.) of a suspension containing 200 x 10^6 bacteria/ml. were injected intranasally. After five days exudate from a consolidated area of lung gave a pure culture of B. pertussis on Bordet-Gengou medium.

Burnet and Timmins found that with large doses of bacteria (200 x 10^6) death of the mice occurred in two to three days; with small doses (8 x 10^6) death was delayed but rarely beyond the eleventh or twelfth day.

Sections from lungs removed five to seven days after inoculation showed a general interstitial pneumonia with cellular infiltration and oedema of the alveolar walls. The bronchiolar epithelium showed evidence of proliferation and, judged by the number of goblet cells, was actively secreting mucus The regular arrangement of the cilia-bearing surface was completely lost.

Other animals which have been studied after intranasal challenge were monkeys (North et al., 1940) and rabbits (Spruht et al., 1938).

The intracerebral route of challenge with Bordetella pertussis was developed between 1943 and 1946 by Kendrick et al.

(1947). Their method was an adaptation of the procedure described by Norton and Dingle (1935) who used the intracerebral route of challenge in the mouse protection tests for S. typhi vaccines.

Standfast (1958b) assayed fourteen vaccines in the laboratory by both the intracerebral challenge (I.C.) and the intranasal challenge (I.N.) techniques. The I.C. test arranged the vaccines in a different order of potency from the I.N. test. Field trials showed that the mouse potency test using the I.C. challenge corresponded very well with the field results (M.R.C. Reports 1951, 1956, 1959) whereas the I.N. challenge did not. All recent work on B. pertussis has concentrated on the I.C. challenge route.

2.2 Bacterial Growth Intracerebrally in Normal Mice

2.2.1. High Virulence Strains

Dolby and Standfast (1961) studied the I.C. infection with the mouse-brain high virulence strain 18/323. They found that immediately after injection of the challenge suspension, contained in 0.025 ml., about 90% disappeared from the brain.

Similar observations have been made by others, Schlesinger (1949) observed that only 3-10% of the inoculum could be found in the mouse brain one hour after injection with equine encephalomyelitis virus.

Similarly Cairns (1950) could only recover 2-8% of a

bacteriophage inoculum from the mouse brain less than five minutes after injection. In most of his experiments 0.3 ml. of the suspension was injected, however he could not show a smaller loss into the blood stream when only 0.005 ml. was injected using a micrometer syringe.

Mims (1960a) injected Indian ink intracerebrally into mice and found that it was distributed throughout the subarachnoid space, in the Virchow-Robin spaces and in the ventricles. The injected material was deposited within the cerebrospinal fluid spaces and did not come into contact with the cerebral matrix (Schaeffer and Muckenfuss, 1938).

Mims also observed, in brains that were removed after one or two days, glial cells in the subependymal area and the pia laden with Indian ink. It was suggested that they had migrated into the cerebral matrix from the subarachnoid space and ventricles.

Blyth (1955) studied the course of infection with the mouse high virulence B. pertussis strain 18/323 at short time intervals after injection of the inoculum. He showed an 80% loss of bacteria fifteen minutes after challenge. The bacterial count rose slightly and then fell over the next few hours; this was followed by a rapid increase in the count which reached or even exceeded the original inoculum in twenty-four hours. The viable count increased by approximately one logarithm daily for three or four days after which the

multiplication rate slowed down, the mice dying after six to seven days with a bacterial count of $10^{7.5}$ to 10^{8} live bacteria.

Dolby and Standfast (1961) showed by injection of graded challenge doses of the high virulence strain 18/323 that the growth curves were approximately parallel for all inocula and the time to death depended on the size of the inoculum. Unlike challenge with virulent strains by the I.N. route where small doses may lead to a sublethal infection (Dolby et al., 1961) all intracerebral infecticns by high virulence strains are lethal. It seemed that one surviving organism was capable of multiplying and eventually bringing about the death of the mouse; the terminal brain count was independent of the challenge size. Similar results were obtained by Berenbaum et al. (1960) and Iida et al. (1962) also using the high virulence strain 18/323 and by Andersen (1952b, 1957a) using the high virulence strain 2-Atox.

2.2.2. Low Virulence Strains

Recent work has been mainly concerned with the three most used high virulence strains 18/323, G353 and 2-Atox. However a few brief reports have appeared in the literature concerning mouse - low virulence strains. The low virulence strains seemed to consist of two types, one of which multiplied in the mouse brain for a few days and then rapidly disappeared and a second type which never multiplied in the brain and with

which the brains were sterile within twenty-four hours.

Blyth (1955) challenged intracerebrally groups of six mice with 2 x 10^3 viable organisms of an attenuated strain H154. The brain count reached a maximum by the fifth day and then decreased until the brains of all mice were sterile by the twenty-first day. All the mice remained alive and well throughout the experiment.

Andersen (1957a) injected mice with a freshly isolated strain 1528. During the following two or three days the organisms multiplied rapidly and there was little variation in the individual brain counts. After this time the number of organisms varied considerably, the brains of some mice rapidly becoming sterile while in others multiplication continued and the mice died.

Brown (1958) described an intracerebral infection with a strain 1011 which had an LD_{50} of 2 x 10^5 organisms. An inoculum of 10^3 organisms multiplied in the mouse brain for three to four days and then disappeared.

Further investigations with a Tohama Phase I strain showed that on injection of 10^4 viable organisms the viable count had reached a maximum by the first day and remained at this level for a further six days, no bacterial counts were made after this time. When 2 x 10^7 viable organisms were injected all mice were dead by the third day with a terminal viable count of $10^{7.5}$ organisms (Iida et al., 1962).

The same workers also injected 10^4 organisms of a Tohama Phase III strain; all mice had sterile brains within twenty-four hours, no increase in viable count having been detected. Even after injection of 7×10^9 organisms of this strain, it was difficult to find any bacteria after five hours.

No visible distinction can be observed in mice when challenged with any of these three types of strains during the first hour following challenge. Whereas the Tohama III strain continues to decrease in number after this time, both the high and low virulence strains multiplied approximately tenfold for the twenty-four hours after challenge and no distinction by viable count could be made at this time. However during the next twenty-four hours a real difference was observed, the high virulence strain multiplied a further tenfold whereas the low virulence strain only increased slightly or may even have decreased in number. Little attempt has been made to account for these differences. An antibacterial response due to the formation of local antibody has been suggested (Andersen, 1957a) and phagocytosis has also been implicated (Brown, 1958). No concrete evidence has been given to support either of these theories.

2.3. Bacterial Growth in Actively and Passively Immunised Mice

Although the Pertussis Vaccine Potency assay was described by Kendrick et al. (1947) it was not until fourteen years later that the mechanism of the test was explained.

As early as 1944 Ospeck and Roberts had demonstrated protection against B. pertussis by injecting intracerebrally challenged mice intravenously with a B. pertussis "antitoxin" prepared in rabbits.

Blyth (1955) examined the course of infection in actively and passively immunised mice challenged with the high virulence strain 18/323. He found that the infection became established in all mice, vaccinated and unvaccinated. The intracerebral count increased more slowly in the vaccinated mice than in the unvaccinated controls until the count reached 2.5×10^4 organisms/brain. After this time there was a very rapid clearance of organisms in the vaccinated group and most mice had sterile brains by the seventh day. He also suggested that the challenge organisms could act as a secondary antigenic stimulus but no attempt was made to explain how the specific antibody reached the infected site.

These observations were later confirmed by Andersen (1957a) who found that some immunised animals died at the same time as the control mice while in others the number of organisms decreased rapidly on the sixth to eighth day after challenge. A more precise examination of the bacterial counts in immunised mice was carried out by Berenbaum et al. (1960) and by Dolby and Standfast (1961). Their observations, which were in close agreement, showed that for the first twenty-four hours after challenge no difference could be found in the bacterial counts in the brains of normal and immunised animals.

From the second to fourth days the counts in the immunised mice fell slightly below those in the normal animals. The maximum count in the brains of immunised mice was reached at about the fourth day, and was approximately 10^6 viable organisms. After this time the number of surviving organisms rapidly declined until most mice had sterile brains between the sixth and fourteenth days.

The four day lag between challenge and the time when organisms began to disappear was quite unlike the immediate reduction in the numbers of organisms in immune mice challenged by the intranasal route (Dolby et al., 1961). This suggested some barrier between the infecting organisms and antibody which was present in intracerebrally challenged mice but not in the intranasally challenged mice.

An immediate fall in the bacterial count in the brain was observed by Spasojevic (1962) who incubated mixtures of organisms and immune sera and injected the mixture intracerebrally. The bacterial counts never increased and the brains of surviving mice were sterile in twenty-four hours. From bacterial counts before and after incubation it was concluded that the sera were not bactericidal in vitro. It also suggested that complement played no part in pertussis immunity in the mouse unless the damage caused by the injection facilitated the entry of complement or that complement was a normal component of the cerebrospinal fluid.

2.4. The Blood Brain Barrier

Damage to the blood-brain barrier was implicated as the means of entry of specific antibody (Berenbaum *et al.*, 1960; Holt *et al.*, 1961). Evidence for the existence of a blood brain barrier has accumulated since the first experiments of Ehrlich (1885) who found that an intravenous injection of coerulein-S left the central nervous system uncoloured. The presence of this physiological barrier seems to be widely accepted but its anatomical basis is not clear. Tschirgi (1952) considers the barrier to be located in the pia-glial membrane while Spatz (1933) favours a capillary endothelium theory.

The breakdown of the blood-brain barrier with *B. pertussis* infections was noted by Berenbaum *et al.* (1960) who used radio-iodinated human serum albumin, injected intraperitoneally into mice at the same time as intracerebral challenge, as a marker for damage. They found that the radioactive levels of the infected brains at the sixth day were about five times higher than those of the control uninfected mice. It was also suggested that the breakdown was due to the release of bacterial endotoxin.

Holt *et al.* (1961) also recorded the breakdown of the blood-brain barrier using Pontamine Sky Blue or Human serum as a marker. These workers showed a change in the brains of mice when the bacterial count in the brain reached 10^5 - 10^6 viable organisms at about the fourth day of infection. The

infection proceeded as normal below this level, the effective challenge dose being constant at 10^5 - 10^6 live microbes when the blood-brain barrier was broken and the organisms were not affected by antibody until this point was reached. In the mouse test, therefore, the proportion of mice surviving challenge with the high virulence strain is dependent only on the degree of specific immunity and is independent of the challenge size.

Experiments with Pontamine Sky Blue showed slight colouring of the brain by the third day, the intensity increasing from this time onwards. The brains of surviving mice were no longer coloured after the sixth to seventh day. A slow seepage of antibody might explain the slightly slower rise in the bacterial count of the brains of immune mice than in the control mice over the first four days of infection as shown by Dolby and Standfast (1961).

2.5. Interference Immunity

Evans and Perkins (1954b) showed that *B. pertussis* vaccine injected intracerebrally either in the form of a killed bacterial suspension or a cell free extract (Pillemer *et al.*, 1954) was able to protect mice against the otherwise fatal infection that followed intracerebral injection with *B. pertussis* strain 18/323. The vaccine could be injected either simultaneously with, or a few days before the challenge. This type of immunity was termed "interference immunity" to distinguish it from specific antibody immunity.

No attempt was made to follow the fate of the injected organisms but it was shown that the brains of surviving mice were sterile four weeks after challenge. Evans and Perkins suggested that this type of immunity may be related to the interference phenomenon found in some virus infections.

Support for these observations was provided by Dalldorf et al. (1947) who showed that vaccinia virus injected intracerebrally gave significant protection against a subsequent challenge with B. pertussis 18/323. No effect could be observed in mice which received the vaccine one, two or three days after challenge.

Blyth (1955) determined the bacterial count at daily intervals in mice receiving vaccine and challenge intracerebrally. Three groups of mice could be recognised, in the first group the organisms were completely eliminated by the third day after challenge and the brain was found to be sterile in all mice killed after this time. In a second group the viable count increased at a slower rate and reached a lethal level two or three days later than the control mice. In the last group the viable count increased at virtually the same rate as the control mice.

Evans and Perkins (1954a) showed that a single intraperitoneal injection of B. pertussis vaccine was able to produce a substantial degree of immunity to an intracerebral challenge of B. pertussis injected a few hours later. Neither

circulating protective antibody nor complement-fixing antibody could be detected at this time and agglutinating antibodies were present only in small quantities in surviving mice. Nor was there evidence that the challenge suspension could act as a secondary antigenic stimulus. This effect was produced neither with vaccine heated to 100°C for 15 minutes nor with vaccines prepared from B. parapertussis, B. bronchisepticus and E. coli, nor with Tetanus Toxoid and Diptheria P.T.A.P. Evans and Perkins considered that this immunity was of the interference type as it was unlikely that enough specific antibody could be formed in this short time. It was not then known that antibody is unable to reach the antigen until four days after challenge and it now seems likely that this early immunity is of the antibody type.

The period between vaccination and challenge was later extended to cover a time of up to forty days (Evans and Perkins, 1955a). Using nearly 1200 mice they were able to show that mice challenged fourteen days after vaccination were more susceptible than those challenged at either ten days or twenty days after vaccination. It was postulated that the fall in protection was due to a waning in interference immunity and a slow build up of antibody immunity. It was suggested that at around the fourteenth day after vaccination an unstable state existed in the mouse and it was not until at least the twentieth day that antibody immunity was at its maximum. They suggested that in the mouse test mice should not be challenged until at

least twenty days after vaccination.

Later Andersen (1957b) found no difference in the ED_{50} of vaccines whether mice were challenged fourteen or thirty days after vaccination.

2.6. Pathology of B. pertussis Infections

Reports in the early literature (Mallory and Horner, 1912; McDonald and McDonald, 1933) implicated the ciliated epithelium of the respiratory tract as the site of infection in B. pertussis infections. In children dying from whooping-cough the pertussis bacilli were found mainly in the ciliated lining of the bronchi and trachea.

Burnet and Timmins (1937) showed a massive proliferation of small bacilli in the mucus with destruction of the ciliated surface of the bronchiolar epithelium. In experiments with rabbits and monkeys the organisms were limited to the ciliated surface of the bronchi and bronchioles and were not found in alveoli (Sprunt et al., 1938).

Gallavan and Goodpasture (1937) showed in experiments with chick embryos that infection was restricted to the respiratory tract, and that there was also inflammation and necrosis of the middle and basal layers of the epithelial membrane.

Very few studies have been made of the site of infection in the mouse brain and almost all of these have been made in

mice infected with the high virulence strain 18/323.

As in the respiratory tract infection Berenbaum et al. (1960) and Iida et al. (1962) found in the mouse brain that bacterial multiplication was restricted to the ciliated surface of the ependyma and there was no general invasion of the brain tissue. Infiltration of polymorphonuclear leucocytes and other phagocytic cells was seldom observed until after the fourth day when some organisms were seen within these cells.

Berenbaum et al. reported that some organisms were seen within ependymal cells and Iida et al. mentioned that bacteria seemed to be intracellular in impression smears but these were not apparent in paraffin sections. Brain lesions increased in severity and many haemorrhages were seen in the terminal stages of infection and death of the mice seemed to result mainly from acute hydrocephalus.

Iida et al. (1963) found that cortisone, injected subcutaneously two hours before and one day after challenge, increased the infectivity of a low virulence Tohama Phase I strain. Whereas in untreated mice the LD_{50} for this strain was $>2 \times 10^6$ bacteria, in cortisone treated mice the LD_{50} was reduced to 2×10^3 bacteria. Also when previously immunised mice were treated with 2.5 mgm. of cortisone two hours before and one and two days after challenge with 100 LD_{50} of 18/323, they succumbed to the infection as did the non-immune mice. A marked degree of leucopenia was observed for two days

following the administration of cortisone but the blood leucocyte count was normal by the fifth day. No explanation of this phenomenon was given and the importance of the leucopenia was not clear.

Recently the same workers have studied the pathology of B. pertussis infections by the use of fluorescent antibody to stain the bacteria (Iida et al., 1966). Specific fluorescent antiserum was injected intravenously into normal and cortisone treated mice four days after an intracerebral challenge of B. pertussis 18/323. In normal mice bacterial colonies were located on the surface of the ependymal cells and six hours after injection of immune serum the colonies became loosened and finally detached from the ependyma. The main effect of cortisone was that the colonies continued to adhere to the surface of the ependymal cells. Treatment with cortisone did not affect the appearance of the fluorescence and the degree of leucocytic infiltration was similar to that in the untreated group although previous work (Iida et al., 1963) had shown cortisone to produce an extensive leucopenia.

This work did not go much further in explaining how B. pertussis 18/323 is eliminated from the brain of the immune mouse.

2.7. Biological Activities of Bordetella pertussis

Many biological activities of importance have been described for B. pertussis. The following have been recognised:-

(i) Agglutinogen
(ii) The Heat-Labile Toxin
(iii) The Heat-Stable Toxin
(iv) Haemagglutinogen
(v) The Histamine Sensitising Factor (HSF)
(vi) The Protective Antigen (PA).

2.7.1. Agglutinogen

Until Andersen (1953) demonstrated that freshly isolated strains of B. pertussis may be divided into different serological groups the species had always been considered to be serologically homogenous (Leslie and Gardner, 1931; Standfast, 1951b).

Leslie and Gardner (1931) described the occurrence of four different phases, Phases I and II, which were considered to be the Smooth (S) forms, and Phases III and IV which were considered to be Rough (R) forms. Later it was suggested that more than one agglutinogen/agglutinin system was involved in the Phase I agglutination (North et al., 1939) and various investigators (Smolens et al., 1947; Kendrick et al., 1949) mentioned that freshly isolated strains of B. pertussis may vary considerably in the agglutinability.

Andersen (1952a, 1953) distinguished the thermolabile (O)

antigen and the thermostable (K) capsular antigens a_1 a_2 a_3 a_4 and a_5 by their resistance to two hours heating at 100°C and found that all strains had a common O and a_1 antigen but were divisible according to their content of a_2 a_3 a_4 and a_5 antigens. Eldering et al. (1957, 1962) have recognised at least fourteen different antigens among various Bordetella strains.

Some workers (Flosdorf and Kimball, 1940; Smolens and Mudd, 1943) found that the protective effect of vaccines was closely associated with their agglutinogen content. However Andersen (1955) found that mouse protective sera need contain no agglutinins and that the protective power of vaccines is not associated with agglutinin type specificity (Andersen and Bentzon, 1958).

Preston and Te Punga (1959) suggested that the group specific and not the type specific agglutinogen was responsible for protection and later (Preston and Evans, 1963) found that antisera, which they claimed contained only specific agglutinins, could protect mice against an intracerebral challenge of B. pertussis. Investigations into the reported increased incidence of whooping cough have shown that there was a predominance of the Type 1:3 agglutinogens in strains freshly isolated from whooping cough patients (Preston, 1963, 1965; Mon. Bull. Min. Hlth. 1965). It was suggested that

agglutinogen deficiencies in pertussis vaccines had resulted in failure to give complete protection against infecting strains possessing these agglutinogens. Recently Holt (1967) has shown that the incidence of whooping cough in vaccinated children is no higher than is to be expected from the known failure rate of B. pertussis vaccines.

Other evidence has accumulated against the theory that an agglutinogen is the protective antigen. Horton and Standfast (1953) obtained good protection against B. pertussis infections using inagglutinable vaccines. By sonic disintegration Pillemer (1950) was able to prepare an extract of B. pertussis which when adsorbed onto red cell stromata was highly protective for mice (Pillemer et al., 1954). This preparation when tested in mice gave a high level of protection but the antisera contained little or no agglutinins (Evans and Perkins, 1955b).

Holt (unpublished work) has obtained good protection in mice by modulating usually highly agglutinable strains of B. pertussis on special media to produce highly protective vaccines containing no agglutinogens. There is now a considerable weight of evidence against an agglutinogen being the protective antigen.

2.7.2. Haemagglutinogen

In 1947 Keogh et al. (Keogh, North and Warburton 1947;

Keogh and North 1948) proved the existence of an antigen in B. pertussis, B. parapertussis and B. bronchisepticus able to agglutinate human and animal red blood corpuscles. According to their reports it seemed to be the antigen responsible for protection. The authors further reported that a correlation existed between the haemagglutinogen content of B. pertussis strains, their virulence for mice and their ability to induce protection in mice. Their observations have not been confirmed by later investigators. Winter (1953) showed that anti-haemagglutinin was present in only 14% of convalescent sera.

The lack of correlation between protective antibodies and anti-haemagglutinin has also been shown by the intracerebral challenge technique (Thiele, 1950; Masry, 1952). Masry obtained purified preparations of haemagglutinogen and was able to reduce the nitrogen content without affecting the content of haemagglutinogen. From immunisation experiments with such purified preparations he was unable to demonstrate protection against intracerebral or intranasal infection.

The vaccine prepared by Pillemer et al. (1954) although highly protective did not contain any haemagglutinogen.

2.7.3. Heat Labile Toxin

The first investigations of the toxin of B. pertussis were undertaken by Bordet and Gengou (1906, 1907, 1909). It

is an extremely labile toxin losing its toxicity above 37°C and is easily detoxified with formalin (Evans and Maitland, 1937).

Billaudelle et al. (1960) were able to separate the heat labile toxin into two fractions, one part which was dermonecrotic for rabbits and a second part, the neurotoxin, which was lethal for mice when injected intracerebrally.

Early workers (Evans and Maitland, 1937, 1939; North et al., 1939) failed to discover the antigenic property of the toxin, probably due to its very labile nature, but this was finally established by Evans in 1940 (Evans, 1940). He showed that pertussis antitoxin will neutralise the action of B. parapertussis and B. bronchiseptus toxins in vitro and that rabbits immunised with pertussis toxin were immune to the effects of the other toxins. By cross-neutralisation experiments Andersen (1953) found the toxins produced by the three species to be identical.

Although Ospeck and Roberts (1944) demonstrated protection in mice with an injection of "antitoxin", many workers have shown that the antitoxin does not protect mice against intracerebral challenge with B. pertussis (Verwey and Thiele, 1949, Munoz et al., 1959; Billaudelle et al., 1960).

2.7.4. Heat Stable Toxin

Cruickshank and Freeman (1937) isolated a lipo-

polysaccharide from B. pertussis that protected mice challenged intraperitoneally. It has partly been characterised and constitutes 3.4% of the cell wall (MacLennan, 1960). The lipopolysaccharide as prepared by the method of Westphal et al. (1952) has been found to be non-protective in mice against an intracerebral challenge (Munoz, 1963) but has a high adjuvant activity similar to that of other Gram negative lipopolysaccharides (Farthing, 1961; Farthing and Holt, 1962).

2.7.5. The Histamine Sensitising Factor (HSF)

A factor has been found to be present in B. pertussis Phase I organisms that increased the sensitivity of mice to an intraperitoneal injection of histamine by 50-100 fold (Parentjev and Goodline, 1948).

In preparations of B. pertussis cell walls the histamine sensitising activity and the protective activity were often closely associated. Pittman (1951a) showed in protection tests that more female than male mice survived; the difference was found to be in the same order of magnitude as the difference between the histamine sensitivity of female and male mice. Pittman (1951b) showed a close correlation between histamine sensitising activity and protective activity with fluid vaccines. By prolonged heating the sensitising property was reduced relatively more than the protective activity.

Other workers (Joo and Pusztai, 1960) have shown the

HSF content to parallel the protective potency while Munoz and Hestekin (1963) considered them to identical.

Fishel (1956) showed that X-irradiation destroyed the immune response of his animals but not the histamine sensitising response. Dolby (1958) showed, with fractionated extracts of B. pertussis, the histamine sensitising factor to be different from the antigen protecting mice against an intracerebral challenge.

2.7.6. The Protective Antigen

The protective antigen is the substance which gives rise to antibody able to protect mice against a lethal infection with Bordetella pertussis.

From the previous sections it seems certain that the factor in B. pertussis cells that elicits immunity is not associated with the agglutinogen, the haemagglutinogen, the heat labile toxin, or the histamine sensitising factor. Pillemer et al. (1954) could not detect any of the above factors in his stroma protective antigen complex which was shown to be highly protective.

The possibility of two protective antigens, one antigen protective against the I.N. challenge route and the other antigen protective against the I.C. challenge route was shown by Dolby and Standfast,(1958). They examined many rabbit B. pertussis antisera and found some to be protective against an I.N. challenge, some to be protective against an I.C.

challenge and some to be protective against both challenge routes.

The antigens have been partially purified by fractionation (Dolby, 1958). The I.N. antigen was stable to boiling for one hour whereas the I.C. antigen was destroyed. Strains may possess one or both antigens.

2.8. Virulence of Bordetella pertussis

The weight of the mouse and the volume of challenge dose did not seem to influence virulence by the intracerebral route but by the intranasal route the LD_{50} decreased both with small mice and with large dose volumes (Standfast, 1958a).

Toxin production has often been associated with virulence of micro-organisms. Standfast (1958a) investigated the effect of B. pertussis toxin on sheep lung preparations. He used the distance travelled by injected Indian ink as a measure of ciliary activity and found that a living suspension of B. pertussis, or cell free toxin, inhibited ciliary movement but this effect could be neutralised by the addition of "antitoxic" serum or by heating the bacteria to 100°C for one hour before challenge.

Although he was unable to demonstrate any effect of toxin on virulence in 1951 (Standfast, 1951a) by the intranasal route he later showed (Standfast, 1958a) that toxin enhanced the virulence of strains of medium virulence but had no effect on strains of high and low virulence. Enhancement was only

evident when the toxin was mixed with the challenge and not when given before or afterwards but toxin had no effect on intracerebral virulence.

The possibility that toxin plays any part in intracerebral virulence was virtually ruled out when a highly virulent strain 2-Atox was isolated which produced no demonstrable dermonecrotic toxin (Andersen, 1952b).

Keogh and North (1948) suggested that the haemagglutinogen was connected with virulence by the I.N. route but this was shown not to be so by Standfast (1951a). The relationship of haemagglutinating titre to intracerebral virulence has not been investigated.

A "Quick Killing Factor", the ability of some strains of equal LD_{50} to kill in a shorter time than others could only be demonstrated by the I.N. route (Standfast, 1958a).

Lacey (1960, 1961) used a variety of media to convert virulent strains, X-mode, into avirulent strains, C-mode. This modulation brought about many changes in agglutinogens, haemagglutinogens, toxicity, HSF and antigenicity of the organisms making it difficult to relate any of these to virulence.

There is still no concrete evidence on the virulence factors of B. pertussis organisms and the precise reaction of the mouse brain, in normal and immune mice, to infection with virulent and avirulent strains is still not clear. It is hoped that the present thesis will shed some light on these matters.

3. Materials and Methods

Strains of B. pertussis

Three high virulence and three low virulence strains of B. pertussis have been used throughout this work. In this thesis high virulence strains are defined as those strains which have an intracerebral LD_{50} for mice of 30-60 viable organisms. Injection of approximately 10^3 viable organisms normally results in the death of all mice in 6 to 9 days. Low virulence strains have LD_{50}s in the order of 10^6 viable bacteria. Injection of 10^3 viable organisms gives rise to a transitory infection lasting 3 to 4 days.

The following strains have been studied in some detail and their cultural characteristics, as set out below, can be used as a method of distinguishing them as different strains. Some of these data have been obtained from Dr. L.B. Holt (Unpublished Observations) and the degree of agglutination was determined by the semi-quantitative method described by Preston (1963).

High Virulence Strains

a. 18/323 - this has been derived from the original Washington strain (Kendrick et al., 1947).

Agglutination T1 +++ T2 ++ T3 ++

% Organisms viable on Bordet-Gengou Medium 10%. This is the viable count on B.G. medium as a percentage of the total count calculated from the turbidity of the suspension using Brown's Tube tables.

b. G353

Agglutination T1 +++ T2 ++ T3 -

% Organisms viable on Bordet-Gengou Medium 40%

Produces a dermonecrotic toxin.

c. 2-Atox

Agglutination T1 +++ T2 +++ T3 +++

% Organisms viable on Bordet-Gengou Medium 40%

Does not produce a dermonecrotic toxin.

Low Virulence Strains

These strains were isolated during a whooping-cough epidemic in Belgrade.

a. B2288

Agglutination T1 ++++ T2 +++ T3 +++

% Organisms viable on Bordet-Gengou Medium 35%

% Organisms viable on Blood Agar Medium 0%.

b. B124

Agglutination T1 +++ T2 +++ T3 -

% Organisms viable on Bordet-Gengou Medium 22%

% Organisms viable on Blood Agar Medium 22%.

c. B1772

Agglutination T1 +++ T2 +++ T3 +++

% Organisms viable on Bordet-Gengou Medium 25%

% Organisms viable on Blood Agar Medium 0%.

These factors distinguish the strains used as 6 individual strains.

Media

Bordet-Gengou Medium (BG-D)

A.	Peeled, sliced potatoes	2,500 g.
	'Analar' Glycerin	200 mls.
	Distilled Water	ad 10 litres

The potatoes were dispensed in gauze bags and cooked in the glycerin-water until soft. The bags were removed from the fluid, allowed to drain and the volume made up to 10 litres.

B.	Sodium Chloride (Analar)	170 g.
	Distilled Water	ad 10 litres

The two solutions were mixed and dispensed as required.

Bacto Agar 2.0 g% was added and media autoclaved for 30 minutes at 10 lbs. p.s.i.

Two layer plates were used. The bottom layer consisted of 20 ml. of base and the top layer 12 mls. of a horse blood-base mixture. Defibrinated horse blood (Wellcome Research Labs.) was used at the rate of 50 mls. to 200 mls. base, giving a mixture containing 20% blood. In the experiments on modulation the horse blood was replaced by sheep blood (defibrinated, no preservative) and single layer plates containing 20% blood were used.

Cohen and Wheeler Medium

The following formulations were used:

	Cohen and Wheeler	Cohen and Wheeler containing high magnesium
Metals Solution	1 ml.	1 ml.
Starch (B.D.H. soluble)	1.5 g.	1.5 g.
$MgSO_4 . 7.H_2O$.	0.9 g.	11.9 g.
KH_2PO_4	0.5 g.	0.5 g.
NaCl	4.6 g.	Nil
Cysteine hydrochloride	0.1 g.	0.1 g.
Yeast Extract (Difco)	4.0 g.	4.0 g.
Casamino Acids (Tech)	10.0 g.	10.0 g.
Distilled water	ad 1 litre	ad 1 litre

The pH was adjusted to 7.6 using N.NaOH.

Metals Solution

$CaCl_2$	1 g. %
$FeCl_3 . 6.H_2O$	1.21 g. %
$CuCl_2$	0.04 g. %

Dissolved in 1% HCl solution and autoclaved at 10 p.s.i. for 30 minutes.

Casamino Acids Solution

Casamino Acids (Tech)	40 g.
Sodium Chloride (AR)	16 g.
Distilled Water	ad 4 litres

Adjust pH to 7.4. Autoclave for 20 minutes at 10 lbs. p.s.i.

Bacterial Cultures

All strains were grown from the freeze dried state on Bordet-Gengou (B.G.) medium at 34°C for 72 hours. The second 18 hour subculture from this was used for the mouse challenge.

Suspensions of Organisms

All suspensions and dilutions were made in 1% casamino acid solution.

Challenge Injections

Mice, lightly anaesthetised with ether, were injected intracerebrally at a point likely to bisect the ventricles.

0.025 ml. was injected from a 0.25 ml. syringe through a 0.25 inch 26 SWG needle.

Intracerebral Mixtures of Organisms and Serum

Mixtures of organisms and serum were incubated at 37°C for 60 minutes in a water-bath. Viable counts were estimated after incubation as a check against in vitro killing by complement.

Bacterial Counts from Mouse Brains

Each mouse brain was removed asceptically and added to 2 ml. of 1% casamino acids contained in a 1 oz. universal bottle. The brain was homogenised at half speed in a M.S.E. homogeniser for 1 minute. The homogenate was then centrifuged at 1,500 r.p.m. for 90 seconds in an International PRI centrifuge. Decimal dilutions of the supernatants were made in 1% casamino acids and the bacterial counts estimated by the method of Miles and Misra (1938) on B.G. medium. Three drops of each of three suitable dilutions were spread on each plate and bacterial counts were where possible calculated from drops containing 40 to 80 organisms.

Mice

Two strains of white mice were used throughout these experiments depending upon supply. The strains used were

I.C.I. and Theilers Original (T.O.) mice (Scientific Animal Supplies, Elstree, Herts).

Female mice were used weighing 14-18 g. when challenged.

Two strains of black mice were also used on occasions. These were B10.D2 "Old Line" and B10.D2 "New Line" mice supplied by Dr. A.A. Glynn, Wright-Fleming Institute. These strains of mice are genetically identical except that the "Old Line" are deficient in a component of complement probably corresponding to the human C'_5 component.

Leucocyte Counts

0.05 ml. of blood taken from the brachial artery was diluted in 0.95 ml. of 2% Acetic Acid solution coloured light blue with gentian violet. The diluting fluid lyses the red cells and a total white cell count is made on 0.1 cu. mm. of blood in a Neubauer Cell. The number of cells counted x 200 gives the number of white cells per cu. mm. of mouse blood (Dacie and Lewis, 1963).

Slide Agglutination

Slide agglutinations were determined by the method of Preston (1963). A suspension of Bordetella pertussis containing approximately 5×10^{10} organisms per ml. was mixed with a standard loopful of each of the monospecific sera on a glass slide and rocked gently for 5 minutes.

Degrees of agglutination were recorded as follows:-

++++ Immediate formation of large agglutinates

+++ Formation of large agglutinates in 3 minutes

++ Agglutinates visible within 3 minutes, complete within 5 minutes

+ Slow formation incomplete in 5 minutes

0 No agglutination visible in 5 minutes.

Lysosomal Enzyme Measurements

Free Enzymes

Enzymic measurements were performed on the cytoplasmic extracts. These were prepared by adding 1 mouse brain to 4.5 ml. ice cold 0.32 M Sucrose and disrupting it with a Potter-Elvehjem type homogeniser with a teflon pestle which was rotated slowly for 5 seconds. The homogenates were centrifuged at 800 g. for 10 minutes in a PRI International Refrigerated Centrifuge to remove nuclei, cellular debris and intact cells. The resulting supernatant was designated the cytoplasmic extract.

Total Enzymes

For total enzyme determinations the brains were macerated for 8 minutes at high speed in a M.S.E. blender. Control experiments had found this time to be most suitable as a loss in total enzyme had been found when the brains were macerated for a longer time.

Substrates

Acid Phosphatase - 3 mM Disodium Dinitro Phenyl Phosphate. N-Acetyl-β-D-Glucosaminidase-4mM p-nitrophenyl-acetamido-2-deoxy-D-glucopyranoside. β-Galactosidase - 2.5 mM o-nitrophenyl-β-galactoside. β-Glucuronidase - 1 mM Phenolphthalein glucuronide. All these substrate solutions were prepared in 0.05M Sodium Acetate buffer at pH 5.0.

Buffer Solutions

(a) Sodium acetate 0.05M, pH 5.

(b) 0.4M glycine-sodium hydroxide, pH 10.5, for use in acid phosphatase, β-galactosidase and N-acetyl glucosaminidase determinations.

(c) 0.133M glycine, 0.067M sodium chloride, 0.083M sodium carbonate, pH 10.7, for use in β-glucuromidase determinations.

Free Enzyme Determinations

(a) Acid phosphatase, β-galactosidase, N-acetyl glucosaminidase

A mixture of 1 ml. of the specific substrate, 0.2 ml. cytoplasmic extract and 0.3 ml. acetate buffer was incubated at 37°C for 15 minutes. The reaction was stopped by addition of 1.5 ml. of 2.75% trichloracetic acid and the mixture centrifuged at 2000 g. for 10 minutes. 1 ml. of the supernatant was added to 2 ml. of glycine/NaOH buffer and the optical density

was determined at 420 mμ on a Unicam SP600 Spectrophotometer.

(b) β-Glucuromidase

A mixture of 1 ml. of the specific substrate, 0.2 ml. of the cytoplasmic extract and 0.3 ml. acetate buffer was incubated at 37°C for 15 minutes. The reaction was stopped by adding 6 ml. the glycine/NaCl/Na_2CO_3 buffer and the mixture centrifuged at 2000 g. for 10 minutes. The optical density of the supernatant was determined at 545 mμ on a Unicam SP600 Spectrophotometer.

Total Enzyme Determinations

Total enzyme determinations were carried out on homogenates after treatment in the MSE homogeniser at high speed for 8 minutes and the 0.3 ml. of acetate was replaced with 0.3 ml. of 0.15% Triton-X, a non-ionic detergent (Lennig Chemicals Ltd.) This detergent has been shown to cause complete loss of latency for all the enzymes.

Standards

Standard curves for p-nitrophenol and o-nitrophenol were obtained for calculating the amount of enzyme release for acid phosphatase, N-acetyl-β-D-glucosaminidase and β-galactosidase.

Difficulty was experienced in obtaining a standard curve for phenolphthalein owing to its very low solubility in acetate buffer and the results have been expressed in optical density (O.D.) units.

Irradiation Technique

Mice, anaesthetised with sodium pentabartitone (Nembutal, Abbots) 60 ug/g injected intraperitoneally, were irradiated in groups of 6 arranged radially in the irradiation apparatus. Doses were given at 90-95 R/min. at 220 KV 15 m. amp with 0.5 mm. copper and 1.0 mm. of aluminium as added filtration. Shielding where applicable was with 2.5 mm. lead.

Exposures were continuously monitored with a Baldwin Farmer Substandard Dosemeter, the probe of which was placed in the X-ray field on the same radius as the mice. The probe was covered with a Mix D plastic phantom, this being designed to give a similar scatter and absorption characteristics as tissue.

Histology

Mice were injected subcutaneously with 5 mgm./kg. Colcemid, an antimitotic agent, 4 hours before they were killed. Bryans (1959) showed that this was the optimum time interval for demonstration of mitotic activity.

The parietal bones were removed and the brains fixed in

1% acetic acid in 10% formol saline. After fixation for 24 - 48 hours the brain was dissected out and the cerebral hemispheres divided transversely into 3 equal parts.

These were slowly dehydrated for 24 hours each in 70%, 80, 95% and absolute alcohol. They were cleared overnight in chloroform and embedded in paraffin wax.

Staining

Haematoxylin and Eosin Stain

Sections were treated with xylol to remove wax and then with absolute alcohol to remove the xylol. Slides were dipped in Ehrlich's haematoxylin for 20 minutes, washed in water, differentiated in 1% acid/alcohol and washed until blue. The sections were then stained with 1% eosin for 1-2 minutes and then washed for several minutes in water. Absolute alcohol was applied for 2 minutes which was followed by xylol until transparent.

Mayers Haemalum

Sections are dewaxed with xylol and the xylol removed with absolute alcohol. They are stained for 5 minutes in Mayers Haemalum. The nuclei are stained dark blue and the cytoplasm light blue.

Autoradiograph Technique

1 µC/g. of triated thymidine (H^3thymidine) labelled in

the 6 position (Radiochemical Centre, Amersham) was injected intraperitoneally into female T.O. mice weighing 14-18 g. The mice were killed either 1 or 6 hours later by decapitation. The brains were fixed and dehydrated as described previously, but autoradiograph sections were floated out on distilled water after cutting and picked up on subbed slides.

Sections were dried for 24 hours, dewaxed in xylol, hydrated through alcohol and washed in slowly running water for 30 minutes. The slides were then transferred to distilled water.

Ilford K2 Nuclear Research Emulsion was mixed with an equal volume of distilled water under a safe-red light and maintained at 60°C.

The slides were removed from the distilled water and allowed to dry until just damp. Slides were dipped into emulsion for 2-3 seconds, drained and dried. When completely dry, they were sealed in a light proof box containing "drierite" and stored at 4°C for 3-6 weeks.

The slides were then rinsed in distilled water and developed in Kodak D 19B developer for 5 minutes and washed in 3 changes of distilled water. The slides were fixed until clear using a 1 in 10 solution of Johnson's Fix-sol for 10 minutes, washed in tap water for 30 minutes, stained with Mayer's Haemalum for 5 minutes, dehydrated as before and mounted.

Statistical Analysis

Where this was applicable the X^2 test has been used.

	+	-	
A	a	b	a+b
B	c	d	c+d
	a+c	b+d	a+b+c+d = N

$$X^2 = \frac{(ad - bc)^2 \times N}{(a+b)(c+d)(a+c)(b+d)}$$

In all cases Yates correction for continuity of small numbers has been used. This consists of reducing by 0.5 the values which are greater than expectation and increasing by 0.5 those which are less, i.e. reduce the difference.

EXPERIMENTAL

4. Intracerebral Infections with Bordetella Pertussis in Normal Mice

4.1. Introduction

In order to obtain a comprehensive understanding of Bordetella pertussis infections in the mouse brain it was decided that a number of different strains must be studied. Three low virulence strains and three high virulence strains have been studied in some detail.

The high virulence strains, 18/323, G353 and 2-Atoxic have an LD_{50} for mice of 40-80 viable organisms. The normal challenge dose, 5000 viable organisms, brought about the death of most mice in 6-7 days.

The low virulence strains studied, B2288, B1772 and B124, have LD_{50}s in the region of 10^6 viable organisms. The normal challenge dose gives rise to only a transitory infection and most mice have a sterile brain 5 days after challenge. With larger challenge doses of 10^5 - 10^6 viable organisms the brain of most mice does not become sterile until at least the eighth day after challenge.

In this chapter the course of infection of these high virulence and low virulence strains in normal mice will be described in some detail. The same 2 strains of mouse, I.C.I. and Theilers Original (T.O.), have been used throughout these experiments and no differences in the reaction of these 2 mouse strains to infection have been found.

4.2. Infections with Low Virulence Strains

Groups of 30 I.C.I. mice were challenged intracerebrally with the mouse low virulence strains B2288, B1772 or B124. Six mice were killed at 1 hour, 1, 2, 3 and 4 days after challenge; the brains were removed asceptically and processed as described previously. The challenge doses were 6.4×10^3 viable organisms of strains B2288 and B1772 and 8.7×10^3 viable organisms of strain B124.

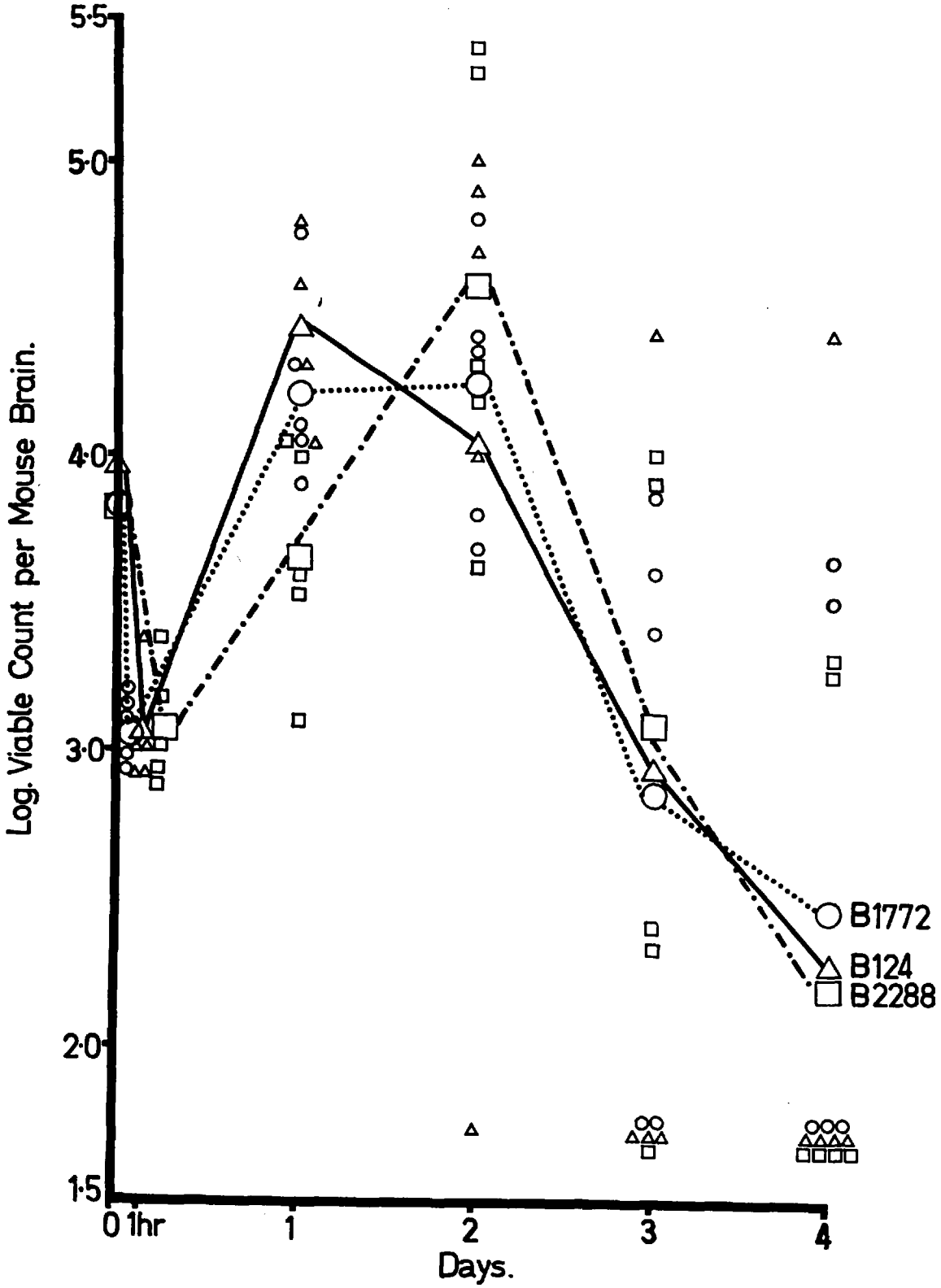
This experiment was extended to study the course of infection following graded challenge doses of a low virulence strain. Three groups of 42 mice were challenged intracerebrally with 5.6×10^3, 5.6×10^5 or 5.6×10^7 viable organisms of the low virulence strain B2288. Seven mice were killed at 1 hour and at days 1 - 5 and the intracerebral viable counts determined as before.

4.2.1. Results

The normal intracerebral infections of 3 low virulence strains were studied in mice challenged with an inoculum of approximately 6000-9000 viable organisms. It must be emphasised that all 3 infections in Fig. 1 are typical of any one of the low virulence strains tested at this dose level.

In no case was the count at 1 hour after challenge more than about 10% of the number of organisms inoculated. After this time 3 basic patterns of infection emerged, which were

Figure 1. Intracerebral infection with the low virulence strains of B. pertussis, B2288, B1772 and B124. The challenge inocula were 6.4×10^3 viable organisms of strains B2288 and B1772 and 8.7×10^3 viable organisms of strain B124.


5·5
5·0
4·0
3·0
2·0
1·5
Log. Viable Count per Mouse Brain.
0 1hr
1
2
3
4
Days.
B1772
B124
B2288

thought to be due to variation in the mice. Sometimes the infection was typified by the B124 infection in Fig. 1. The peak viable count was reached by 24 hours, after which the mean viable count declined and by the fourth day 4 out of 5 brains contained less than 50 organisms. With B1772 the viable count reached its maximum between 1 and 2 days and then declined until 3 out of 5 brains contained less than 50 organisms by the fourth day.

Finally the B2288 infection did not reach the maximum count until the second day, again falling rapidly from this time to give 4 out of 6 brains containing less than 50 organisms by the fourth day.

The results of 24 experiments with low virulence strains are summarised in Table 1. Each figure indicates the average increase in the geometric mean (G.M.) viable count in the number of experiments indicated. In each experiment the viable counts from at least 5 brains were determined at all time intervals.

On average the viable count increased by between $\log_{10}$ 0.8 and 0.9 between 1 and 24 hours and remained at about the same level between days 1 and 2. After this time the viable count fell rapidly, by at least $\log_{10}$ 0.9 between the second and third day and at least $\log_{10}$ 0.6 between the third and fourth days. Brains containing less than 50 organisms were found in a few of the mice killed on the second day but the

TABLE 1. Comparison of the average increase or decrease in geometric mean number of bacteria at daily intervals with various low and high virulence strains of B. pertussis.

Strain	Number of Experiments	Average $\log_{10}$ increase or decrease in the geometric mean number of organisms between			
		1 hour and 1 day	1 day and 2 days	2 days and 3 days	3 days and 4 days
B 2288	10	+0.90	+0.04	-0.98	-0.90
B 1772	8	+0.90	-0.06	-0.91	-0.80
B 124	6	+0.83	-0.35	-0.90	-0.60
18/323	7	+1.06	+1.30	+1.11	+0.35
G 353	4	+1.07	+1.33	+1.05	-0.07
2-Atox	2	+1.00	+1.60	+1.20	+1.00

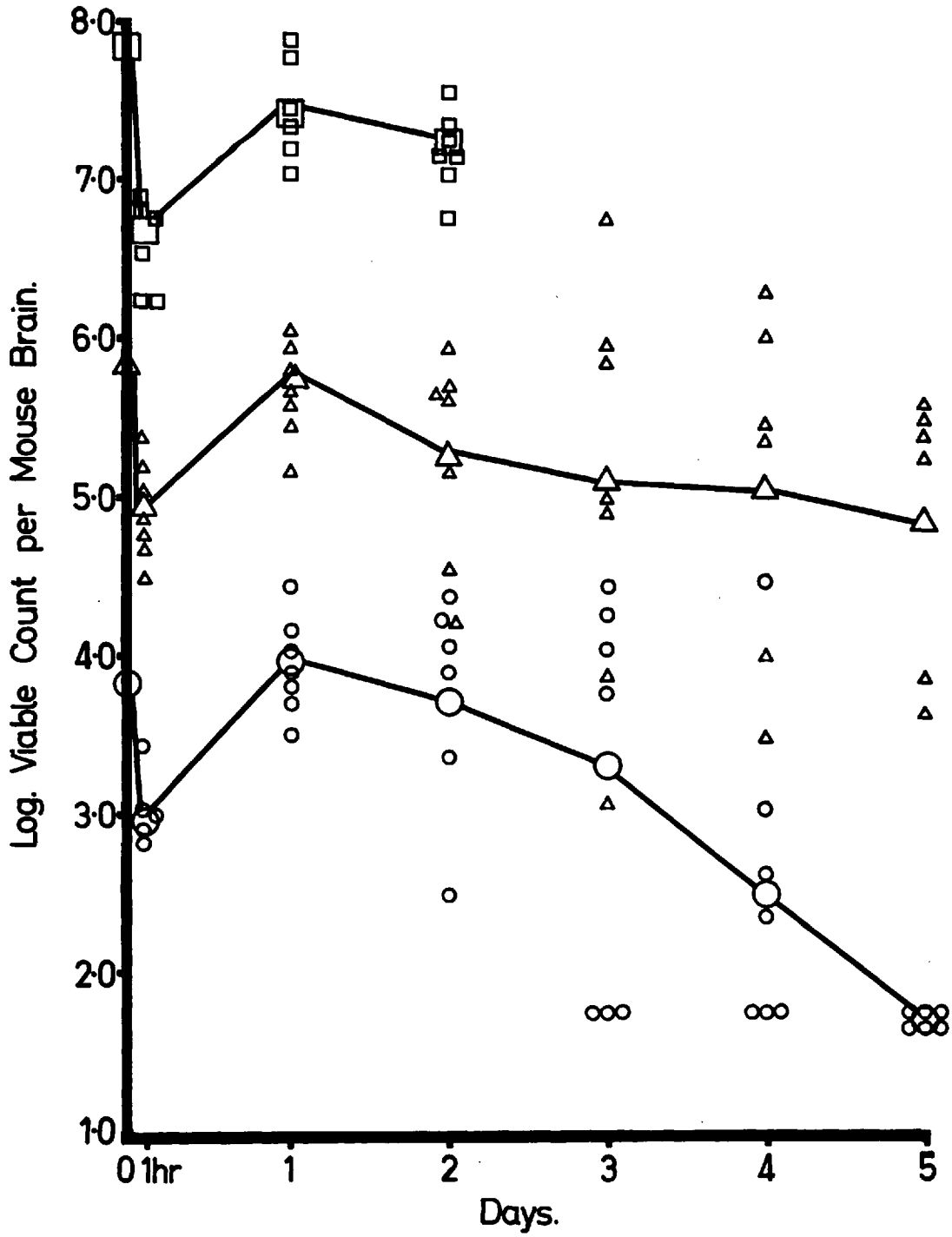
majority of brains did not contain less than 50 organisms until at least the third or fourth days.

When graded challenges of B. pertussis B2288 were injected intracerebrally, 3 distinct courses of infection became evident (Fig. 2).

With the low challenge dose, 5.6×10^3 viable organisms, the course of infection was typical of those described above. The maximum viable count was reached after 24 hours; this was followed by a decline until the brains of all mice contained less than 50 organisms at the fifth day. When the inoculum size was increased 100-fold, to 5.6×10^5 viable organisms, the period of infection was significantly extended and brains containing less than 50 organisms were not detected until the eighth day. At the fifth day, when with lower dose all brains contained less than 50 organisms, the geometric mean viable count was 10^5 organisms which was approximately the same as the 1 hour count.

Even with a challenge dose of 5.6×10^7 viable B. pertussis the course of infection for 48 hours was very similar to those obtained with the lower doses. The count at 1 hour after challenge was approximately 10% of the number injected and the maximum viable count was reached by 24 hours. However, this high level of infection resulted in the death of all mice by the third day. The decline in count at the terminal part of infection with the high challenge dose could be due to a lack of

Figure 2. Intracerebral infection with graded challenge doses of the low virulence strain of *B. pertussis* B2288. The challenge inocula were 5.6×10^3, 5.6×10^5 and 5.6×10^7 viable bacteria.


8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
5
Days.

nutrients in the brain at this terminal stage of infection or, possibly, to an intense cellular reaction. It is interesting, as will be shown later, that this decline in viable count is not as evident with high virulence infections in mice pretreated with cortisone.

It was of interest to examine the range of viable bacterial counts in the brains of the individual mice. The differences between the highest and lowest counts were very similar for all 3 doses at the first day after challenge; they were $\log_{10}$ 0.96, 0.92 and 0.82 for the low, middle and high doses respectively. On the second day the difference was again 0.82 for the high dose but had increased to 1.67 and 1.87 in mice given the middle and low doses and by the fourth day this difference had increased to 2.83 and 2.72 respectively.

These figures indicate a large variation in the response of the mice to these low virulence infections. This was even better emphasised when the bacterial counts at the fourth day in mice challenged with 5.6×10^3 organisms were examined. Three brains contained less than 50 organisms while the counts of the other four were distributed between this figure and 2.7×10^4.

4.3. Infections with High Virulence Strains

Intracerebral infection with the high virulence strain of *B. pertussis*, 18/323, has been well documented (Blyth, 1955;

Berenbaum et al., 1960; Dolby and Standfast, 1961). Andersen (1957a) used the high virulence strain 2-Atox as her challenge strain in experiments on early immunity.

I can find no reference to intracerebral infection with B. pertussis G353 and no evidence that the infections with these 3 strains have been compared. All 3 strains have approximately the same LD_{50} for mice, namely between 40 and 80 viable organisms (Andersen, 1952b; Eldering et al., 1966).

4.3.1. Experimental

A group of at least 35 I.C.I. mice was challenged intracerebrally with 5.7×10^3 viable organisms of the high virulence strain 18/323. Six mice were killed at 1 hour and 6 daily for 6 days after challenge. The brains were removed aseptically, processed as described previously and the G.M. of the viable counts from each group of brains calculated. The experiment was repeated as above in groups of mice challenged with 3.6×10^3 viable B. pertussis G353 and 5.2×10^3 viable B. pertussis 2-Atox.

4.3.2. Results

Typical infections with 3 high virulence strains are shown in Figs. 3, 4 and 5. In all 3 infections approximately 90% of the inoculum disappeared within the first hour after challenge. By 24 hours the number of bacteria in the brains

had reached or even exceeded the number inoculated. During the next 2 days the count increased by at least 10-fold daily. Up to this time, the increase in count had been very similar for all 3 strains (Table 1) and by the third day the geometric mean viable counts, following injection of about 5×10^3 organisms, were slightly in excess of 10^6.

Between the third and fourth days the G.M. viable count in the 18/323 infections increased on average by $\log_{10}$ 0.55 and the 2-Atox infections by $\log_{10}$ 1.0. From the fourth day onwards the rate of increase in count for these strains was between 3 and 8 times daily and in some cases there may even be a slight fall in viable count in the terminal stages of infection.

This was very different from the infection with G353 which in 4 experiments showed a decrease or levelling off in the geometric mean viable count between the third and fourth day. However, this decline was only transitory and the increase in count between days 4 and 5 often compensated for this lag (Fig. 6).

Mice challenged with about 5×10^3 G353 took on average 1 day longer to die than mice challenged with an equal number of 18/323.

The counts in the individual brains were spread over a very small range, rarely greater than 1 on a $\log_{10}$ scale, throughout infection. The only exception to this rule being

Figure 3. Intracerebral infection with the high virulence strain of B. pertussis 18/323.

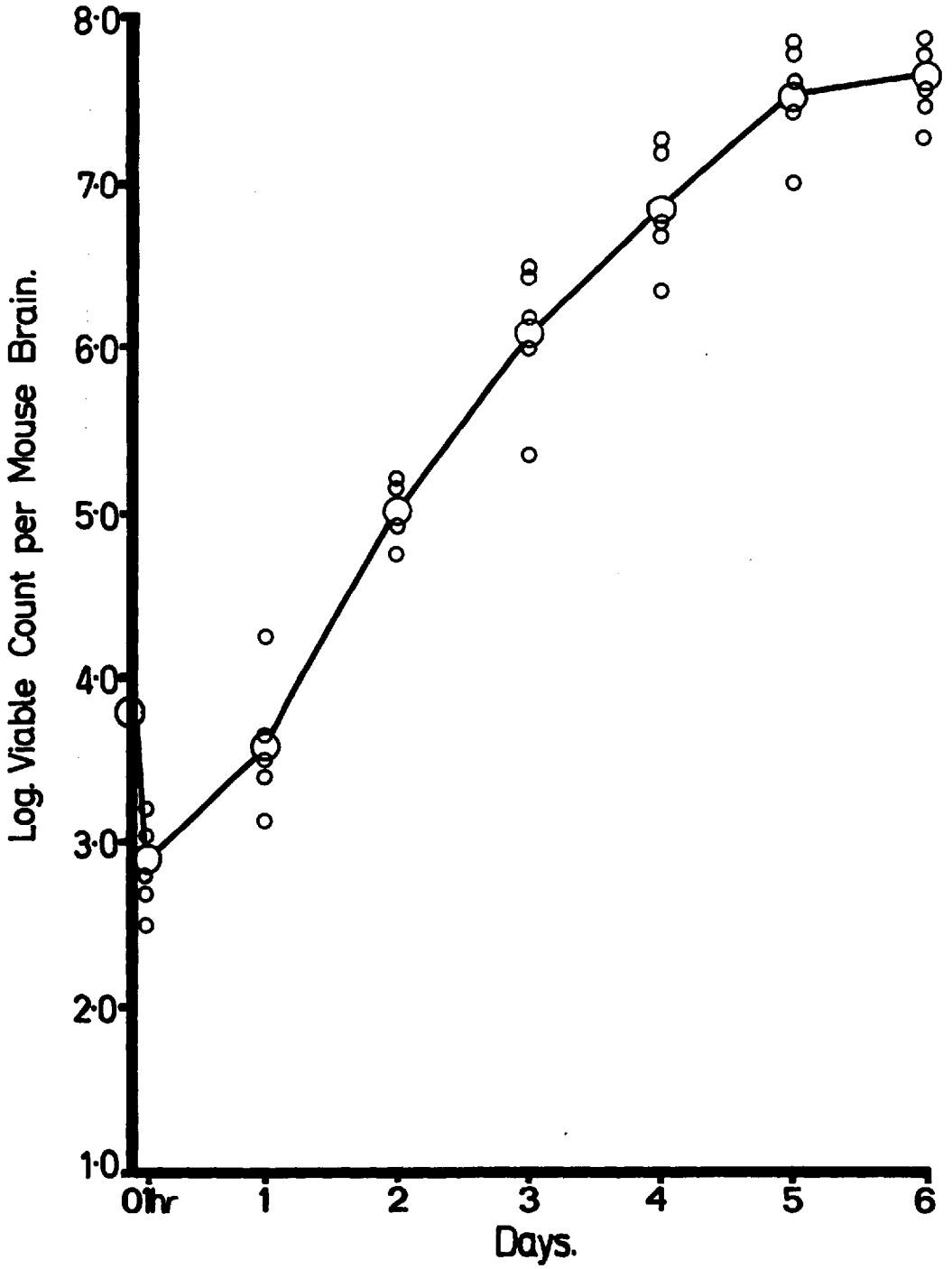

8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0hr
1
2
3
4
5
6
Days.

Figure 4. Intracerebral infection with the high virulence strain of B. pertussis G353.

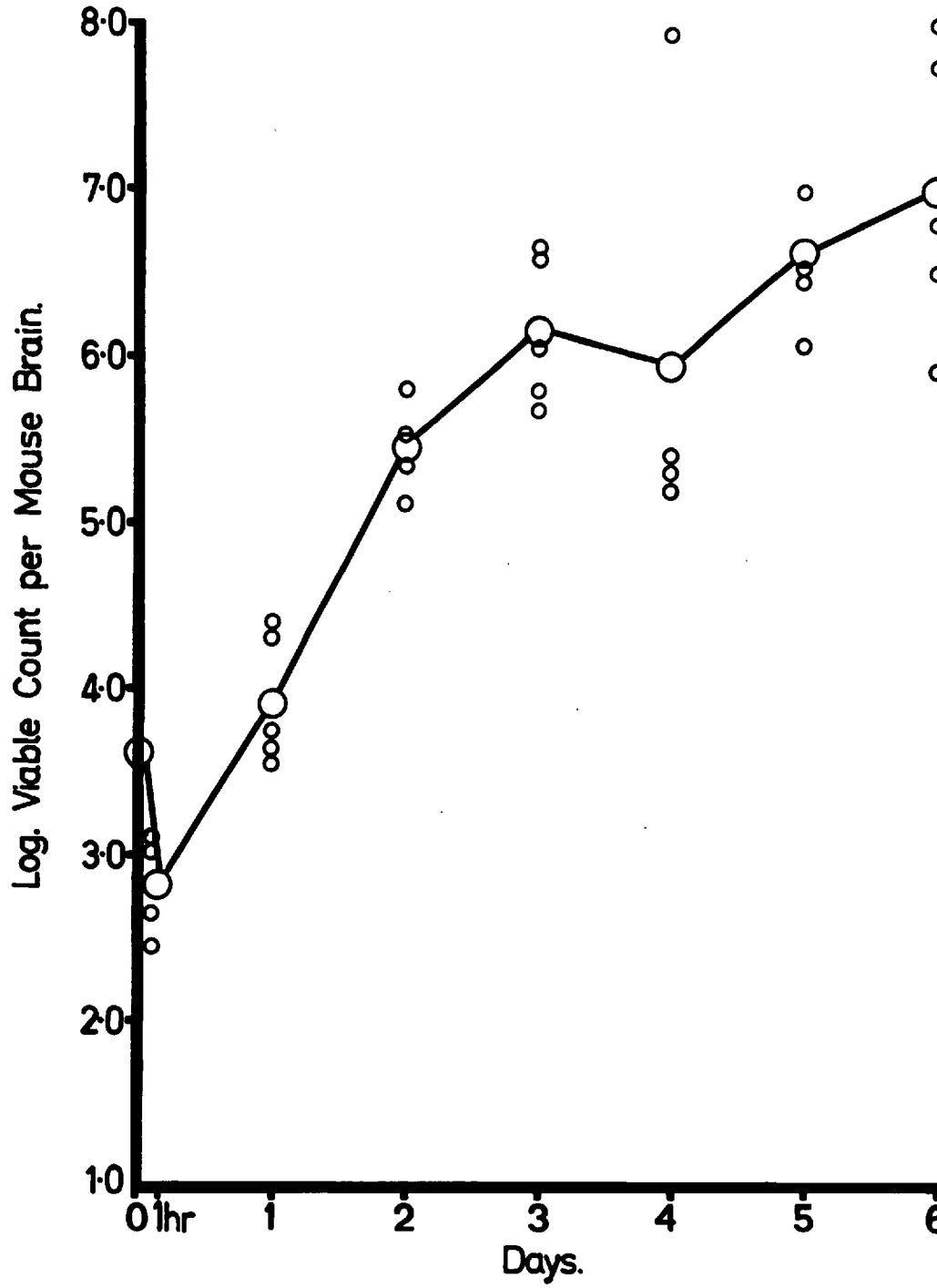

8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
5
6
Days.

Figure 5. Intracerebral infection with the high virulence strain of B. pertussis 2-Atox.

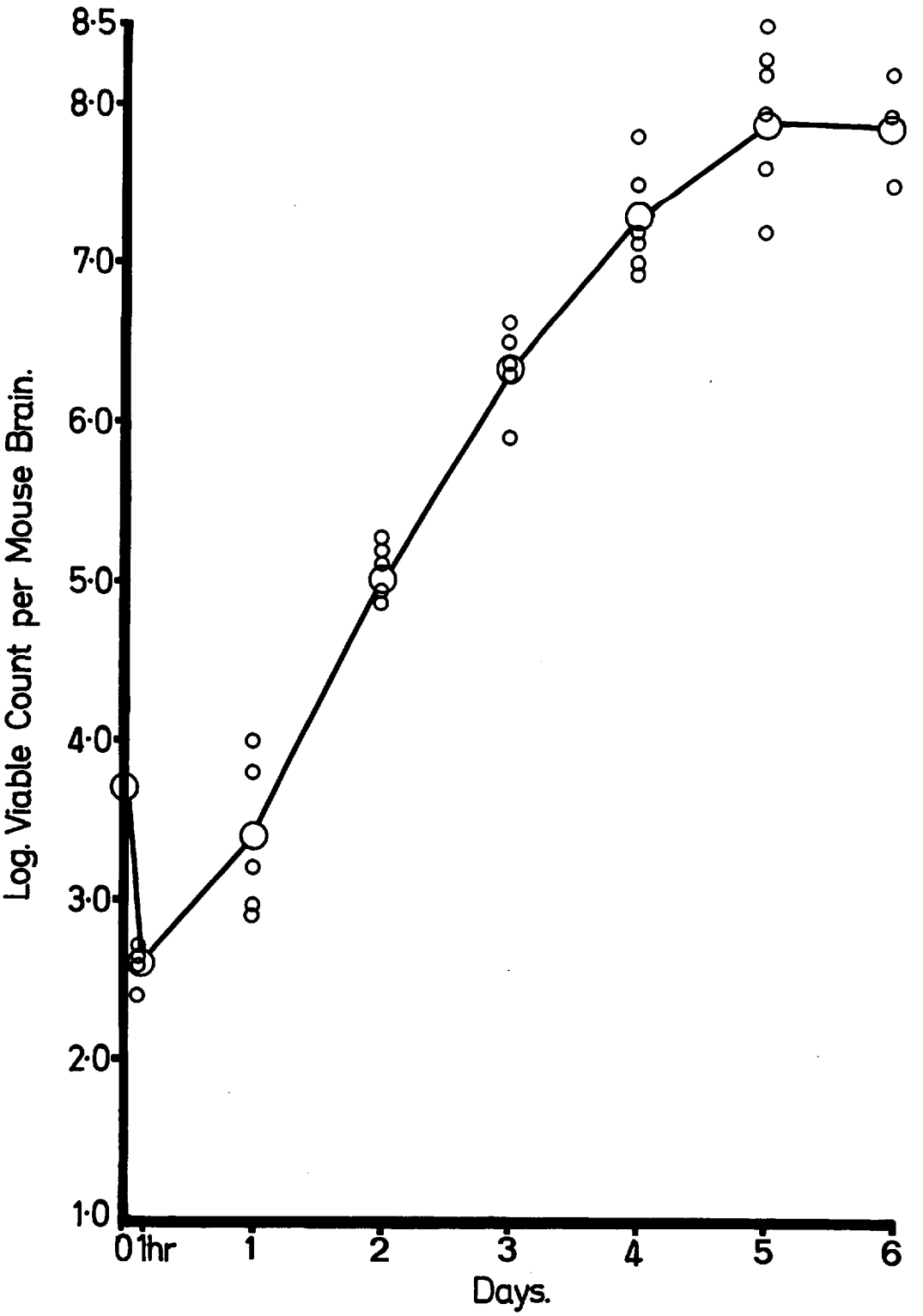
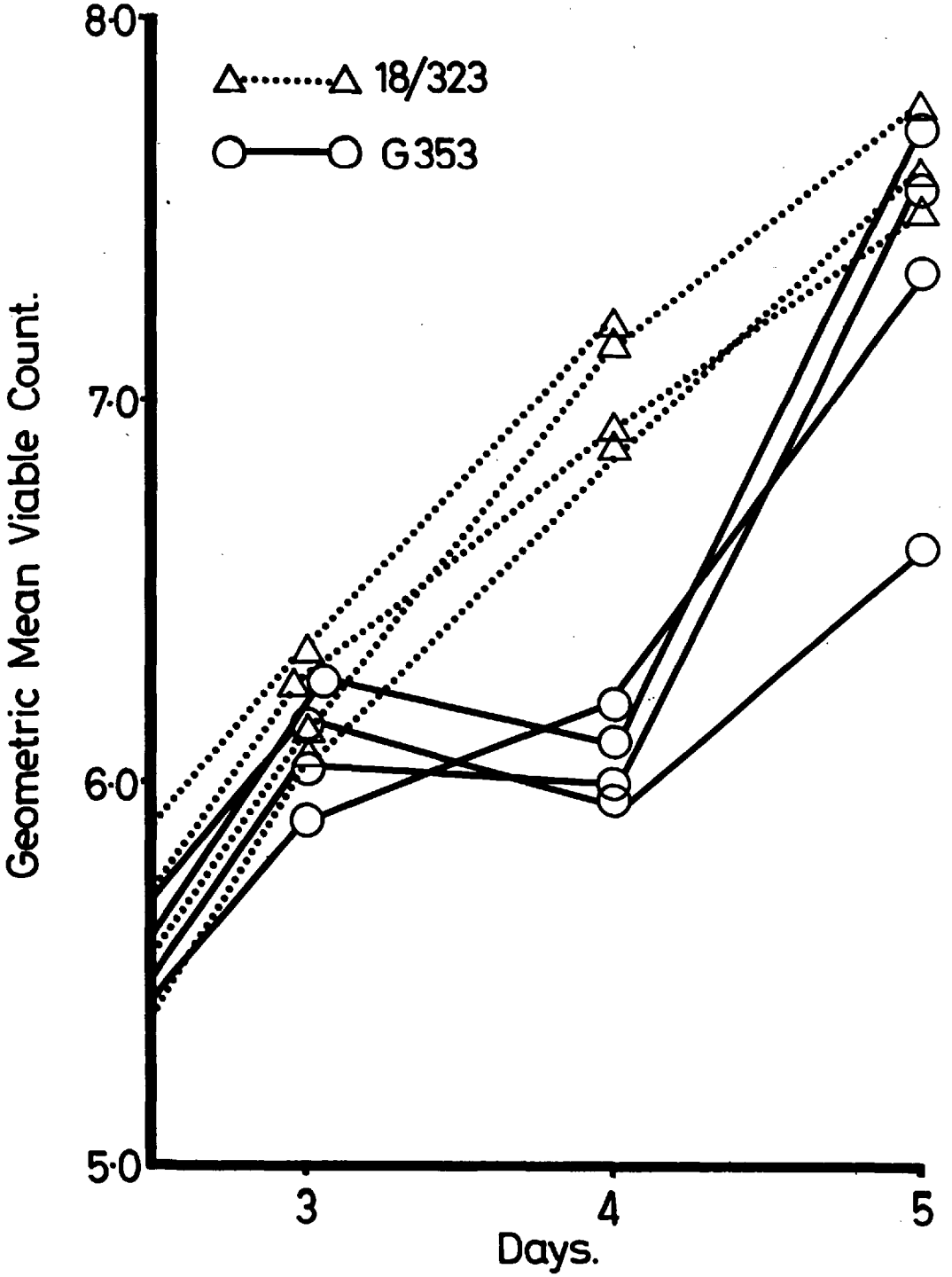

8·5
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
5
6
Days.

Figure 6. Intracerebral infection on the 3rd, 4th and 5th days with the high virulence strains of B. pertussis 18/323 and G353. Each point is the geometric mean viable count from at least 5 brains.


8·0
7·0
6·0
5·0
18/323
G353
Geometric Mean Viable Count.
3
4
5
Days.

in the terminal stages of G353 infections, where the range of counts was often greater than 2 on a $\log_{10}$ scale.

The lag and spread of counts may indicate some change in response to this infection when the count is greater than 10^6 viable organisms. This is the count indicated by Holt et al. (1961) as being necessary for damage to the blood-brain barrier. Thus some factors external to the brain may be coming into play at this stage of infection.

4.4. Infection with a Low and High Virulence Strain during the 24 hours after Challenge.

It has been reported in the previous sections that in numerous experiments the increase in viable count during the 24 hours after challenge was never as great with low virulence strains as it was in infections with high virulence strains. This may have been due to a number of factors: the loss of organisms after challenge may have been greater with the low virulence strains than with the high virulence strains or the time at which multiplication commenced may have been delayed with the low virulence organisms. Other factors involved may be the multiplication time and animal response. There was no evidence in vitro that the low virulence strains of B. pertussis multiplied at a slower rate than high virulence strains.

Examination of counts at short time intervals after

challenge with low or high virulence strains may give some indication as to the nature of this difference.

4.4.1. Experimental

Forty-five T.O. mice were challenged intracerebrally in each experiment with B. pertussis 18/323 or B. pertussis B1772. Five mice were killed at 3 minutes, 30 minutes, 1, 2, 4, 6, 8, 10 and 24 hours after challenge. The brains were removed aseptically and processed as described.

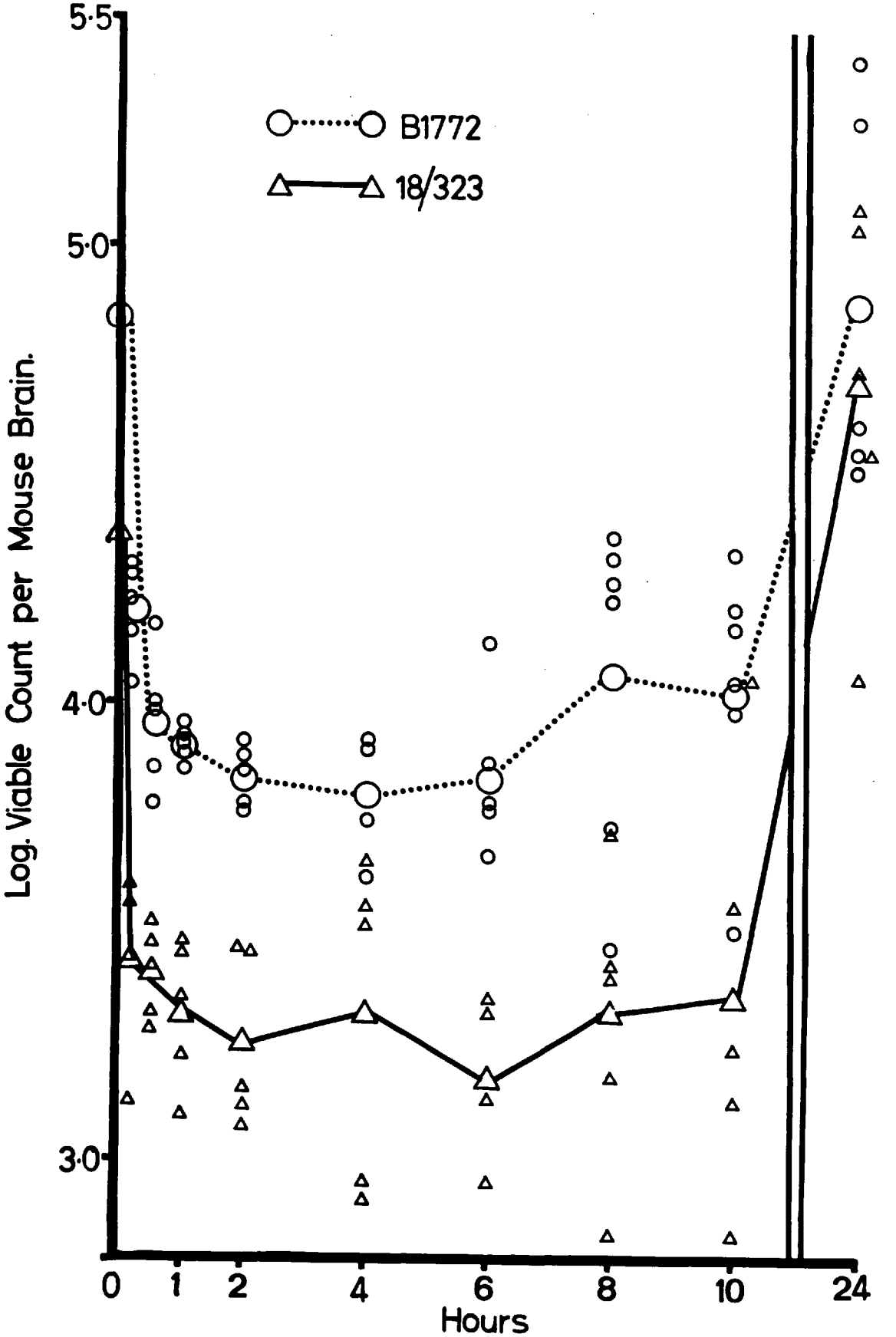
One group of mice was challenged with 2.38×10^4 viable bacteria of strain 18/323 and the second group with 7×10^4 viable bacteria of strain B1772.

4.4.2. Results

The results of this experiment are shown in Fig. 7. It can be seen that the courses of the two infections were almost identical. The counts reached a minimum between 2 and 6 hours and increased after this time to reach a level at 24 hours approximately equal to the inoculum. Again evidence was provided in this experiment, which confirmed the results shown in Table 1, that multiplication of low virulence organisms was not quite as rapid as that of high virulence organisms in the first 24 hours. There was no evidence that the size of the inoculum affected the rate of increase in viable count during the 24 hours after challenge.

Comment has previously been made on the approximate 90%

Figure 7. Intracerebral viable counts at short time intervals following challenge with either the high virulence strain 18/323 or the low virulence strain B1772.


5·5
5·0
4·0
3·0
B1772
18/323
Log. Viable Count per Mouse Brain.
0
1
2
4
6
8
10
24
Hours

loss of organisms within the first hour after challenge; it was evident from this experiment that this loss was instantaneous. It was found, as early as 3 minutes after challenge, that there was a 88.5% loss of the inoculum of strain 18/323 and a 77% loss of the inoculum of strain B1772. This very short time seems far too early for this degree of killing by phagocytes. By 1 hour the percentage of the inoculum remaining was 8.5% of the 18/323 challenge dose and 11.5% of the B1772 challenge dose and fell to as low as 6.8% and 8.7% respectively.

4.5. The percentage of Organisms remaining in the Mouse Brain one Hour after Challenge.

The percentage of organisms remaining in the brain 1 hour after challenge was calculated from many experiments as a function of the strain, number of bacteria and dose volume to evaluate the role of any of these factors in virulence.

4.5.1. As a Function of the Challenge Strain

The results of 63 experiments where the challenge strains were injected into normal mice have been tabulated (Table 2). In all cases the figures for the proportion of organisms remaining after 1 hour were spread over a very small range, from 10.5% to 12.9%, with a grand mean of 11.7%. No significant differences could be found in the percentages of

TABLE 2. Percentage of the inoculum of various strains of *B. pertussis* remaining in the mouse brain 1 hour after challenge.

Strain	Number of Experiments	% of the inoculum detectable at 1 hour
18/323	19	11.7
G 353	3	11.0
2-Atox	2	10.5
B 2288	17	12.0
B 1772	15	12.9
B 124	7	11.7
TOTAL	63	11.7

organisms remaining at 1 hour and the number remaining seemed to be independent of the challenge strain.

4.5.2. As a Function of the Number of Bacteria

The above 63 results have been re-arranged as a function of the challenge size (Table 3). Although the number of results was unevenly distributed over the ranges of doses there was no evidence that the proportion of the challenge remaining was dependent on dose.

4.5.3. As a Function of Inoculum Volume

In these experiments groups of 10 mice were challenged intracerebrally with the high virulence strain 18/323. In the first experiment the mice were challenged with 9.2×10^3 viable organisms and in the second experiment with 5.9×10^3 viable organisms.

The dose volumes, 0.025 ml. and 0.0025 ml. were injected with a micrometer syringe.

The results (Table 4) show that when 0.0025 ml. of the challenge suspension was injected a greater proportion of the inoculum was detectable in the brain 1 hour later than when 0.025 ml. was injected. The ratios of the amounts remaining after injection with 0.025 ml. and 0.0025 ml. were found to be almost identical in both experiments: 1:1.49 and 1:1.45.

Statistical analysis shows that neither result was

TABLE 3. Percent number of bacteria remaining in the mouse brain 1 hour after challenge with a range of inocula sizes.

Log_{10} number of bacteria injected	Number of Experiments	% of bacteria remaining 1 hour after challenge
2.5 to 3.5	11	10.0
3.5 to 4.5	44	12.6
4.5 to 5.5	6	12.0
Greater than 5.5	2	10.3

TABLE 4. Percent number of bacteria remaining in the mouse brain 1 hour after challenge with various inoculum volumes.

Experiment Number	% of bacteria remaining at 1 hour after injection of		Ratio of the % of organisms remaining
	0.025 ml.	0.0025 ml.	
1	12.7	19.0	1 : 1.49
2	11.5	16.1	1 : 1.45

Photograph 1. The distribution of Pontamine Sky Blue in the brain and on the reflected skin 1 hour after intracerebral infection.



significant at the P=0.02 level and only the results of experiment 1 were significant at the P=0.05 level.

The combination of these three experiments indicated that the approximate 90% loss of the inoculum was probably mechanical resulting from the pressure of the inoculated fluid.

In a further experiment, 0.025 ml. of a 1% solution of Pontamine Sky Blue was injected intracerebrally, the mice were killed 1 hour later and the brains exposed. A blue area on the reflected skin (Photograph 1) suggests that most of the 90% loss of organisms was due to washback of the inoculum along the needle track.

4.6. Summary of High and Low Virulence B. Pertussis Infections

Two main forms of infection have emerged from these experiments. With all high and low virulence infections 90% of the organisms injected disappeared within a few minutes of challenge and the multiplication phase commenced about 6 hours after challenge. From this time until the 24th hour, the increase in count with the high virulence strains was at least 1 log unit, ranging from 1.00 with 2-Atox to 1.07 with G353. This compared with an increase of only 0.83 for B124 infections and 0.9 for B2288 and B1772 infections. It seemed that the first reaction in the mouse brain occurred as early as 6 hours after challenge. Between days 1 and 2 the difference between high virulence and low virulence strains

was more marked; whereas the count of the high virulence strain 18/323 increased by $\log_{10}$ 1.3, 2-Atox by 1.6 and G353 by 1.33, the count with the low virulence strains only increased slightly, as with B2288 by $\log_{10}$ 0.04, or even decreased, as did B1772 - by $\log_{10}$ 0.06 and B124 - by $\log_{10}$ 0.35.

From the second day the mean viable count with low doses of the low virulence strains declined rapidly until the brains of all mice killed on the fifth day contained less than 50 organisms. With higher doses of the low virulence strains the bacterial count declined at a slower rate and brains containing less than 50 organisms were not observed until at least the sixth day.

From the second to third days the viable count of the high virulence strains increased at least 10-fold. From the third day the rate of increase declined to 3- to 8-fold per day with 2-Atox and 18/323 until the terminal viable count of approximately 5×10^7 organisms was reached on the sixth or seventh days. The only exception to this was G353 which actually showed a decrease in viable count between the third and fourth days. It was interesting that the slow down occurred when the viable count reached 10^6, the count indicated by Holt et al. (1961) as being necessary to produce breakdown of the blood-brain barrier.

With all infections the bacterial counts of the

individual brains were all closely grouped up to the first day of infection. From the second day onwards the counts with low virulence infections were spread over a wide range, sometimes there being up to 1000 times difference between the most and least infected mice. This indicated a very varying response by individual mice to the infections with low virulence strains. This was very different from infections with high virulence strains where the individual counts were very closely grouped, there was rarely more than 10-fold difference between the highest and lowest counts.

The only exception to this was noted in the terminal stages of infections with G353, the wide spread of individual counts occurring once the bacterial count has exceeded 10^6 viable organisms.

5. Experiments on the Mechanism of Infection of Bordetella pertussis in the Mouse Brain.

Lacey (1960) showed that the growth of a B. pertussis strain H5 on a medium in which the sodium chloride was replaced by magnesium sulphate led to an increase in the LD_{50} by the intranasal route from 10^7 to 10^{10} organisms.

Later he pointed out that the surface characters of B. pertussis could be changed by varying the growth medium or incubation temperature (Lacey, 1961). He described this non-genetic variation as "modulation".

As will be shown later, I was able to modulate many strains of B. pertussis under various conditions and significantly increase the LD_{50} of the high virulence strains G353 and 2-Atox.

Thus it seemed possible that the fall in count at 2 days in mice infected with low virulence strains might have been due to the ability of these strains to modulate, the high virulence strains being resistant to modulation.

The other important possibility was the ability of the mouse brains to change physiologically or structurally when infected with B. pertussis or other agents. A change of this kind might lead to a preferential removal of low virulence strains.

5.1. Transfer to B. pertussis B2288 from Infected Brains to previously Uninfected Brains

To investigate the possibility of modulation in low virulence infections of B. pertussis, organisms from the brains of mice which had been challenged $2\frac{1}{2}$ days earlier were transferred to previously uninfected brains.

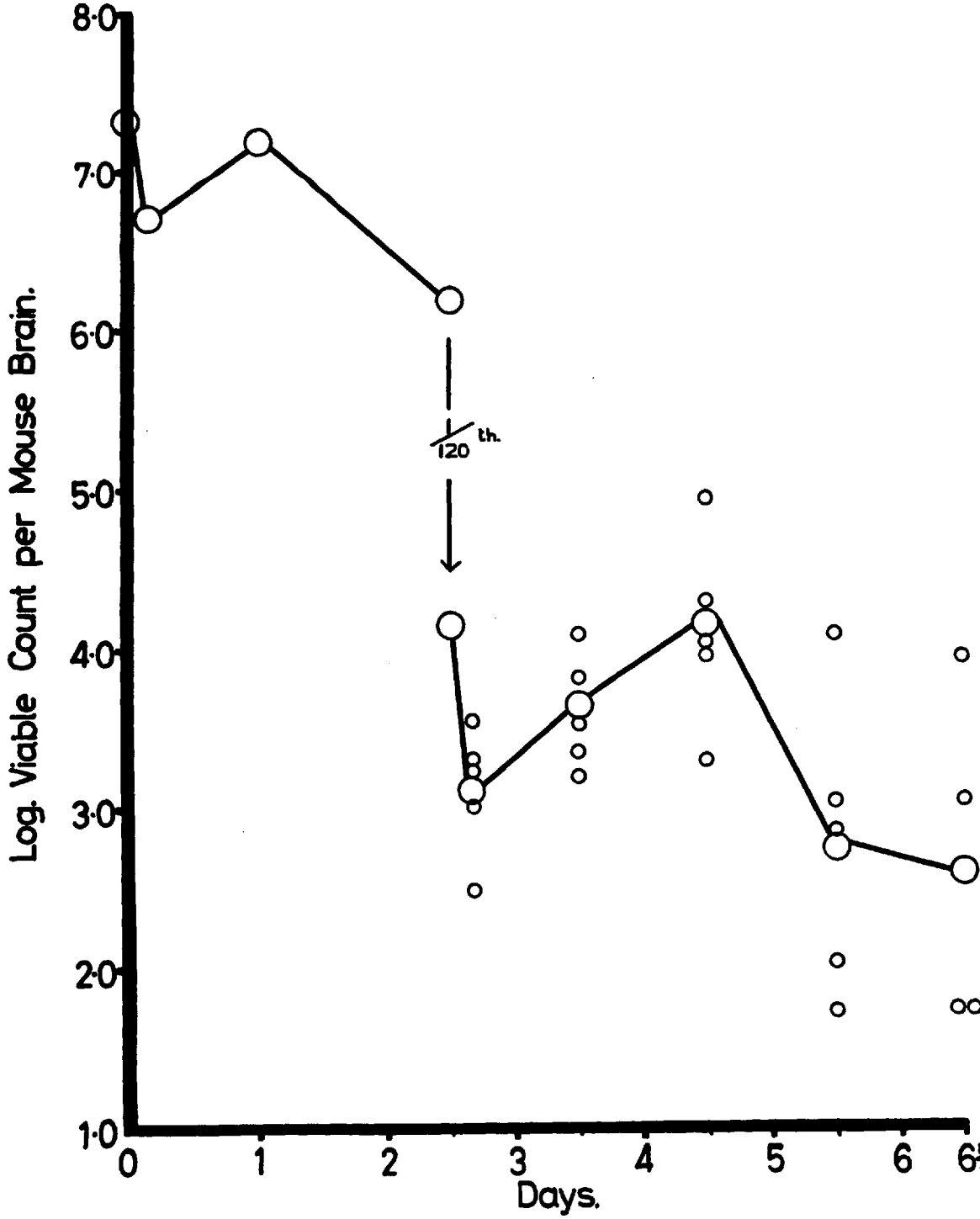
5.1.1. Experimental

Fifteen mice were challenged intracerebrally with 2×10^7 viable organisms of a low virulence strain B2288 and five mice killed at 1 hour, 1 and $2\frac{1}{2}$ days. At this latter time 0.025 ml. of this brain suspension containing bacteria was injected intracerebrally into 25 normal mice. This amount was equivalent to a 120th of a mouse brain and contained 1.45×10^4 viable organisms.

5.1.2. Results

One hour after challenge only 10% of the inoculum was detectable in the brain (Fig. 8). From this time the count increased to reach a maximum of 1.27×10^4 bacteria at the second day, which was followed by a rapid decline until 2 of 4 brains contained less than 50 organisms at the fourth day. This type of infection was very similar to those low virulence infections described earlier. The distribution of individual counts was also very similar to previous experiments (Sect.4.2.2)

Figure 8. Transfer of bacteria of the low virulence strain B2288 from infected brains to previously uninfected brains at $2\frac{1}{2}$ days. The amount transferred was equivalent to $^{1}/_{120}$th of a brain.


8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
1/120 th.
0
1
2
3
4
5
6
6½
Days.

being spread over $\log_{10}$ 1.0, 1.75, 2.38, and 2.2 on the first to fourth days.

Since the course of this infection following injection with organisms taken from the infected mice $2\frac{1}{2}$ days after challenge was no different from the usual course of a low virulence infection, it was thought that the decline in count from the second day onwards was not due to modulation of the low virulence strain.

5.2. B. pertussis B2288 infections in mice previously infected with B. pertussis B2288

In the last section (5.1.) it was shown that the decline in infection with low virulence strains in the mouse brain after the second day was apparently not due to modulation of the infecting organisms. It was decided that the most probable alternative was either an alteration in brain structure, e.g. damage to the ependymal surface, or to a stimulation of some defensive mechanisms.

These postulates were examined by reinfecting mice with the same low virulence strain of B. pertussis. In the previous section organisms had been transferred from infected brains to normal brains at $2\frac{1}{2}$ days but here it was necessary to reinfect the same mice at $4\frac{1}{2}$ days as this had been shown previously to be the time when most brains contained less than 50 organisms after challenge with a low dose of low virulence

strain. Also, at this time any effects due to interference immunity would be minimal.

5.2.1. Experimental

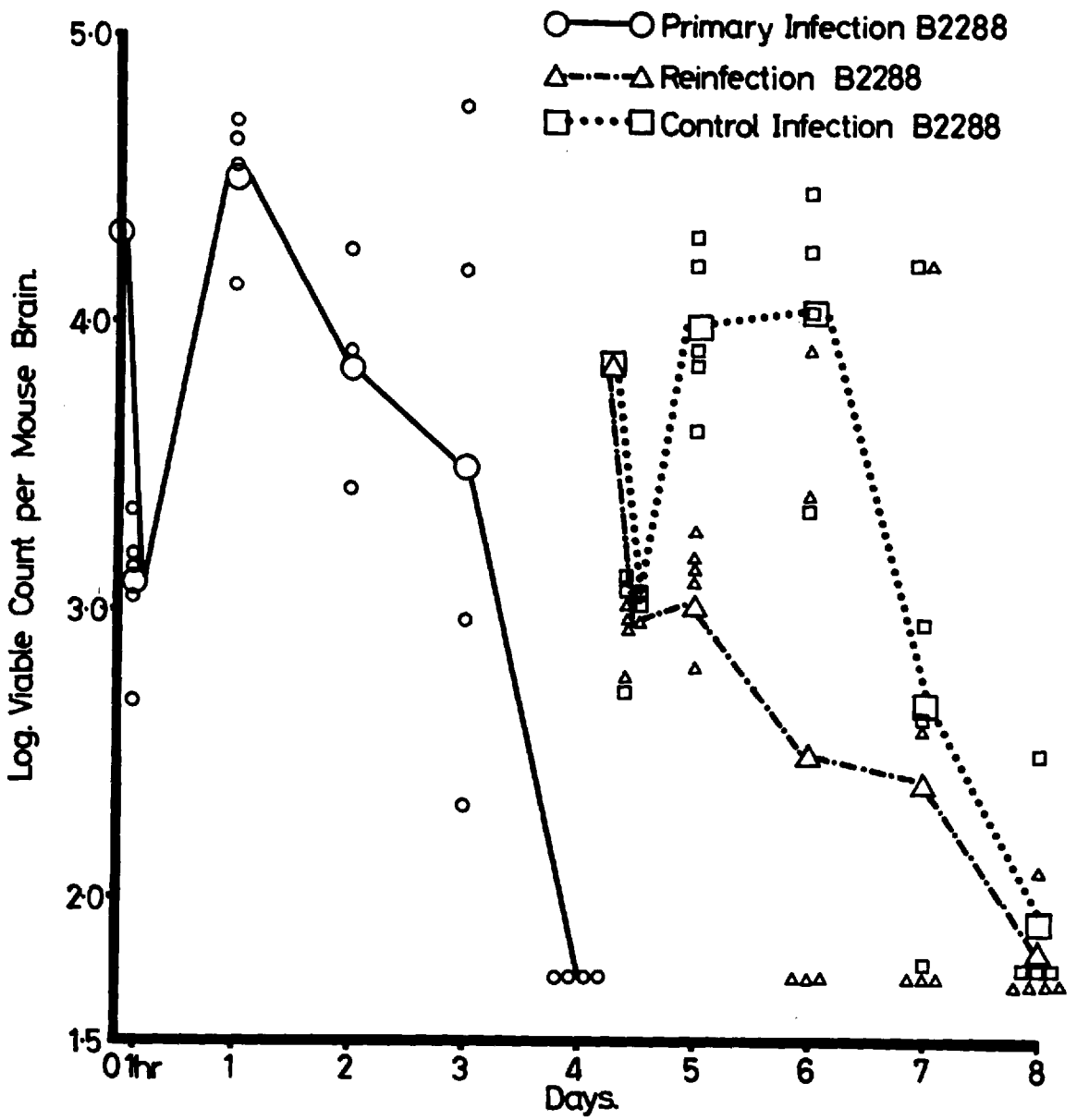
Fifty mice were challenged with 2×10^4 viable B. pertussis B2288. Five mice were killed at 1 hour, 1, 2, 3 and $4\frac{1}{2}$ days. At day $4\frac{1}{2}$ the 25 mice remaining and 25 normal mice were rechallenged with 6.9×10^3 viable B. pertussis B2288. Brain counts on 5 mice from each group were made daily.

5.2.2. Results

The results (Fig. 9) show a normal course of infection in the 25 mice killed after challenge with 2×10^4 B. pertussis B2288. At $4\frac{1}{2}$ days all the brains contained less than 50 organisms. A very similar infection is presumed to have occurred in the 25 mice remaining at the end of this part of the experiment.

When these mice were rechallenged with 6.9×10^3 viable B. pertussis B2288, there was an 87% loss of organisms in the first hour which was little different from the control infection. However, at days 1 and 2, there was a clear difference between the infection in the rechallenged mice and the control mice. In the control group the count exceeded the inoculum by the first day and by the second day the

Figure 9. Reinfection with the low virulence strain B2288 into mice previously infected with the same low virulence strain.


Primary Infection B2288
Reinfection B2288
Control Infection B2288
Log. Viable Count per Mouse Brain.
5·0
4·0
3·0
2·0
1·5
0
1hr
1
2
3
4
5
6
7
8
Days.

geometric mean was slightly greater than 10^4 organisms. In the reinfected group, however, the count at the first day was only marginally greater than at 1 hour and at the second day had declined to 3.2×10^2 organisms, less than $^1/_{30}$th of the count in the control mice.

In this experiment the brains of 3 out of 5 reinfected mice contained less than 50 organisms at the second day compared with none of 5 in the control group. In a similar experiment with B. pertussis B1772 as the infecting strain the brains of 4 out of 6 mice contained less than 50 organisms at the second day compared with none of 6 in the control group.

These results give a clear indication that mice infected with low virulence strains were less easily infected when challenged with the same strain a few days later. This experiment provides further evidence that the decline in infection at the first or second day with low virulence strains was not due to modulation of the organisms but to some type of response in the mouse.

5.3. B. pertussis 18/323 infections in Mice infected 2 or 4½ days previously with B. pertussis B2288 or B1772

In the previous section (5.2.) it has been shown that mice brains previously infected with a low virulence strain had an increased resistance to reinfection by a second low virulence strain. Evans and Perkins (1954b) showed that mice were

protected against an intracerebral challenge of B. pertussis 18/323 by an intracerebral injection of killed pertussis vaccine given at the same time or 3 hours before the challenge dose. They could detect no effect of the vaccine when injected after the challenge dose. In my experiments mice were rechallenged with the high virulence strain 18/323 2 days after challenge with the low virulence strain B2288 or 4½ days after challenge with the low virulence strain B1772. These experiments were designed to examine whether low virulence strains will promote "interference" immunity to high virulence strains injected at the peak of the low virulence infection and if the brains are still uninfectable with 18/323 when they contain few organisms of the low virulence infection.

5.3.1. Experimental

A group of I.C.I. mice were challenged intracerebrally with 7.5×10^3 B. pertussis B2288, a low virulence strain. Thirty of these mice were rechallenged 2 days later with 2.1×10^3 viable B. pertussis 18/323, a high virulence strain, and five mice killed daily for bacterial count determinations. A control group of 25 mice was also challenged with 2.1×10^3 18/323 and five mice killed daily.

A differentiation of the two strains in one brain was possible as B2288 does not grow on Blood Agar (BA) medium whereas 18/323 grows on both BA and BG media. A total count of organisms was made on BG medium and a count of the 18/323

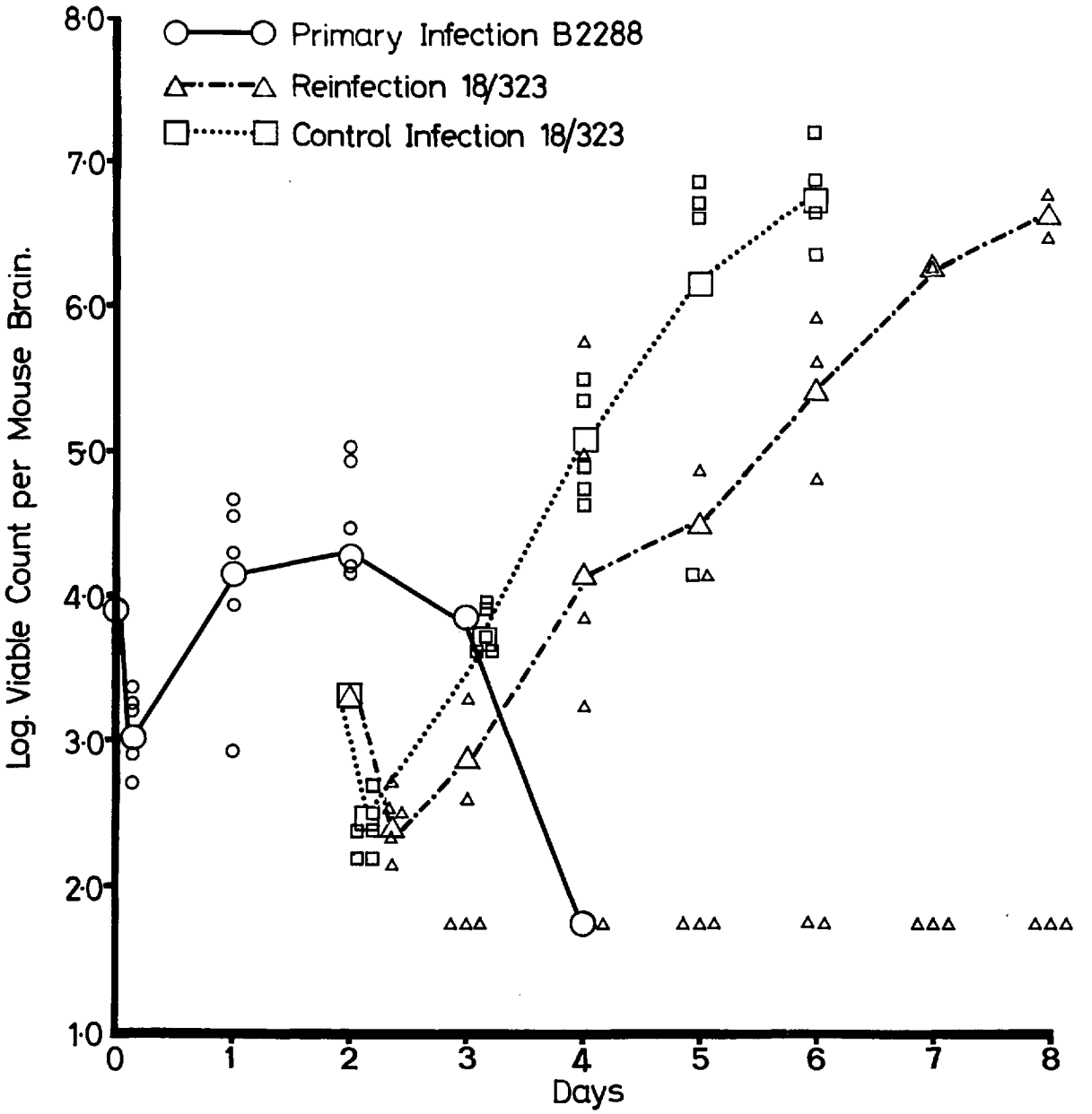
on BA medium. The difference in the two counts was taken to indicate the number of B. pertussis B2288 present in the brain.

Secondly, a group of T.O. mice were challenged with 3.5×10^4 viable B. pertussis B1772, a low virulence strain. Six mice were killed at 1 hour, 1, 2, 3 and $4\frac{1}{2}$ days to determine the course of infection with the low virulence strain. At $4\frac{1}{2}$ days the remaining 25 mice and 30 control mice were rechallenged with 1.8×10^3 viable B. pertussis 18/323, a high virulence strain. Viable counts were determined on 5 or 6 brains at daily intervals.

5.3.2. Results

As shown in Fig. 10, the mice were challenged at the peak of the low virulence infection on the second day. One hour after challenge with the high virulence strain there was no difference between the G.M. counts in either the control or the reinfected groups of mice. However, at 1 day the brains of 3 out of 5 reinfected mice contained less than 50 organisms and the counts in the other 2 were far less than the counts in the control infection. Brains containing less than 50 organisms have not been included in calculating the geometric means of the reinfected brains. Between 1 and 3 brains contained less than 50 organisms daily until the termination of the experiment at the eighth day. The geometric

Figure 10. Reinfection with the high virulence strain 18/323 at the peak of infection with the low virulence strain B2288. Brains containing less than 50 organisms were not included in the geometric mean calculations.


Primary Infection B2288
Reinfection 18/323
Control Infection 18/323
Log. Viable Count per Mouse Brain.
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
1
2
3
4
5
6
7
8
Days

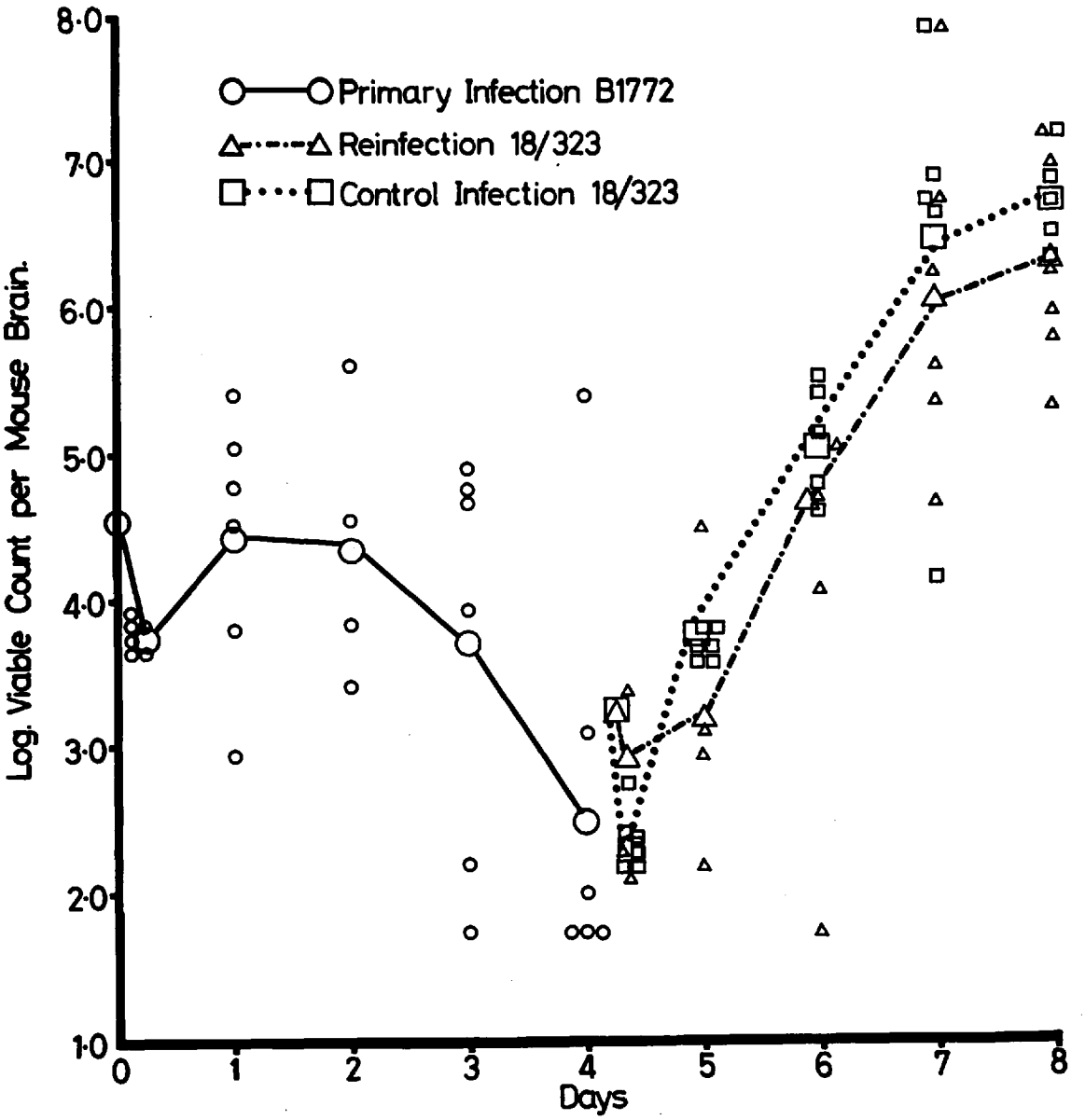
mean of the viable counts in the infected rechallenged brains at each day was over 10 times less than the geometric mean in the control group.

It was clear that mice, challenged with a high virulence strain at the second day peak of an infection with a low virulence strain, were far less susceptible to the high virulence strain than when this dose was injected into the control group of mice. This was very similar to the interference immunity phenomenon described by Evans and Perkins (1954b) between vaccines and high virulence organisms. When the high virulence strain was injected at $4\frac{1}{2}$ days, however, an entirely different picture of events emerged.

The characteristic type of infection with a low virulence strain was again found (Fig. 11). At $4\frac{1}{2}$ days it was found that the majority of brains contained less than 50 bacteria or contained only small numbers of the low virulence strain.

The mice were rechallenged at $4\frac{1}{2}$ days with the high virulence strain 18/323. With the control mice there was an 87% loss of organisms within 1 hour of challenge but with the reinfected group of animals the brains of 2 mice contained many thousands of organisms from the low virulence infection and were not included in the 1 hour count. Of the remaining 3 mice, 2 had expected brain counts and the other 1 contained 2.2×10^3 organisms, some of these obviously remaining from the low virulence infection. The mean of these 3 counts is shown

Figure 11. Reinfection with the high virulence strain 18/323 into mice previously infected with the low virulence strain B1772. Brains containing less than 50 18/323 bacteria were not included in the geometric mean calculations.


Primary Infection B1772
Reinfection 18/323
Control Infection 18/323
Log. Viable Count per Mouse Brain.
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
1
2
3
4
5
6
7
8
Days

in the graph and explains the higher than expected G.M. at 1 hour in the reinfected mice.

After this time there was little evidence that a large number of low virulence organisms remained in the brains. The one sterile brain found at day 6 may have resulted from the high virulence strain being injected into a brain containing many low virulence organisms.

Throughout the experiment the high virulence infection in the previously infected mice lagged slightly behind the infection in the control mice, but there was a considerable overlap of the individual counts. It was concluded therefore that there was no evidence that the animals' response to the low virulence infection had altered their response to the high virulence infection.

These results were quite unlike those described in Section 5.2. where it was shown that brains infected $4\frac{1}{2}$ days previously with low virulence strains were less easily infected with the same low virulence strain. It was evident from these 3 experiments that although the animals' response to the high virulence and low virulence infections may be identical, the mice were more easily able to eradicate low virulence strains than they were able to eradicate high virulence strains. It can be seen in Section 5.2. that the animal response to low virulence infections, which probably reached a peak between days 1 and 2, was still evident at day $4\frac{1}{2}$ although this may be variable as evidenced by the increase

in count in the normal way in some of the mice reinfected with low virulence strains. However, it was quite clear that the response induced by the low virulence strains did not alter the course of infection by high virulence strains. Thus, although a defence mechanism has been stimulated, no effect of this defence can be observed on high virulence strains, injected 4½ days after the start of the first low virulence infection.

5.4. B. pertussis Infections in Mice infected with Influenza or Vaccinia viruses

Evans and Perkins (1954a) suggested that their interference phenomenon might be similar to the interference described for viruses. Further support for this hypothesis is provided by the earlier experiments of Dalldorf et al. (1947) who showed that vaccinia virus induced increased resistance to B. pertussis infections.

Berenbaum et al. (1959) and Iida et al. (1962) have implicated the ciliated lining of the ependyma as the sole site of infection with B. pertussis and none of these workers showed any invasion of the brain substance by this organism.

Henle and Henle (1946) and Mims (1960b) have shown that the main pathological change in mouse brains infected with influenza virus was the complete destruction of the ependymal cells.

In this section infections with B. pertussis 18/323 have been examined in mice pretreated with influenza virus and vaccinia virus and also with ~~B. pertussis~~ B1772 in influenza virus infected mice.

5.4.1. Experimental

Mice were injected intracerebrally with 0.025 ml. of a suspension of influenza virus A_2 Singapore cultivated on egg allantoic fluid. Three days later these mice and a control group were challenged intracerebrally with 1.96×10^3 viable B. pertussis 18/323. Mice were killed at 1 hour and 1 to 6 days after challenge and 2 groups of mice were left to determine the number of survivors. Influenza virus infected mice were also challenged 3 days later with 9.2×10^3 viable B. pertussis B1772, a low virulence strain, and brain viable counts determined at 4 hours and 1 day.

Other mice were injected intracerebrally with 0.025 ml. of a vaccinia virus suspension, Connaught Laboratories Strain, containing 10^7 pock forming units (pfu) per ml. Three days later these mice and a control group of mice were challenged intracerebrally with 6×10^2 viable B. pertussis 18/323. Mice were killed at daily intervals for 4 days for determination of viable counts and other mice remained for determining the number of survivors.

5.4.2. Results

The effects of influenza virus infections on intracerebral infections with B. pertussis 18/323 and B1772 are shown in Figs. 12 and 13 respectively.

When 1.9×10^3 organisms of the high virulence strain 18/323 were injected into the virus infected mice, only 7.1% of the inoculum was found 1 hour later compared with 13% in the control mice. This greater loss of organisms early in the virus infected brains was even more marked with B1772 challenge. Four hours after challenge only 1.9% of the bacterial inoculum was cultured from 6 virus infected brains compared with 12.3% in the control mice.

By the first day after challenge 7 of 10 brains were found to contain less than 50 B. pertussis 18/323 and the geometric mean count in the other 3 mice was $^1/_{10}$th that in the control mice. Approximately the same number of brains, 5 to 7, were found to contain less than 50 bacteria daily and the G.M. count of the remaining mice was found to be between $^1/_5$th and $^1/_{20}$th of that in the control mice. The bacterial counts in the individual virus treated brains nearly always lagged behind the counts in the control group of brains. Thus, although all these mice would eventually die of B. pertussis infections at a later time than the controls, the influenza virus has clearly had some effect on the brains of all the mice. This

Figure 12. Intracerebral infection with B. pertussis 18/323 in mice infected 3 days previously with Influenza Virus A_2 Sing. Brains containing less than 50 bacteria were not included in the geometric mean calculations.

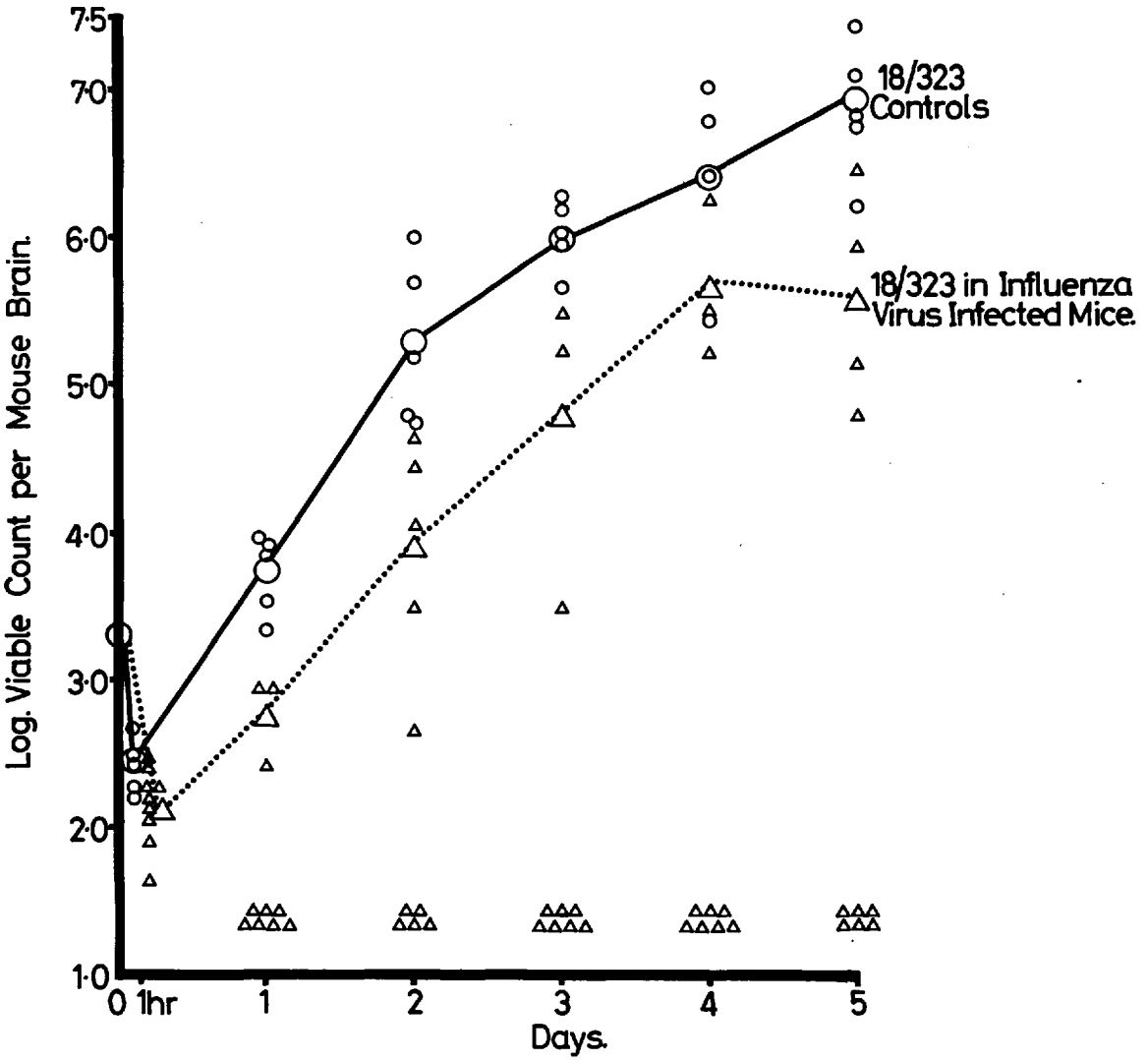

7·5
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
5
Days.
18/323
Controls
18/323 in Influenza
Virus Infected Mice.

Figure 13. Intracerebral infection with B. pertussis B1772 in mice infected 3 days previously with Influenza Virus A_2 Sing. Brains containing less than 50 bacteria were not included in the geometric mean calculations.

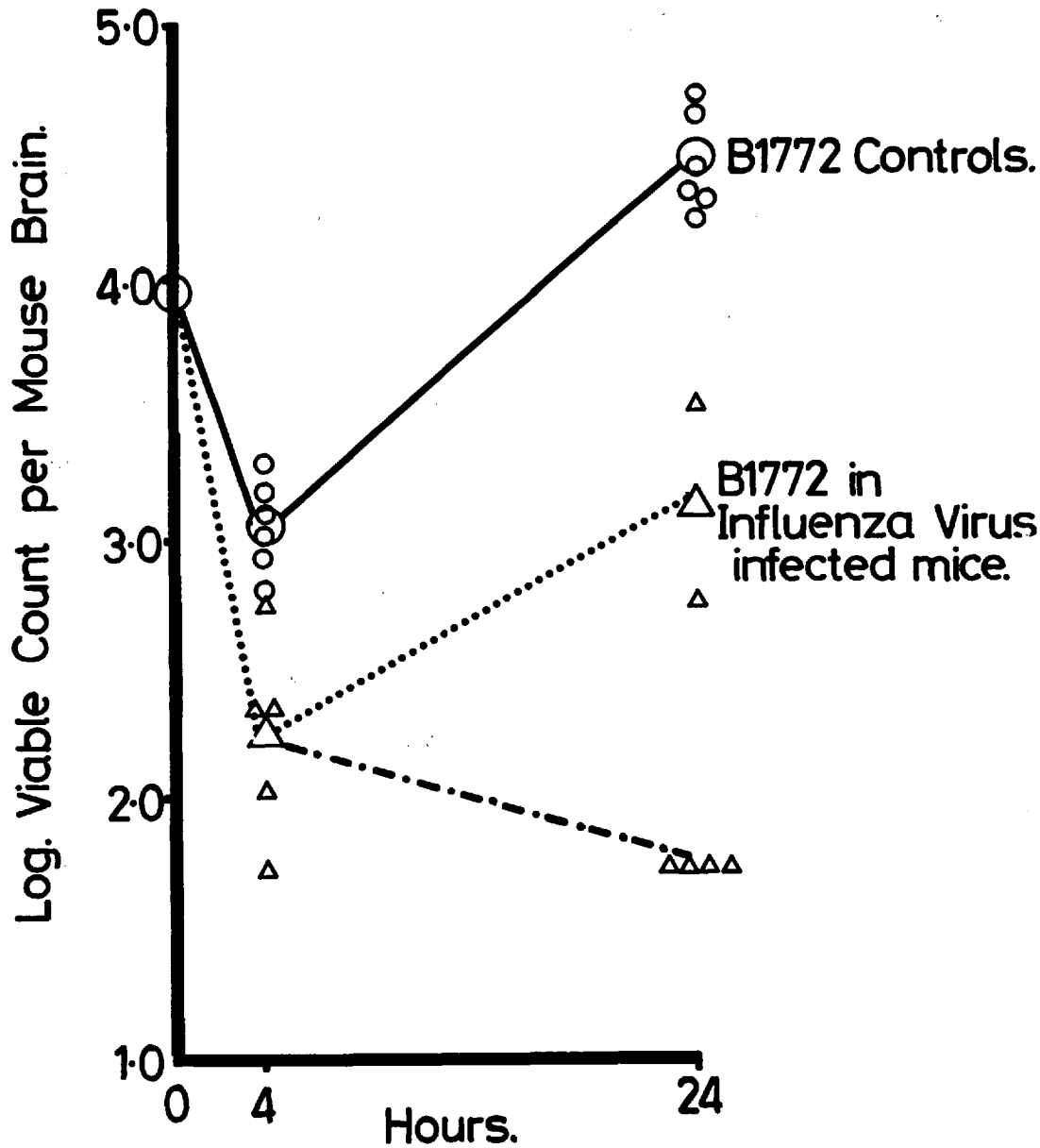

5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
4
24
Hours.
B1772 Controls.
B1772 in
Influenza Virus
infected mice.

TABLE 5. The effect of intracerebral infection with Influenza or Vaccinia Virus on the number of mice surviving challenge with B. pertussis 18/323.

Virus	Interval between virus and challenge	Challenge inoculum of 18/323	Number of mice	Day of death	Average day of death	D/T
Influenza	3	1470	1[illegible]	7	7	9/10
"	2	"	10	7,12,13	10.6	7/10
"	1	"	9	8,8,9,9,9,14	9.5	3/9
None	-	"	11	8,8,8,8,8,9,9,9,10,10	8.7	1/11
Vaccinia	3	720	9	10,10,11,12,12	11	4/9
"	0	"	10	6,7,7,7,8,8,8,10,11	8	1/10
None	-	"	6	7,7,8,8,8,8	7.7	0/6

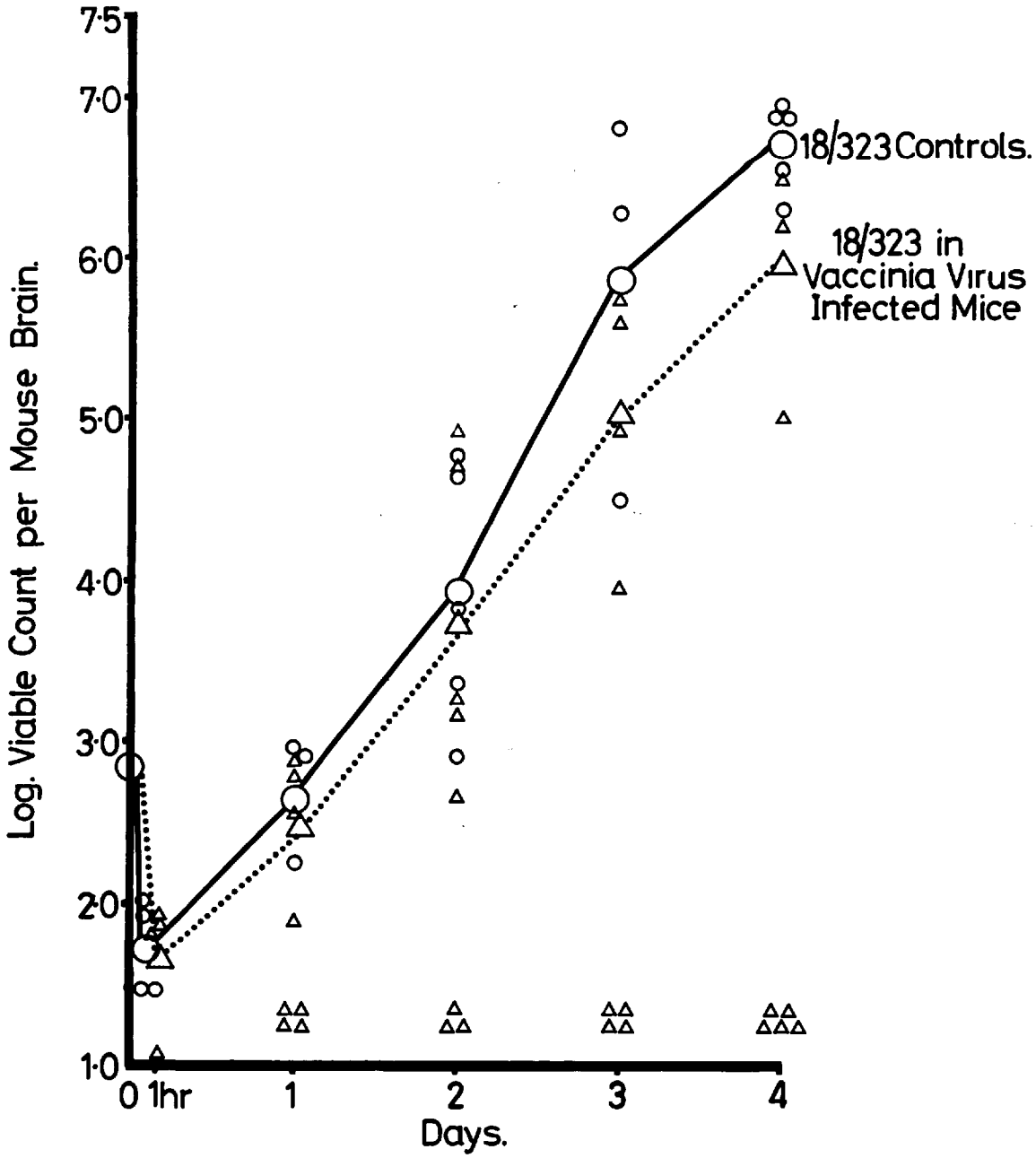
was further emphasised in another experiment in which mice infected with the same dose of influenza virus were challenged either 1, 2 or 3 days later with 1.47×10^3 viable B. pertussis 18/323 (Table 5). In the control group of mice, 1 of 11 mice survived, whereas in the mice challenged with B. pertussis 1 day after injection of virus 3 of 9 mice survived; when this period was extended to 2 days, 7 out of 10 mice survived the challenge and when further extended to 3 days, 9 of 10 mice survived. The average day of death for the control animals was 8.7 days, for the mice challenged 1 day after the virus 9.5 days, for the mice challenged 2 days after the virus 10.6 days and the mice challenged 3 days after the virus 7 days.

In the experiment with B. pertussis B1772 (Fig 13), it was found that 4 hours after challenge considerably fewer organisms were found in the virus infected mice than were found in the control group of mice. By the first day 4 of 6 brains contained less than 50 bacteria and the counts in the remaining 2 were much lower than in the control mice.

The results obtained with vaccinia virus were very similar to those obtained with the influenza virus (Fig. 14).

Although the bacterial counts at 1 hour were slightly lower in the vaccinia infected mice (8.1%) than it was in the control mice (9.0%) by the first day after challenge 4 out of 8 brains in the virus infected group yielded less than 50

Figure 14. Intracerebral infection with B. pertussis 18/323 in mice infected 3 days previously with Vaccinia Virus, Connaught. Labs. strain. Brains containing less than 50 bacteria were not included in the geometric mean calculations.


7·5
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
Days.
18/323 Controls.
18/323 in Vaccinia Virus Infected Mice

B. pertussis 18/323. This proportion remained very similar throughout the experiment, range 3 to 5, and the G.M. count in the remaining mice was always less than that in the control group.

A lag in the death time of the mice similar to that in the influenza virus experiments was also found. The average day of death in mice challenged with B. pertussis 18/323 3 days after injection of the vaccinia virus was 11 days compared with 8 days in mice injected with a virus/bacteria mixture and 7.7 days in the B. pertussis control mice. (Table 5).

5.5. Intracerebral infection with Modulated Organisms

Lacey (1960) reported that the intranasal LD_{50} of a B. pertussis strain H5 was increased from 10^7 to 10^{10} organisms following growth on a medium where the sodium chloride content was replaced with magnesium sulphate.

The same author (Lacey, 1961) also reported that culture of the organisms at 25°C instead of 35°C resulted in the growth of C-mode organisms.

The effect of growth temperature and medium composition on virulence has been studied for several high virulence strains of B. pertussis.

The three high virulence strains of B. pertussis, 18/323 G353 and 2-Atox have been studied under various growth conditions.

The three strains have been grown on B.G. medium at 35°C and 25°C, B.A. medium at 35°C and Cohen Wheeler medium with added blood and magnesium sulphate (see Materials and Methods).

The strains were subcultured on the same medium from the freeze-dried state and the fourth subculture used for injection of the mice.

In Table 6 the results of various experiments with strains grown on the special media or at low temperatures are listed. The number of viable organisms injected and the degree of agglutination (Preston, 1963) are included in the table.

Throughout the experiments, as expected, growth on B.G. medium at 35°C produced highly virulent organisms, doses as low as 400 viable bacteria killing all the mice. All strains were highly agglutinable with antiserum when grown on this medium at 35°C, and also cultures grown on B.A. medium at 35°C were highly virulent and as agglutinable as the B.G. grown strains.

However, when the 3 strains were subcultured on B.G. medium at 25°C, both G353 and 2-Atox became less virulent and challenge doses greater than 2000 viable organisms killed only 1 or 2 mice of each group. The degree of agglutination was also reduced, both these strains being inagglutinable with T1, T2 or T3 antisera. This was very different from 18/323 cultured on B.G. medium at 25°C. These organisms lost none

TABLE 6. Effect of medium and incubation temperature on the agglutination and virulence of various high virulence strains of *B. pertussis*.

Strain	Medium	Incubation Temperature	Degree of Agglutination T1	T2	T3	Number of viable bacteria injected	S/T
18/323	BG	35°C		NT		400	2/8
"	BG	25°C		NT		400	0/8
18/323	BG	35°C	+++	++	++	1150	0/11
"	BG	25°C	+++	+++	+++	1400	0/10
"	BA	35°C	+++	++	+	1900	0/12
"	CW High Mg	35°C	+++	++	++	1300	1/10
G 353	BG	35°C		NT		450	0/8
"	BG	25°C		NT		450	8/8
"	BG	25°C		NT		2200	9/10
G 353	BG	35°C	+++	++	-	500	2/10
"	BG	25°C	-	-	-	1000	9/10
"	BA	35°C	+++	++	+	400	5/10
"	CW High Mg	35°C	-	-	-	600	10/10
2-Atox	BG	35°C	+++	+++	+++	1150	2/10
"	BG	25°C	-	-	-	1450	9/11
"	BA	35°C	+++	++	++	1500	2/9
"	CW High Mg	35°C	-	-	-	3000	7/10

NT = Not tested.
BG = Bordet-Gengou medium.
BA = Blood Agar medium.
CW High Mg = Cohen and Wheeler medium with added magnesium sulphate. Degree of agglutination determined by the semi-quantitative method of Preston (1963.)
S/T = $\frac{\text{Survivors}}{\text{Total Challenged}}$

of their agglutinability and did not lose their virulence (Table 6).

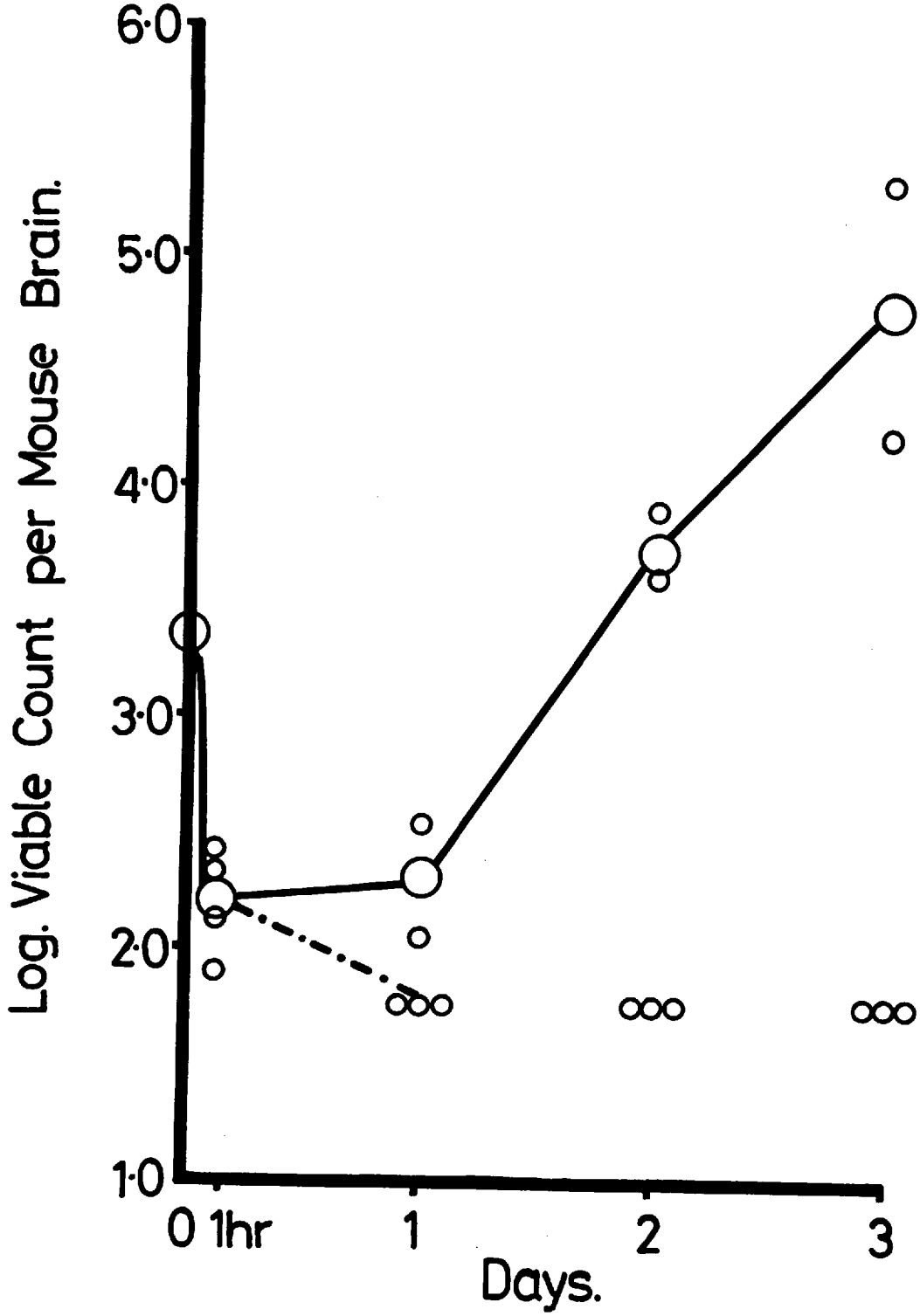
These results were repeated with the strains grown on Cohen and Wheeler medium containing blood and the equivalent amount of magnesium sulphate replacing the sodium chloride. Both G353 and 2-Atox lost their agglutinability and their virulence was reduced. 18/323, however, grown on this medium maintained its agglutinability and virulence.

A typical infection with modulated organisms is shown in Fig. 15. Here mice were challenged with 2.2×10^3 B. pertussis G353 cultured on B.G. medium at 25°C and only 7% of the inoculum remained 1 hour after challenge. Twenty-four hours after challenge the brains of $^3/_5$ mice contained less than 50 organisms and the count in the other two mice was only marginally higher than the count at 1 hour. However, from the first day onwards the count increased as normal by about 10 times daily.

Presumably the lag between 1 hour and 24 hours was due to a modulation of the organisms back to normal or the inoculum may have contained a few organisms which had not undergone modulation on the medium.

Following on from this, one group of normal mice and one group of mice injected with 2.5 mgm. of cortisone 2 hours before challenge and 1 day after challenge were injected with 10^3 B. pertussis G353 cultured on B.G. medium at 25°C.

Figure 15. Intracerebral infection with the strain G353 grown *in vitro* on B.G. medium at 25°C. Brains containing less than 50 organisms were not included in geometric mean calculations.


6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
Days.

Six out of nine of the normal mice survived the challenge whereas none of ten mice survived the challenge in cortisone treated mice. Viable counts on cortisone treated mice dying at the sixth day were in the range 2.63×10^7 to 3.67×10^8 organisms. The role of cortisone in neutralising the modulating effect of the medium is not clear.

Loss of agglutination is not thought to be connected with virulence. Many other properties were also lost, including toxicity, histamine sensitising and efficacy as a vaccine (Holt - unpublished observations).

5.6. Sensitivity of B. pertussis to complement

The possibility that the difference in virulence was due to the varying sensitivity of the strains to bactericidal complement was examined *in vitro* using normal mouse and normal guinea pig serum and *in vivo* using mice of the B.10D2 "Old Line" and "New Line" strains. The "Old Line" mice are genetically identical with the "New Line" except that they are lacking in a component of complement probably corresponding to the human C'_5 (Terry *et al.*, 1964).

Although Rosenberg and Tachibana (1962) have shown that normal mouse serum has a high haemolytic activity tested *in vitro* no one has been able to demonstrate any bactericidal activity of mouse serum *in vitro*.

Recently Glynn and Medhurst (1967) showed that mouse

serum was bactericidal *in vivo* for *E. coli* injected into B10.D2 mice. Rother and Rother (1965) also showed that the absence of the C'_5 component affected only the action of bactericidal complement and had no effect on phagocytosis by the reticuloendothelial system.

5.6.1. Experimental

(a) Collection of normal guinea pig serum (NPS). Normal guinea pigs were bled by cardiac puncture and the blood from each animal stored separately in an ice bath. The cells were spun down in a refrigerated centrifuge at 3000 r.p.m. for 20 minutes and the serum from several animals bulked.

(b) Collection of Normal mouse serum (NMS). Blood was collected from the brachial artery using siliconised dropping pipettes and transferred immediately into a small tube maintained at 0°C. Each tube contained a few polystyrene crystals which prevent the formation of fibrin clots when the tubes were shaken.

The serum and cells were separated by centrifugation at 3000 r.p.m. for 20 minutes at 2°C; the serum could then be pooled without undue loss of haemolytic complement activity.

(c) Antibacterial activity of the sera. Suspensions of the *B. pertussis* strains were prepared in 1% Casamino Acid

solution and adjusted to give a density of 3×10^6 viable organisms/ml. 0.5 ml. of this suspension was mixed with 0.5 ml. of 1% Casamino Acid solution and with 0.5 ml. of NMS or NPS. In another section of this experiment, 0.5 ml. of the bacterial suspension was mixed with 0.5 ml. of NMS and 0.5 ml. of NPS. The mixtures were incubated at 37°C for 30 minutes. Decimal dilutions of the incubation mixtures were made in casamino acids and the bacterial count estimated by the method of Miles and Misra (1938) on Bordet-Gengou medium.

5.6.2. Results

The sensitivities of the strains to the various sera are shown in Table 7. The sensitivities are given as the percentage of the viable organisms sensitive to the bactericidal action of serum. This shows that the high virulence strains of B. pertussis, 18/323, G353 and 2-Atox, were slightly ~~more~~ less resistant to the bactericidal activity of normal guinea-pig serum than were the low virulence strains. Despite the special precautions taken with the processing of the normal mouse serum no bactericidal activity could be detected against any of the four strains of B. pertussis tested. It would seem, in fact, that the mouse serum had become anti-bactericidal as when it was mixed in equal parts with normal guinea-pig serum the bactericidal action of this latter serum was

TABLE 7. Percent number of organisms sensitive to normal guinea pig serum (NPS) and normal mouse serum (NMS) in vitro.

Strain	Percentage of viable organisms sensitive to:-		
	NPS	NMS	NMS + NPS
18/323	>99.99%	0%	0%
G 353	>99.99%	0%	NT
2-Atox	99.97%	NT	NT
B 2288	95.60%	0%	0%
B 1772	95.06%	0%	NT
B 124	95.03%	NT	NT

NT = Not tested.

completely inhibited for two strains of B. pertussis tested.

These results give an indication that the virulence of B. pertussis is not dependent on the resistance of the cells to the bactericidal action of complement.

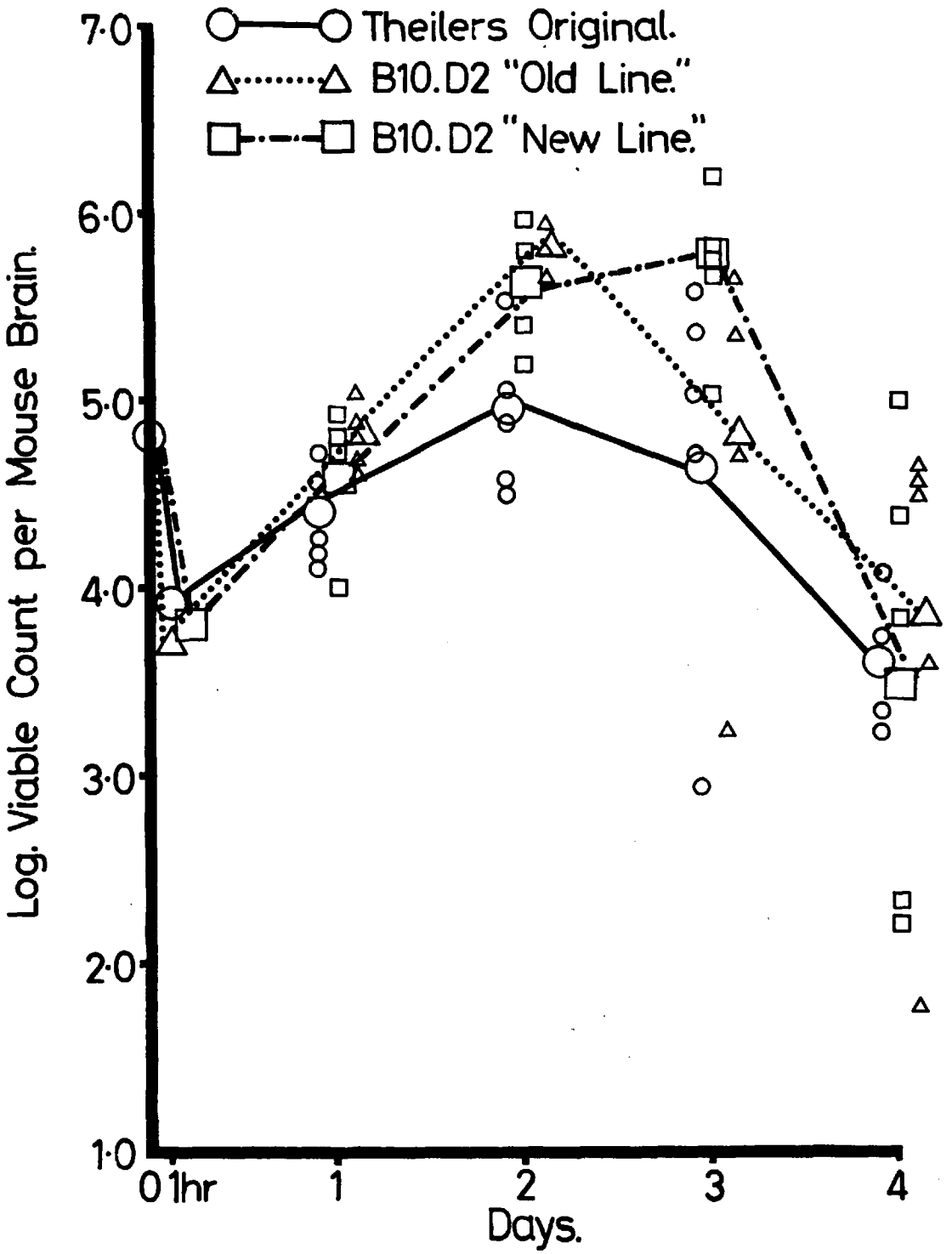
The finding of a lack of any bactericidal action of normal mouse serum against B. pertussis is similar to the results of Rosenberg and Tachibana who found that although the mouse serum was highly haemolytic in vitro, no bactericidal killing against various species of bacteria could be detected in vitro.

Further evidence that the virulence B. pertussis strains were not dependent on their sensitivity to bactericidal complement was provided by the results of intracerebral infection with a low virulence strain B1772 in the B10.D2 strains of mice. Infections in the "Old" and "New" lines of these mice were compared with the infection in the usual strain of white mouse, Theiler's Original.

Intracerebral infection after an injection of 8×10^3 viable organisms is shown in Fig. 16.

One hour after challenge only 10% of the inoculum could be detected in any of the strains of mice. There was little difference between any of the infections at day 1 but on the second day the count in both strains of B10.D2 mice was greater than in the T.O. mice, but they were very similar to each other. On the third and fourth days the bacterial

Figure 16. Intracerebral infection with B. pertussis B1772, a low virulence strain, in Theilers Original, B10.D2 "Old Line" and B10.D2 "New Line" mice.


Theilers Original.
B10.D2 "Old Line."
B10.D2 "New Line."
Log. Viable Count per Mouse Brain.
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
1hr
1
2
3
4
Days.

counts were fairly evenly distributed and there was thought to be no significant difference in the infections in any of the three strains of mice.

If bactericidal complement played an important role in determining the degree of multiplication of the bacteria in the brain it might be reasonably expected that the viable counts in the "Old Line" C'_5 negative mice would be much greater than in the "New Line" C'_5 positive mice.

This was not found to be so and, taken in combination with the *in vitro* results with NPS and NMS already recorded, it can be concluded that bactericidal complement plays little or no part in determining the course of *B. pertussis* infections in the mouse brain.

6. Intracerebral infection with B. pertussis following the Treatment of the Mice with various Substances

Iida et al. (1963) showed that treatment of the mice with 2.5 mgm. of cortisone acetate 2 hours before and 1 and 2 days after challenge greatly enhanced the intracerebral infection produced with a B. pertussis Tohama Phase I strain of low mouse virulence. The LD_{50} of this strain was reduced from more than 2×10^6 organisms to 5×10^3 and, whereas only one mouse died in the control group challenged with 2×10^6 organisms, all the mice died in the cortisone treated group which were challenged with 2×10^4 organisms.

When cortisone-treated mice were challenged with the high virulence strain 18/323 no enhancement of infection could be detected and no significant difference in the mortality nor the average survival time between the treated and untreated control groups was observed.

It was also found that immunity could be neutralised by administration of cortisone at the above times to mice immunised either actively 14 days earlier with vaccine or passively with immune serum 24 hours after challenge. In all experiments a marked leucopenia was found but no correlation of this with the enhanced infection was made.

De Duve et al. (1953) recognised the presence, in many types of cells, of small inclusions designated lysosomes which readily stained for many enzymes. Fell (1962) and Weissman

(1964) showed that hydrocortisone stabilised the lysosomal membrane preventing release of the enzymes. Wolf et al. (1943) and Naidoo and Pratt (1951) showed that the choroid plexus and ependymal cells stained deeply for acid phosphatase and later Becker et al. (1960) showed that the lysosomes of the ependymal cells of rats were concentrated in the apical region of the cells. It therefore seemed possible that release of lysosomal enzymes into the brain might play an important role in the course of B. pertussis infections.

The release of enzymes under various conditions of infection has been measured but no clear correlation could be found between release of enzymes and infection.

It has also been reported (Weissman, 1964) that many substances were able to labilise the lysosome system. If the susceptibility of the low virulence strains to lysosomal enzymes was dependent on their release it was thought that pretreatment with labilisers would lower the degree of infection still further. Labilisers tested were streptolysin O and vitamin A. It was found that, in fact, both these substances enhanced infection.

6.1. Intracerebral infection with Bordetella pertussis in cortisone treated mice

6.1.1. Experimental

Mice were injected subcutaneously with 2.5 mgm. of

cortisone acetate (Cortisyl-Roussel) 2 hours before and 1 and 2 days after challenge with various strains of B. pertussis. Bacterial counts of at least five brains were estimated at daily intervals.

6.1.2. Results

The intracerebral infection of three low virulence strains in cortisone treated mice has been studied in some detail. The results are shown in graphic form (Fig. 17) for strain B1772 and in tabular form (Table 8) for strains B2288 and B124.

The G.M. viable counts at 1 hour were very similar in the control and cortisone treated mice. With all three strains the challenge dose was approximately 1.3×10^4 viable organisms and there was no evidence in the 1 hour counts that cortisone enhanced the retention of organisms in the brain more than the 11.7% calculated in Table 2.

At 24 hours the viable counts in the cortisone treated mice were only marginally greater than those in the untreated control mice for strains B1772 and B2288, but with strain B124 there was a marked difference in viable count of the cortisone treated and untreated mice at 24 hours.

Generally, however, it was not until 48 hours after challenge that the enhancing of infection by cortisone became evident. Whilst the control infection declined the viable counts in the cortisone treated mice increased by 5 to 10 times

Figure 17. Intracerebral infection with B. pertussis B1772 in normal mice and in mice injected subcutaneously with 2.5 mgm. of cortisone acetate 2 hours before and 1 and 2 days after challenge.

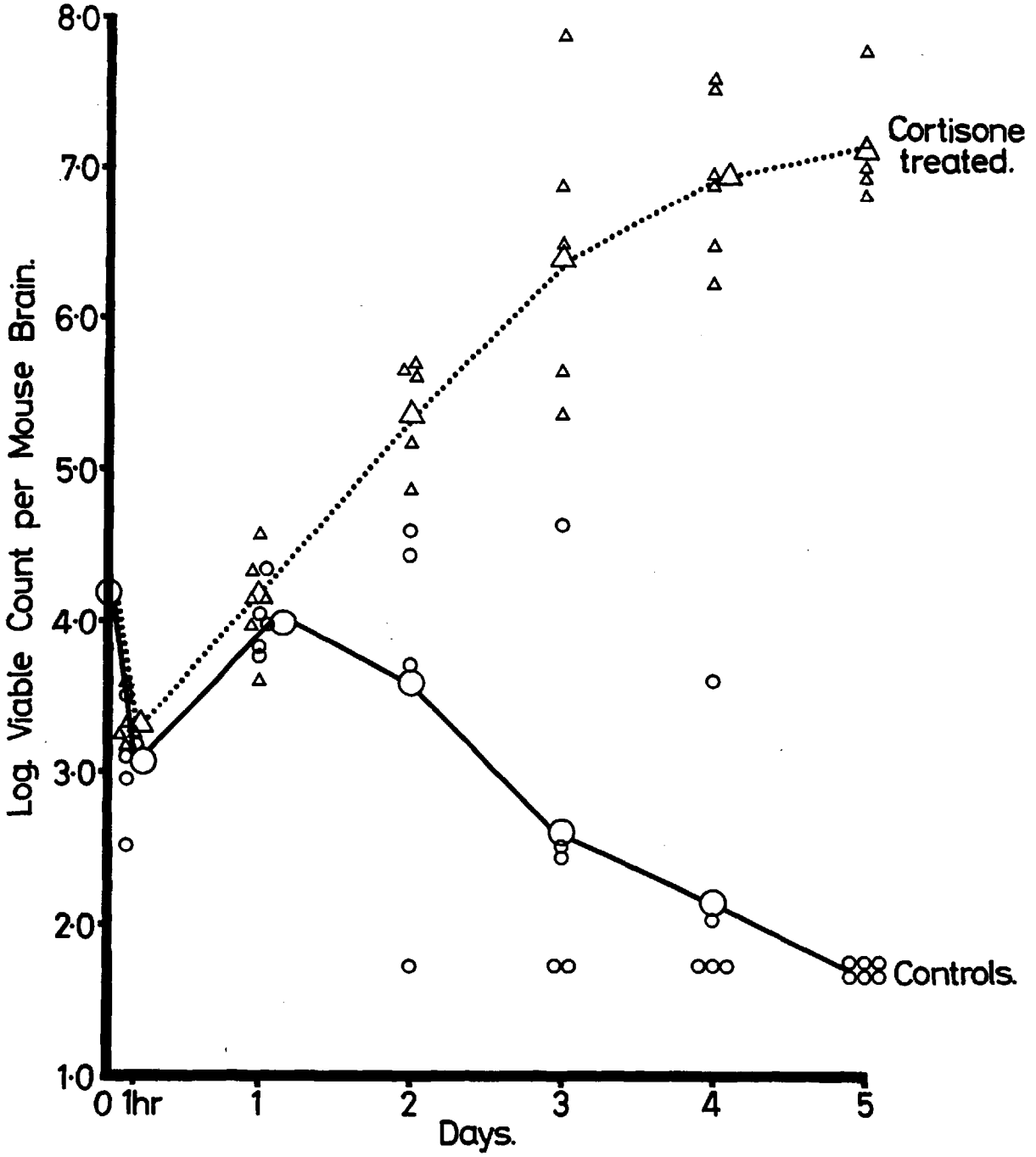

8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
5
Days.
Cortisone treated.
Controls.

TABLE 8. Log. geometric mean viable counts of the low virulence strains of *B. pertussis* B 124 and B 2288 in normal and cortisone treated mice compared with an infection of the high virulence strain 18/323 in normal mice.

Strain	Log. Geometric Mean Viable Count				
	B 124	B 124	B 2288	B 2288	18/323
Time	Normal mice	Cortisone treated mice	Normal mice	Cortisone treated mice	Normal mice
Inoculum	4.12	4.12	4.14	4.14	3.8
1 hour	3.09	3.12	3.33	3.35	2.85
1 day	3.41	4.17	4.35	4.53	3.6
2 days	3.09	5.25	3.35	4.92	5.0
3 days	2.25	6.10	3.36	5.12	5.88
4 days	1.78	6.34	1.70	5.91	6.85
5 days	1.70	7.38	1.70	6.4	7.55

daily. The terminal viable count of the low virulence strains in the cortisone treated mice was in the region of 10^7 - 10^8 viable organisms. This count was approximately the same as that attained by the virulent strains in normal mice. The geometric mean viable counts of a typical 18/323 virulent infection have been included in Table 8 for comparison. Although this is a slightly smaller dose than the low virulence strains, the courses of infection were very similar and only in the terminal stages of infection does the high virulence count exceed that of the low virulence strain in the cortisone treated mice. It is clear therefore that the low virulence strains injected into cortisone treated mice closely mimic the high virulence infection in normal mice.

Figures 18 and 19 show that cortisone treatment of mice even enhanced the infections with high virulence strains. Again, viable counts at 1 hour and 24 hours were very similar in all mice. From the second day onwards in both the infections shown the G.M. viable counts of the cortisone treated mice always exceeded those in the control group of animals.

Table 9 analyses a series of experiments with high virulence and low virulence strains in cortisone treated mice. The average time to death with low virulence strains in cortisone treated mice was approximately the same as that for equal doses of high virulence strains in normal mice. The terminal viable counts were also found to be similar in a range

Figure 18. Intracerebral infection with B. pertussis 18/323 in normal mice and in mice infected subcutaneously with 2.5 mgm. of cortisone acetate 2 hours before and 1 and 2 days after challenge.

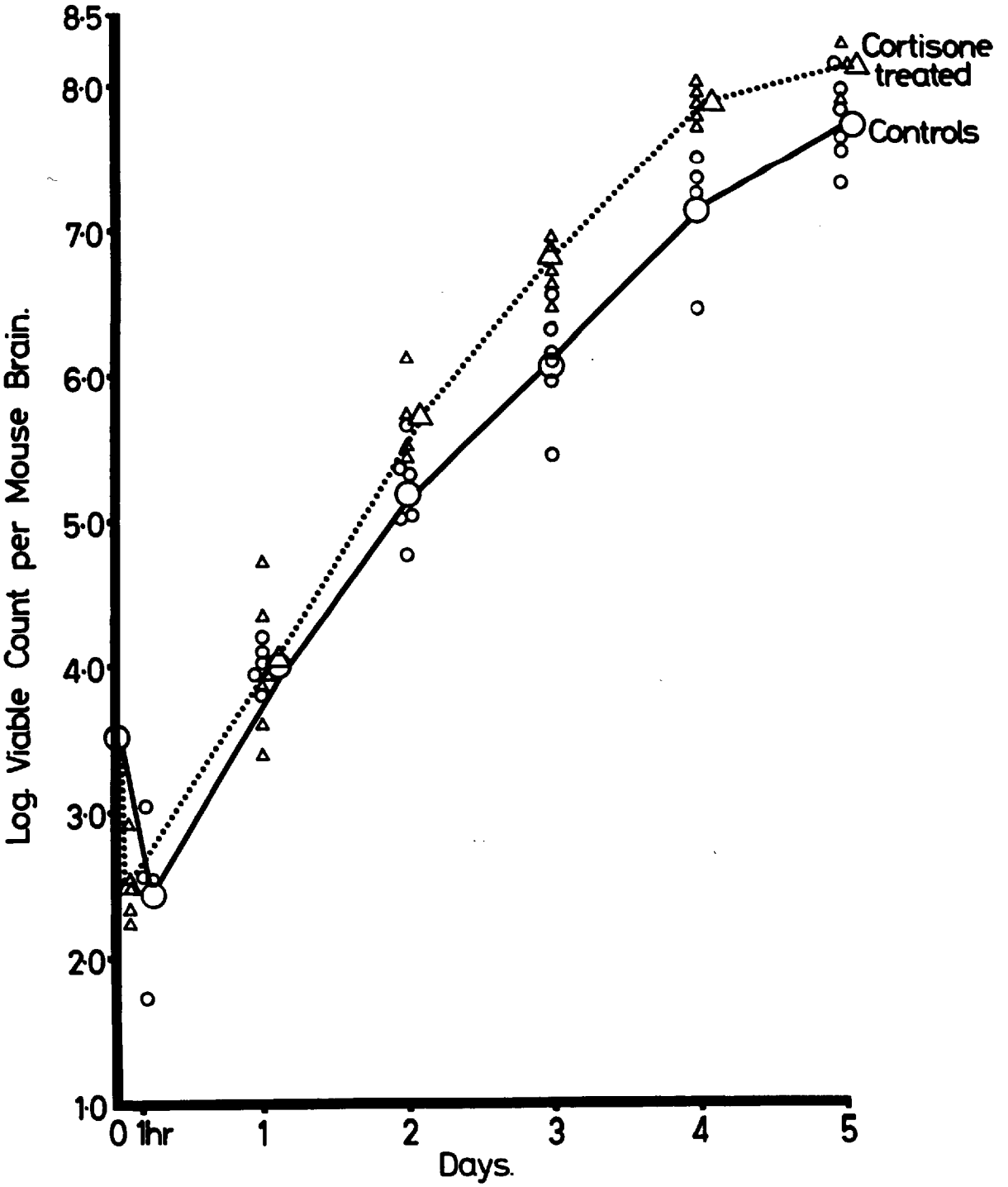

Log. Viable Count per Mouse Brain.
8·5
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
1hr
1
2
3
4
5
Days.
Cortisone treated
Controls

Figure 19. Intracerebral infection with B. pertussis G353 in normal mice and in mice injected subcutaneously with 2.5 mgm. of cortisone acetate 2 hours before and 1 and 2 days after challenge.

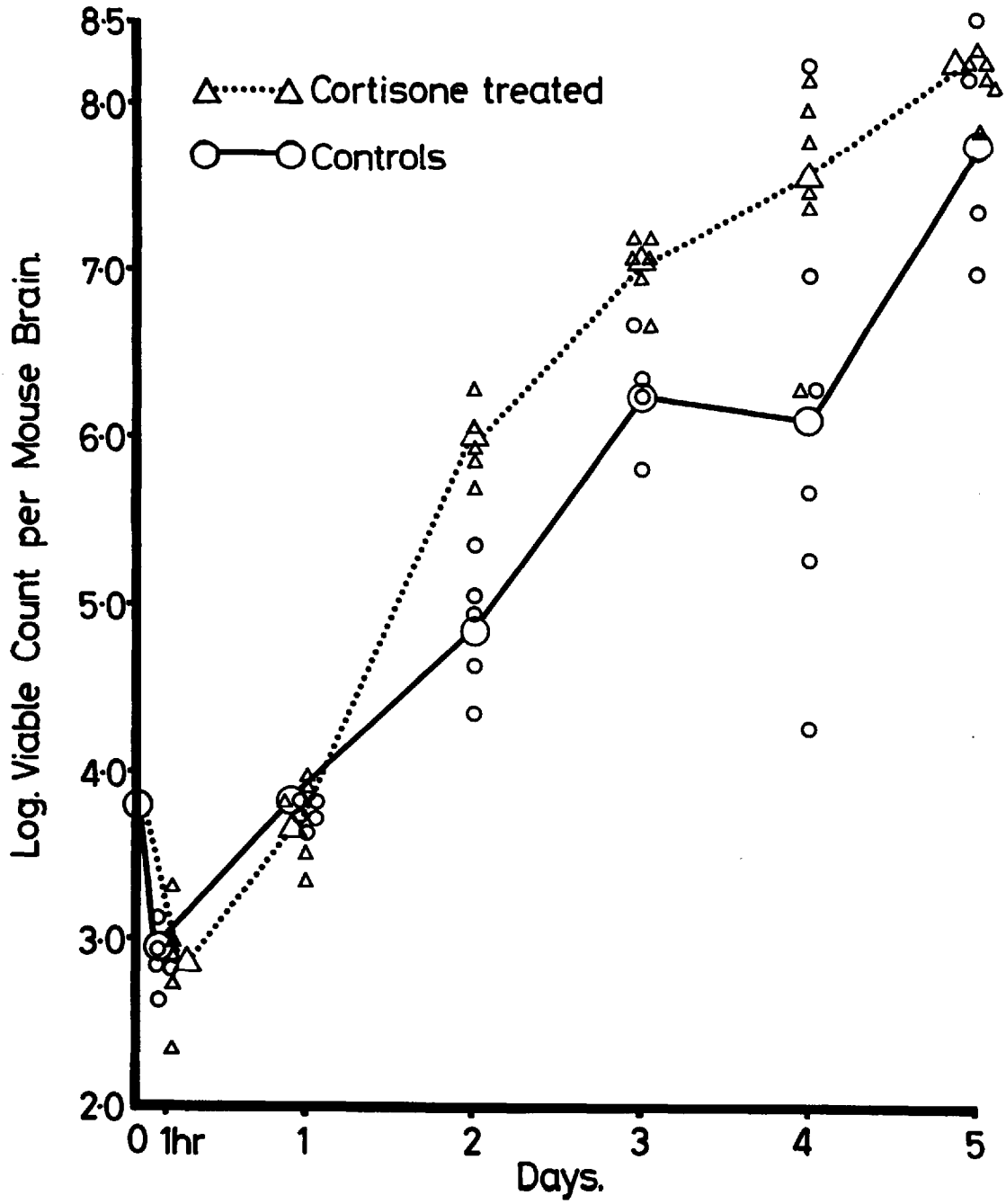

Cortisone treated
Controls
Log. Viable Count per Mouse Brain.
8·5
8·0
7·0
6·0
5·0
4·0
3·0
2·0
0
1hr
1
2
3
4
5
Days.

TABLE 9. The influence of cortisone acetate injected subcutaneously on the number of survivors, average day of death and terminal viable counts in mice challenged with high or low virulence strains of B. pertussis.

Strain of B. pertussis	B2288	B2288	B2288	B1772	B1772	B1772	18/323	18/323	G353	G353	Controls
Number of viable organisms injected	10^4	10^5	10^5	$9x10^3$	$9x10^4$	$9x10^4$	$7.6x10^3$	$7.6x10^3$	$5.7x10^3$	$5.7x10^3$	Nil
Number of mice injected with cortisone acetate 2 hours prior and 1 and 2 days after challenge	7	7	-	8	8	-	8	-	12	-	4
Number of mice receiving no cortisone	-	-	8	-	-	8	-	9	-	12	-
S/T	0/7	1/7	8/8	1/8	0/8	8/8	0/8	0/9	0/12	0/12	4/4
Average day of death	6.6	6.0	>14	6.3	5.6	>14	4.9	6.3	5.5	7.0	>14
Typical viable counts in dying mice	$2.4x10^7$ $2.2x10^7$ $9.5x10^6$	$3.67x10^7$ $5.77x10^7$ $8x10^6$	-	$1.6 x10^8$ $3.9x10^7$ $2.83x10^6$	$3.15x10^7$ $4.72x10^7$ $5.25x10^7$	-	$2x10^8$ $1.6x10^8$ $8x10^7$	$2.05x10^7$ $3.2 x10^7$ $7.6x10^7$	$2x10^8$ $1.4x10^8$ $1.9x10^8$	$2x10^7$ $5.3x10^7$ $9.5x10^7$	-

from 10^7 to 10^8 viable organisms.

However, when cortisone treated mice were infected with high virulence strains the average time to death was reduced by 1.4 and 1.5 days for 18/323 and G353 respectively. With G353 the almost stationary phase in multiplication between days 3 and 4 was not evident in cortisone treated mice. The terminal viable counts in cortisone treated mice infected with high virulence strains often exceeded 10^8 organisms compared with the range 10^7 to 10^8 in normal mice.

6.2. Intracerebral infection with Bordetella pertussis in vitamin A and streptolysin-O treated mice

Lucy et al. (1961) and Dingle (1961) showed that excess vitamin A caused the degradation of cartilage matrix in culture by liberating proteases from the lysosomes of the cells. Later Becker and Sutton (1963) and Sutton (1965) showed that excess vitamin A caused an increase in the number and polarisation of lysosomes in the choroidal epithelial cells of the rat.

Streptolysin-O has also been reported to be a lysosome labiliser (Weissman et al.,1963).

6.2.1. Experimental

The effect of vitamin A injected either intracerebrally or intraperitoneally was investigated. 10^5 international

units (I.U.) of vitamin A acetate (BDH) was injected intraperitoneally 2 hours before and 1 and 2 days after challenge with 9.4×10^4 viable B. pertussis B1772.

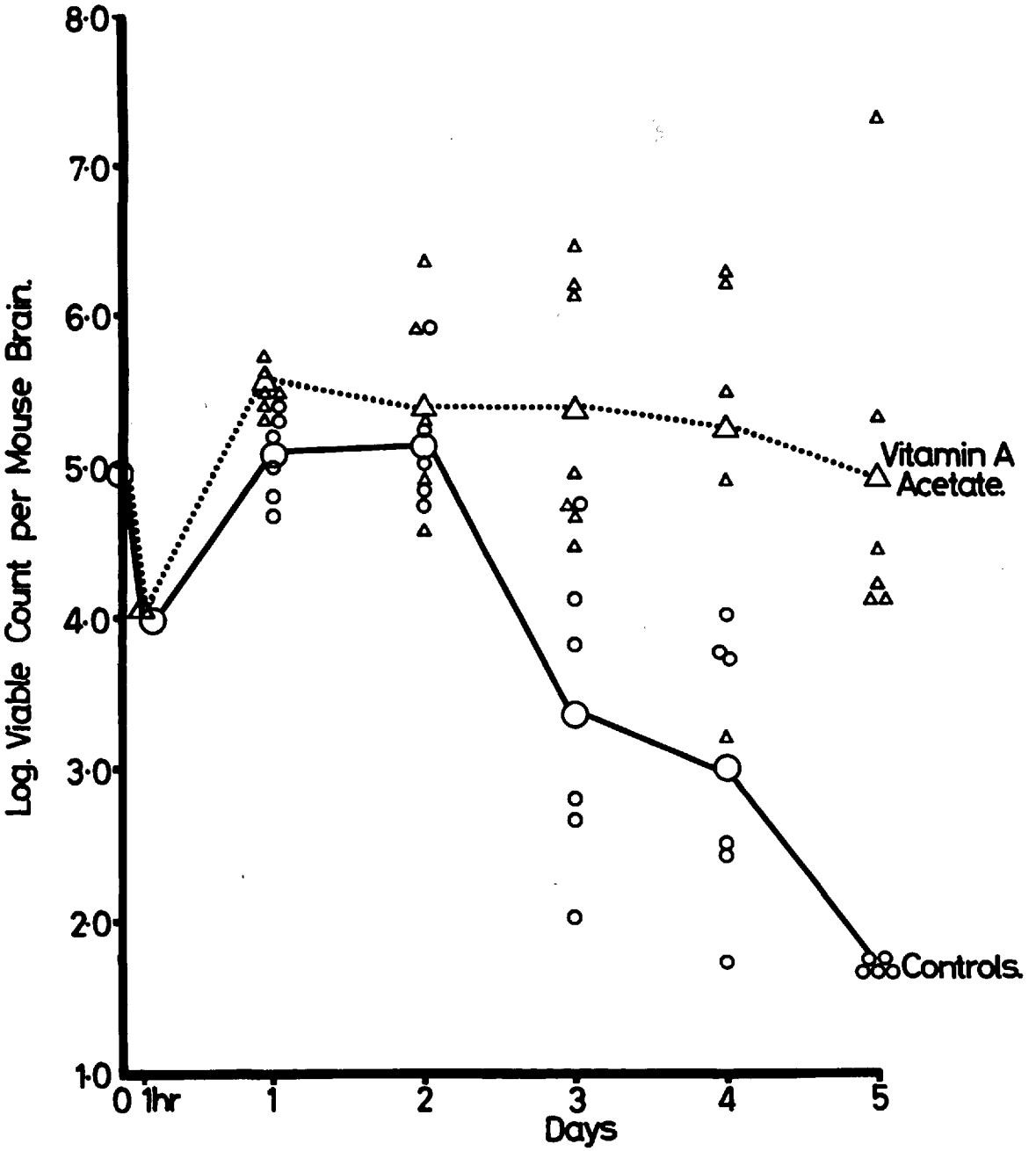
In a second part of this experiment 100 mgm. of vitamin A alcohol (Koch Light Labs.) containing 3.3×10^6 I.U. per g. were dissolved in 4 ml. of almond oil and 0.025 ml. of this solution equivalent to 2000 I.U. was injected intracerebrally into mice 2 hours before challenge with 2.1×10^4 B. pertussis B1772.

Lastly, streptolysin-O, unreduced, dried (B.W. and Co.), diluted in phosphate buffer saline to contain 690 I.U. per ml., was activated with an equal amount of thioglycollate solution and further diluted with phosphate buffer saline to contain 1 I.U. in 0.025 ml. This latter amount was injected intracerebrally 2 hours before and 1 day after challenge with 2.1×10^4 viable B. pertussis B1772.

6.2.2. Results

If labilisation of lysosomal enzymes played an important role in B. pertussis infection, it would be expected that pretreatment with vitamin A would reduce the level of infection. This was not found, there was little difference in the geometric mean counts at 1 hour, 1 and 2 days (Fig. 20). From the second day onwards, when the control infection declined, the infection level in the treated mice remained approximately the

Figure 20. Intracerebral infection with B. pertussis B1772 in mice injected intraperitoneally with 10^5 international units of vitamin A acetate 2 hours before and 1 and 2 days after challenge.


8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
5
Days
Vitamin A Acetate.
Controls.

same, the geometric mean viable count at the fifth day being 9.4×10^4 organisms, the same as the inoculum, and the brains of all the control mice contained less than 50 organisms at this time.

Fell et al. (1962) showed that the free alcohol was a much more potent labiliser of lysosomes than the esters. In our experiments after an inoculum of 2.1×10^4 organisms the geometric mean count in the vitamin A alcohol treated mice was approximately 10^6 viable bacteria at the fourth day, which was almost 10^4 times greater than the count in the control mice (Fig. 21).

A significant rise in the infection in mice treated with streptolysin-O was observed in comparison to the control mice (Fig. 22). The viable counts in the brains of 15 of 18 treated mice were greater than viable counts in the control mice from the second days onwards. In another series of experiments with mice treated with the same dose of streptolysin-O the geometric mean counts at the second day were at least 10 times greater than the counts in the control mice: the figures for streptolysin-O treated and control mice were for B1772 - 4.73 and 3.56, B124 - 4.67 and 3.61, and B2288 - 4.10 and 3.09.

6.3. Lysosomal enzymes in normal and B. pertussis infected mouse brains

Four lysosomal enzymes acid phosphatase, N-acetyl-β-D-

Figure 21. Intracerebral infection with B. pertussis B1772 in normal mice and in mice injected intracerebrally with 2000 I.U. of vitamin A alcohol 2 hours before challenge.

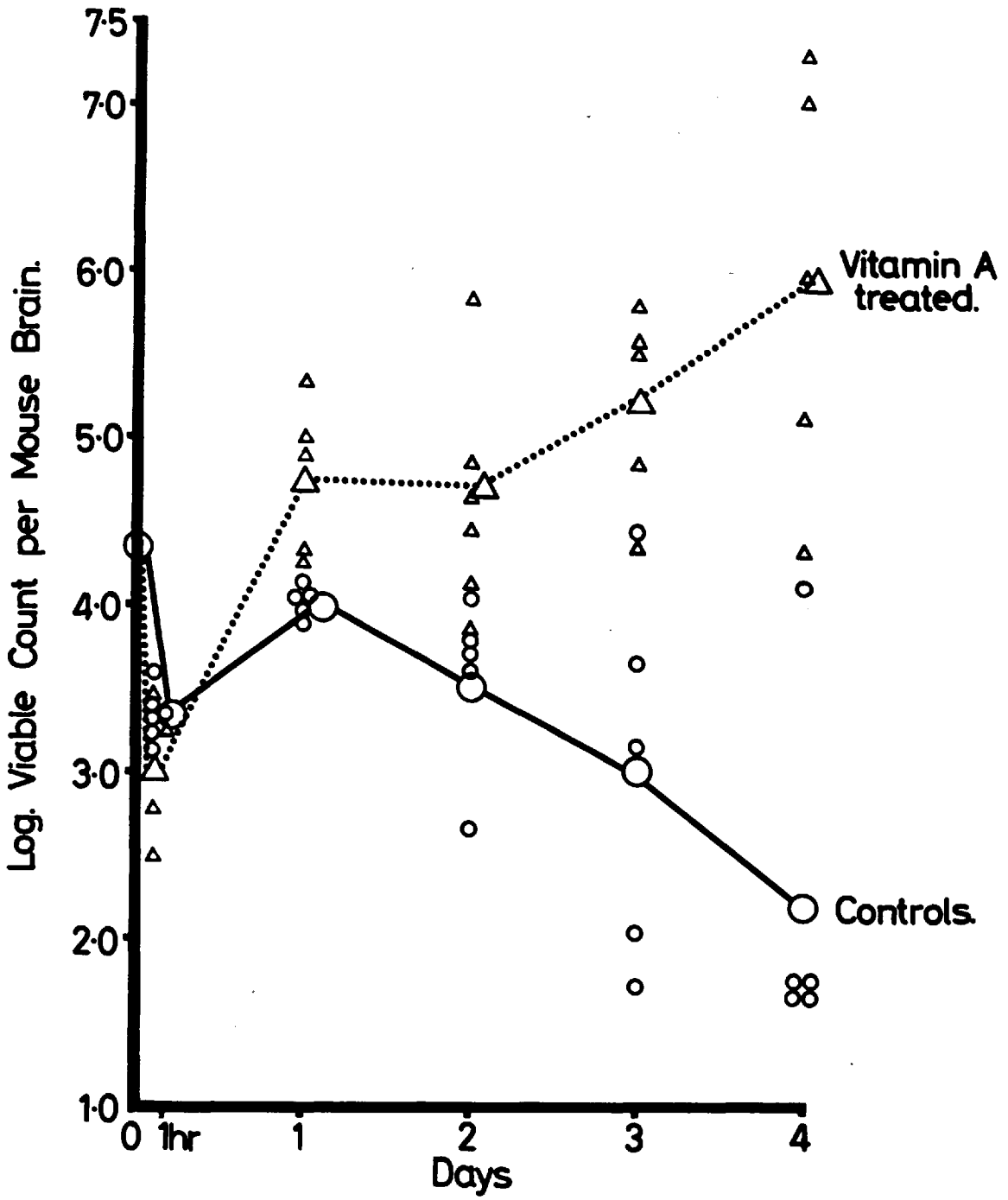
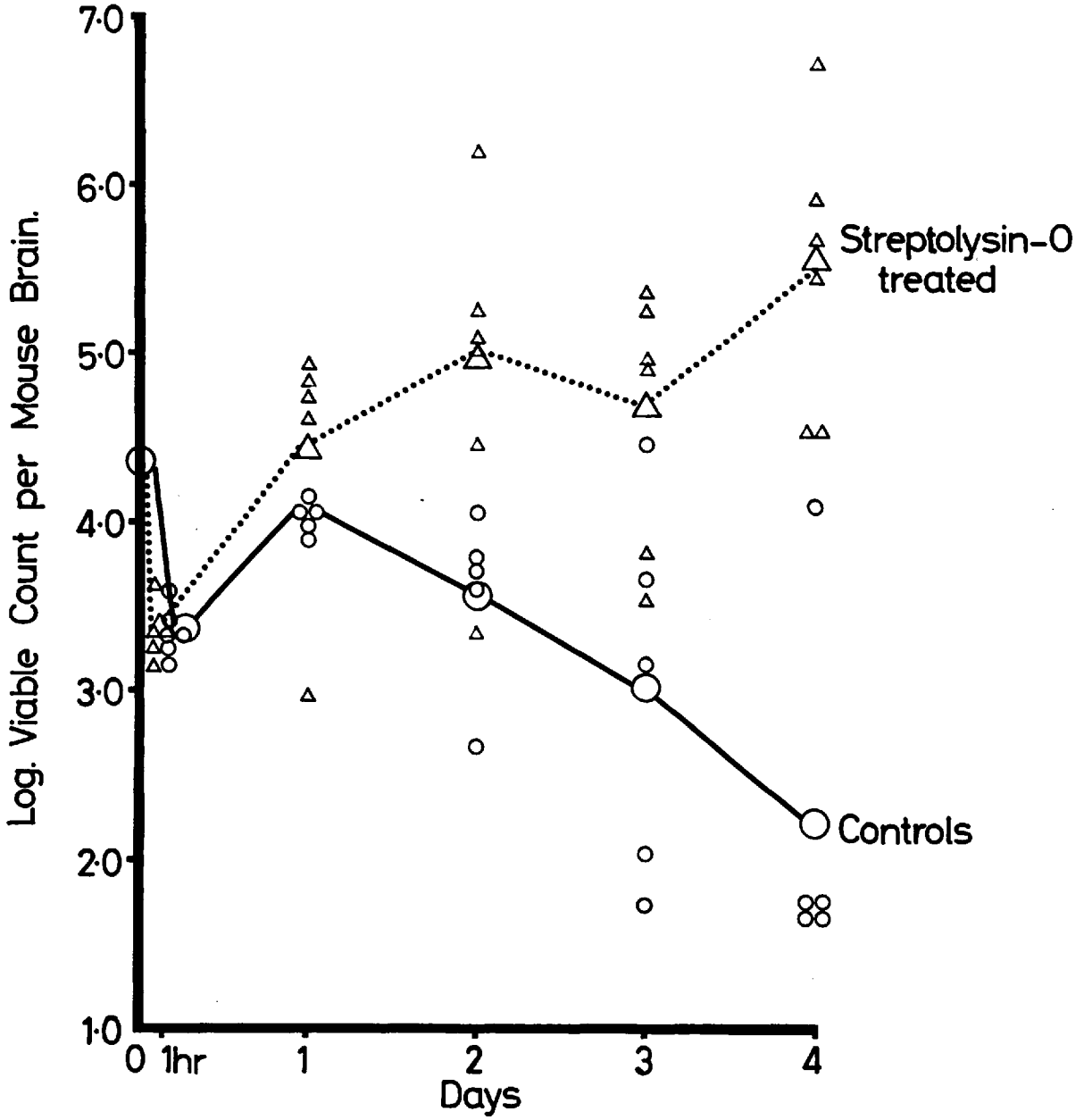

7·5
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
Days
Vitamin A treated.
Controls.

Figure 22. Intracerebral infection with B. pertussis B1772 in normal mice and in mice injected intracerebrally with 1 international unit of streptolysin-O 2 hours before and 1 day after challenge.


7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
Days
Streptolysin-O treated
Controls

glucosaminidase, B-galactosidase and B-glucuronidase, have been studied in an attempt to find out whether any of these enzymes play a role in B. pertussis infections. It has been suggested earlier that cortisone may enhance the infection of a low virulence strain by preventing the release of lysosomal enzymes from the choroid plexus and ependyma into the ventricles. It was also postulated that if this was so, pretreatment of the mice with certain substances such as vitamin A and streptolysin-O, which have been shown to be lysosomal labilisers, might result in a much reduced infection.

Although cortisone enhanced infections (Section 6.1), it was found that vitamin A and streptolysin-O (Section 6.2) also enhanced infections and did not reduce them as expected.

In view of these results, it was decided to examine the free and total amounts of these enzymes in the mouse brain during infections with high and low virulence strains and after treatment with cortisone, vitamin A and streptolysin O.

The results of the enzyme measurements at days 1, 2 and 7 are shown in Tables 10, 11 and 12. Each figure is the mean of enzyme measurements in six mouse brains. The free enzyme measurements were made on the cytoplasmic extract. One of the major difficulties with this preparation was obtaining an equal pressure for all homogenisations and this is emphasised by examination of the amount of the free enzymes in the control preparations, there being at least a 40% difference between the

lowest and highest control reading for all four enzymes.

At either 1, 2 or 7 days there was never greater than a 40% increase in free or total enzymes in the infected mice, in comparison to the control mice. Although my results for amounts of free B-galactosidase and N-acetyl-B-D-glucosaminidase in normal mice were in close agreement with those of Millson (1965) and Hunter and Millson (1965), these workers recorded a 3 times increase in the amount of free B-glucuronidase and a $2\frac{1}{2}$ times increase in free N-acetyl-B-D-glucosaminidase in scrapie agent infected mouse brains. Thus mice seem capable of producing large amounts of free enzyme in some circumstances and it seems that release of lysosomal enzymes is of little importance in B. pertussis infections.

The effect of cortisone, vitamin A and streptolysin-O on the amount of free enzymes was also examined. There was little difference between the results of the various treatments in either of the enzymes (Table 13). Treatment with cortisone did not deplete the amount of free enzyme and treatment with vitamin A and streptolysin-O did not release a measureable increase in amounts of enzymes. Released enzymes may have been rapidly washed away with the flow of cerebrospinal fluid, but this is thought to be unlikely.

TABLE 10. Amount of Free and Total Lysosomal Enzymes detected one day after challenge with various strains.

Strain \ Enzyme	Acid Phosphatase		N-Acetyl β-D-Glucosaminidase		β-Galactoside		β-Glucuronidase	
	Free	Total	Free	Total	Free	Total	Free	Total
B 1772	37,670	83,200	11,300	54,470	1235	1787	70	117
Controls	30,300	78,000	9,520	48,800	1195	1780	84	112
B 1772	38,530	90,000	12,270	43,900	1510	2516	53	80
Controls	29,040	69,000	NT	35,400	947	1960	46	83
B 124	35,250	115,600	11,800	57,300	1377	2750	38	80
Controls	34,100	92,800	11,030	64,500	1452	2680	52	118
B 2288	29,870	NT	18,915	NT	NT	NT	NT	NT
Controls	27,000	NT	14,950	NT	NT	NT	NT	NT
B 124 in cortisone treated mice	36,400	110,000	12,530	56,730	1590	3350	81	105
Cortisone treated controls	26,830	85,600	9,630	50,800	1530	2750	67	108
18/323	28,430	67,670	9,100	42,430	1295	2175	70	74
Controls	29,030	69,600	NT	39,800	980	1960	47	84

Acid Phosphatase, N-Acetyl-β-D-Glucosaminidase — µgp-Nitrophenol released/hour/G wet weight brain.
β-Galactosidase — µgo-Nitrophenol released/hour/G wet weight brain.
β-Glucuronidase — O.D. units released/hour/G

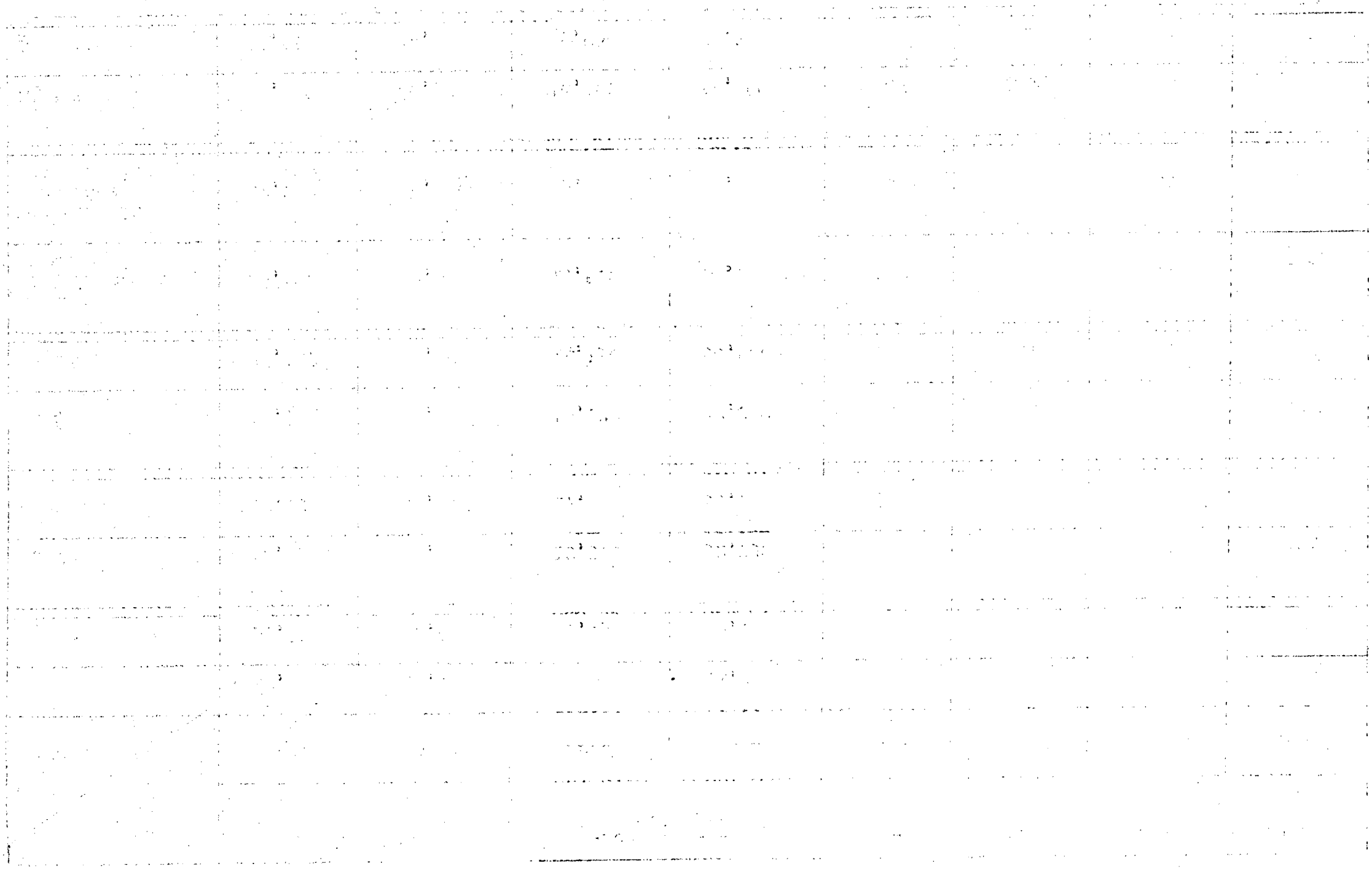


TABLE 11. Amount of Free and Total Lysosomal Enzymes detected 2 days after challenge with various strains.

Enzyme / Strain	Acid Phosphatase		N-Acetyl β-D-Glucosaminidase		β-Galactosidase		β-Glucuronidase	
	Free	Total	Free	Total	Free	Total	Free	Total
B1772	29,200	86,200	10,000	51,760	NT	NT	NT	NT
Controls	37,370	92,000	9,250	46,470	NT	NT	NT	NT
B1772	44,300	63,300	18,300	52,830	1300	2010	62	104
Controls	37,730	60,870	17,000	55,700	1500	3100	68	89
B124	41,700	94,400	16,630	62,800	NT	NT	60	86
Controls	38,100	93,600	14,130	56,500	NT	NT	70	82
B124 in cortisone treated mice	28,600	87,300	13,800	68,000	NT	NT	79	105
Controls in cortisone treated mice	37,000	89,200	15,200	59,460	NT	NT	65	83
18/323	39,530	66,530	16,500	59,000	1367	2260	60	88
Controls	37,830	64,000	15,960	56,200	1500	3112	67	88

Acid Phosphatase)
N-Acetyl-β-D-Glucosaminidase) µgp-Nitrophenol released/hour/G wet weight brain.

β-Galactosidase µgo-Nitrophenol released/hour/G wet weight brain.
β-Glucuronidase

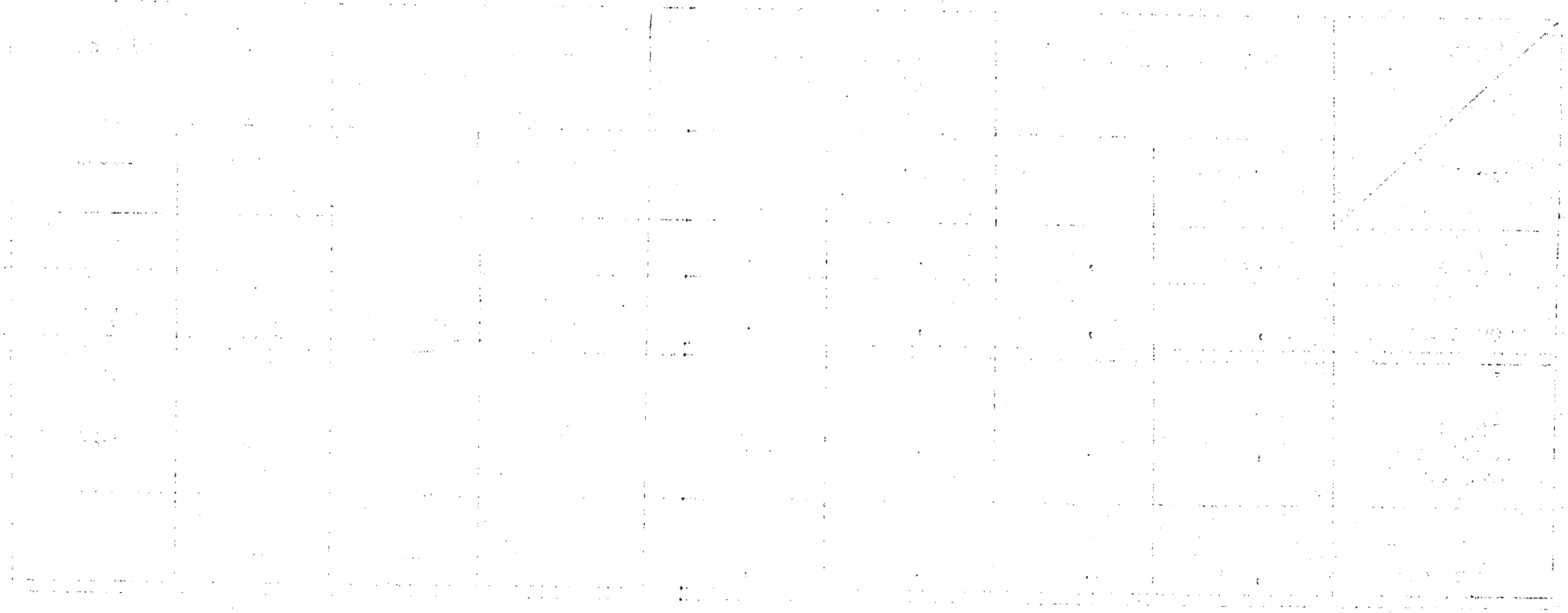


TABLE 12. Amount of Free and Total Lysosomal Enzymes detected 7 days after challenge with various strains.

Enzyme / Strain	Acid Phosphatase		N-Acetyl β-D-Glucosaminidase		β-Galactoside		β-Glucuronidase	
	Free	Total	Free	Total	Free	Total	Free	Total
18/323	28,500	90,200	13,400	66,900	1580	2280	53	147
Controls	30,900	91,000	9,600	52,700	1600	2530	55	110
B124 in cortisone treated mice	30,000	93,500	10,750	59,850	NT	NT	73	143
Controls	30,100	86,000	11,310	45,200	NT	NT	76	79

Acid Phosphatase)
N-Acetyl-β-D-Glucosaminidase) μgp-Nitrophenol released/hour/G wet weight brain.

β-Galactosidase μgo-Nitrophenol released/hour/G wet weight brain.

β-Glucuronidase O.D. units released/hour/G wet weight brain.

TABLE 13. Amount of Free Lysosomal Enzymes 2 hours after treatment with various substances.

Enzyme / Treatment	Acid Phosphatase	N-Acetyl-β-D Glucosaminidase
Cortisone	26,830	15,200
Vitamin A	24,800	14,150
Streptolysin-O	19,000	12,000
Controls	27,000	14,950

Acid Phosphatase
N-acetyl-β-D-glucosaminidase } μg p-nitrophenol/released/hour/G wet weight brain.

7. Bordetella pertussis infections in X-irradiated mice

The X-irradiation experiments in this section and histological work in Section 10 has been done in conjunction with J.W. Hopewell of the Department of Experimental Pathology, St. Mary's Hospital.

In the previous sections intracerebral infections with Bordetella pertussis have been examined under various conditions. It has been established that no differences in the counts could be detected in the first few hours after challenge with either high or low virulence strains. Small doses of low virulence strains multiplied in the mouse brain for 24 to 48 hours before rapidly disappearing, most brains containing less than 50 organisms by the fourth day whereas small doses of high virulence strains multiplied continuously in the brain until the mice died with viable counts of approximately 5×10^7 organisms.

Secondly, it was found that mice previously infected with a low virulence strain were more resistant to a second infection with the same strain but not to a second infection with a high virulence strain.

No relation could be demonstrated between virulence and complement sensitivity either in vivo or in vitro or sensitivity

to lysosomal enzymes *in vivo*, but cortisone injected subcutaneously 2 hours before challenge greatly enhanced intracerebral infections with both high and low virulence strains. Various other substances, especially vitamin A and streptolysin O, enhanced infections with low virulence strains.

Berenbaum *et al.* (1960) and Iida *et al.* (1962) reported that no marked leucocytic infiltration into the ventricles occurred until the third day of infection or at the times when signs of illness were visible with the high virulence strain 18/323. It was interesting to note that in their experiments the bacterial counts were approximately 7.5×10^6 viable organisms at the third day and that visible signs of illness are not apparent until the count has reached 10^6 organisms. These counts are higher than those reported by Berenbaum *et al.* (1960) and Holt *et al.* (1961) as being necessary to cause a breakdown of the blood brain barrier in mice. These workers have shown that radio-iodinated serum (RIS) and Pontamine Sky Blue (PSB) did not enter the brain in large amounts until a count of $10^5 - 10^6$ was reached. It was thought that leucocytic infiltration into infected brains would be minimal until severe damage to the cerebral blood vessels had occurred at the times when the count had exceeded $10^5 - 10^6$ organisms.

It has been found that after injection of even moderately large doses of *B. pertussis* B2288, a low virulence strain, no breakdown of the blood brain barrier was noted when using PSB as a marker for damage. It has also been noted that in cortisone-

treated mice the blood brain barrier was damaged when the mice were infected with low virulence strains.

Iida et al. (1966) found no decrease in the number of leucocytes in the ventricles of immune mice treated with cortisone and infected with B. pertussis 18/323.

In the light of this evidence, it was thought unlikely that white blood cells played the most important role in determining the course of high and low virulence infections.

This hypothesis was tested by irradiating mice with small doses of X-rays. Little enhancement of the infection was found in mice irradiated only to the body, but when either the heads alone or the heads and bodies were irradiated a very marked enhancement of infection with low virulence strains was detected.

7.1. Comparison of body and whole mouse irradiation on infections with low virulence strains

Preliminary experiments indicated that female T.O. mice were resistant to radiation doses of up to 400 Roentgens (R) given to the body, no deaths occurring in mice observed for 12 days after irradiation.

This dose was selected for irradiation to heads and bodies or bodies alone, but a dose of 1000 R could be used for head irradiation without any evidence of gut damage for at least three months. The bodies were adequately shielded in these

experiments and white cell determinations indicated that radiation scatter was negligible.

7.1.1. Experimental

Two groups of 30 mice were irradiated with a single dose of 400 R. One group received the radiation to the body only, the heads being shielded by 2.5 mm. lead, and the second group received radiation to the head and body. These mice and a control group were challenged three days later with 3.2×10^4 viable B. pertussis B1772, a low virulence strain. A three-day interval was chosen to allow the mice time to recover from the stress of the irradiation procedure. The W.B.C. count was also determined in blood taken from the brachial artery.

7.1.2. Results

A marked difference was observed between the effects on infection of irradiating either the body alone or the head and body of mice (Fig. 23). The white blood cell (WBC) count is also recorded in Table 14.

As in many of the previous experiments where the infections have been modified by pretreatment of the mice with various substances, no differences in the viable counts in irradiated and normal mice could be found at 1 hour and 1 day after challenge. However, a small effect was seen at the

second day, the count in mice having had head and body irradiation being 15 times greater than that in the controls and the count in the group given only body irradiation was 5 times greater than the control count.

By the third day the count in the mice having had head and body irradiation was greater than the count in the other two groups by a factor of 100 and by the fourth day the difference had increased to 1000.

From the second day onwards the counts in the individual mice of the head and body irradiated group were generally greater than those of the control group or body only irradiated group.

Reference to Table 14 shows that irradiation had drastically reduced the number of circulating white blood cells. Although the number of cells in the mice having had head and body irradiation was about half that in the mice having had body only irradiation, it was thought unlikely that this difference resulted in enhancement of infection in the first group.

It seems that enhancement of infection of low virulence strains occurred only when the head as well as the body was irradiated and further experiments were carried out to examine various aspects of this idea.

7.2. B. pertussis B1772 infections in head irradiated Mice

Hopewell and Wright (1967) showed that irradiated rat

Figure 23. Intracerebral infection with B. pertussis B1772 in normal mice, mice irradiated with 400 R to the body only (head protected) and in whole mice irradiated with 400 R 3 days before challenge.

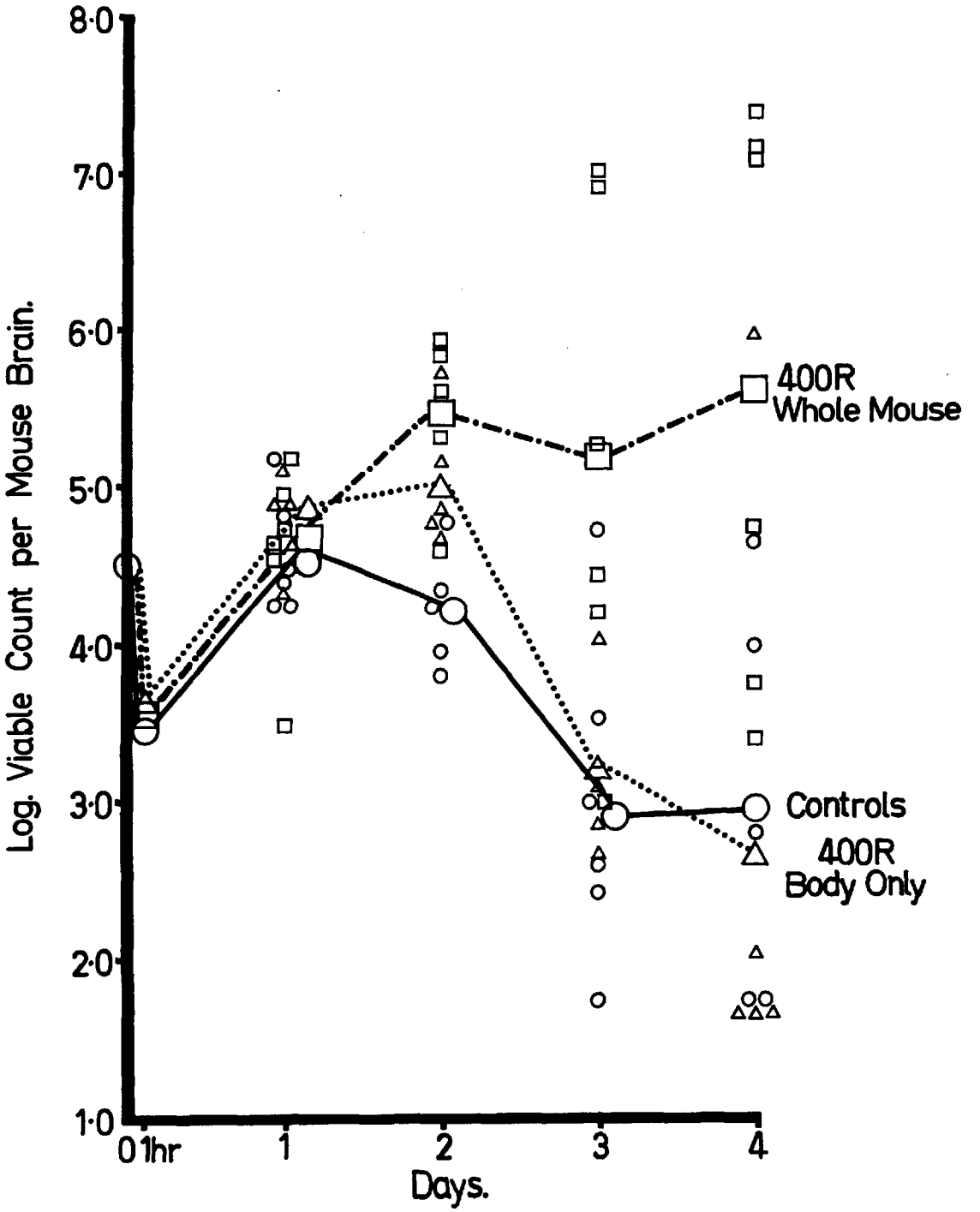

8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0 1hr
1
2
3
4
Days.
400R
Whole Mouse
Controls
400R
Body Only

TABLE 14. W.B.C./cu.mm.* in the peripheral blood of Normal and Irradiated B. pertussis B 1772 Infected Mice.

Group	Days after challenge				
	0	1	2	3	4
Controls	3950	4700	4266	5608	5950
400R whole mice	280	210	290	190	350
400R bodies only	310	470	580	630	710

Mice were challenged 3 days after irradiation.

*Average count for 6 mice.

R = Roentgen.

brains were more susceptible to injury than normal brains for at least six months after irradiation. These workers suggested that glial cells stored up radiation damage until stimulated to divide when they either failed to divide or died.

If the control mechanism of B. pertussis infections in the mouse brain is dependent on division of and phagocytosis by glial cells and if radiation damage is "stored up", then irradiation three months before challenge should also greatly enhance infection.

7.2.1. Experimental

Mice were irradiated with 400 R or 1000 R to the heads only 3 days, 3 weeks or 12 weeks before challenge with B. pertussis B1772. The bodies were adequately shielded with 2.5 mm. of lead. In the mice challenged 3 days after irradiation the W.B.C. count was determined in blood taken from the brachial artery.

7.2.2. Results

When heads of mice were irradiated with 400 R and 1000 R and challenged with 3×10^5 viable B. pertussis B1772, the viable counts were very closely grouped for 24 hours after challenge (Fig. 24). However, from the second day onwards, the bacterial counts in the irradiated mice generally exceeded the counts in the control mice. By the fourth day the viable counts in all irradiated mice were greater than those in the

control mice.

In a comparable experiment mice whose brains had been irradiated with 400 R and 1000 R were challenged three days later with 2.4×10^5 viable B. pertussis B1772. Deaths of mice were recorded daily (Table 15) and it can be seen that of 12 mice receiving 1000 R to the heads 10 (83%) died of infection and of 12 mice receiving 400 R to the heads, 7 (58%) died of infection, whereas only $^1/_{12}$ (8%) of the control mice died. In a later experiment $^{11}/_{12}$ (91%) mice died of infection after irradiation with 1000 R and challenge with 3×10^5 B. pertussis B1772.

Examination of W.B.C. counts (Table 16) in 1000 R irradiated mice showed a small drop in total number for 1 or 2 days after infection, but after this time there was little difference between the count in the control or irradiated infected groups of mice.

When mice were irradiated 3 weeks before challenge with 8.4×10^4 viable B. pertussis B1772 the same general picture was obtained as described for mice irradiated 3 days before challenge (Fig. 25). The G.M. counts were closely grouped at 1 hour and 1 day but from the second day onwards, when the control infection was declining, the bacteria in the irradiated mice were multiplying rapidly. By the fourth day the G.M. count in the 1000 R and 400 R irradiated mice had reached 4×10^6 and 3×10^5 organisms respectively, compared with

Figure 24. Intracerebral infection with B. pertussis B1772 in normal mice and in mice irradiated with 400 R or 1000 R to the head only (the body shielded) 3 days before challenge.

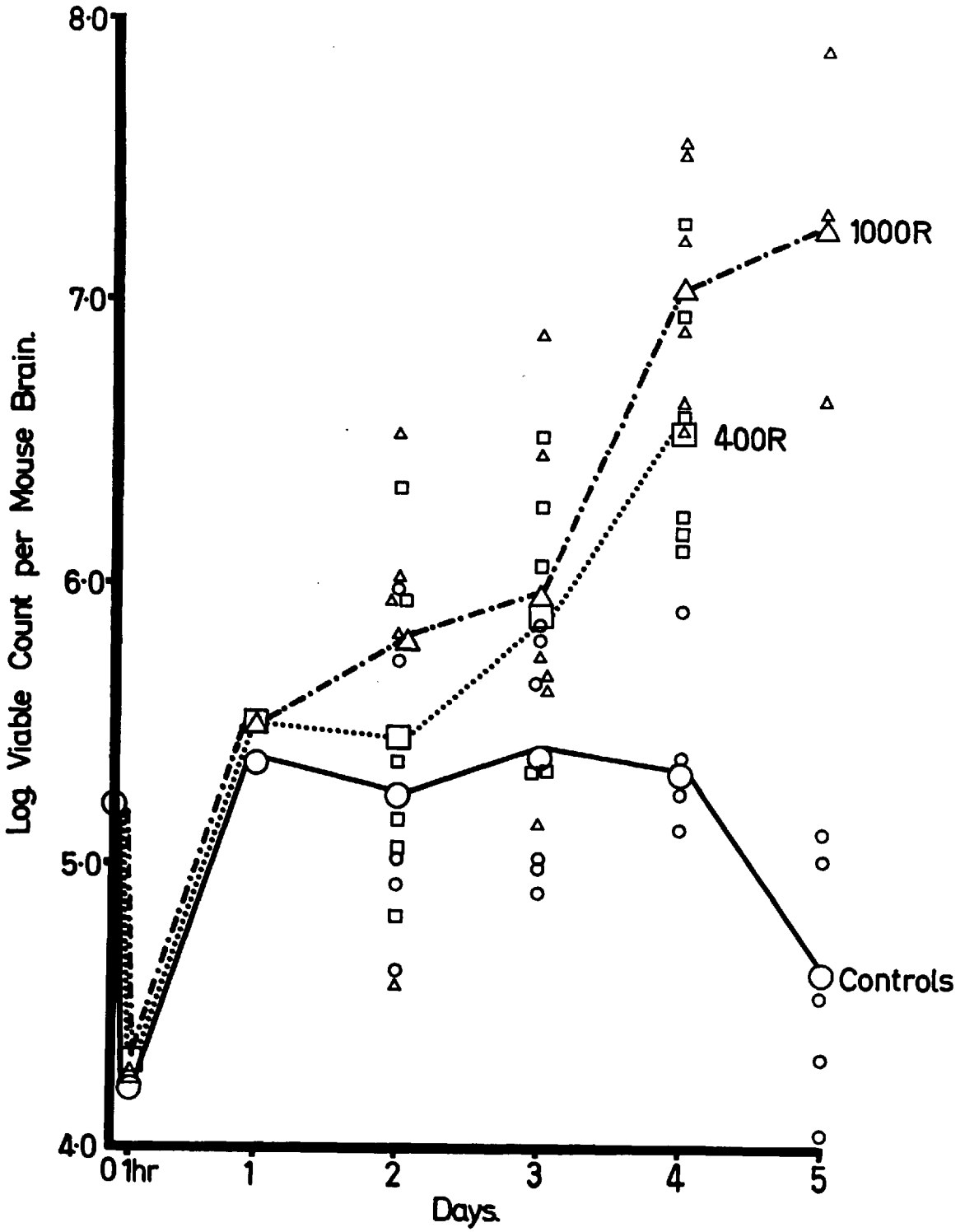

8·0
7·0
6·0
5·0
4·0
Log Viable Count per Mouse Brain.
0 1hr
1
2
3
4
5
Days.
1000R
400R
Controls

TABLE 15. Number of mice dying from B. pertussis B 1772 infection following head irradiation.

Irradiation dose	Challenge inoculum	Day of Death	D/T	%D/T
400 R	2.4×10^5	4,4,5,5,6,8,10	7/12	58%
1000 R	2.4×10^5	4,5,6,7,8,8,8,12,14,14	10/12	83%
1000 R	3×10^5	5,5,6,6,6,6,7,8,9,9,9	11/12	91%
None	2.4×10^5	10	1/12	8%

R = Roentgen.

D/T = $\frac{\text{Number of Dead Mice}}{\text{Number of Mice Challenged.}}$

TABLE 16. White Blood Cells per cu. mm.* in the peripheral blood of normal mice and mice irradiated to the heads only 3 days prior to infection with *B. pertussis* B 1772.

Irradiation Dose	Days after challenge			
	1	2	3	4
Nil	2900	3800	3500	2560
1000 R	1600	2600	4300	2540

*Average of six mice.

R = Roentgen.

Figure 25. Intracerebral infection with B. pertussis B1772 in normal mice and in mice irradiated with 400 R or 1000 R to the head only (the body shielded) 3 weeks before challenge.

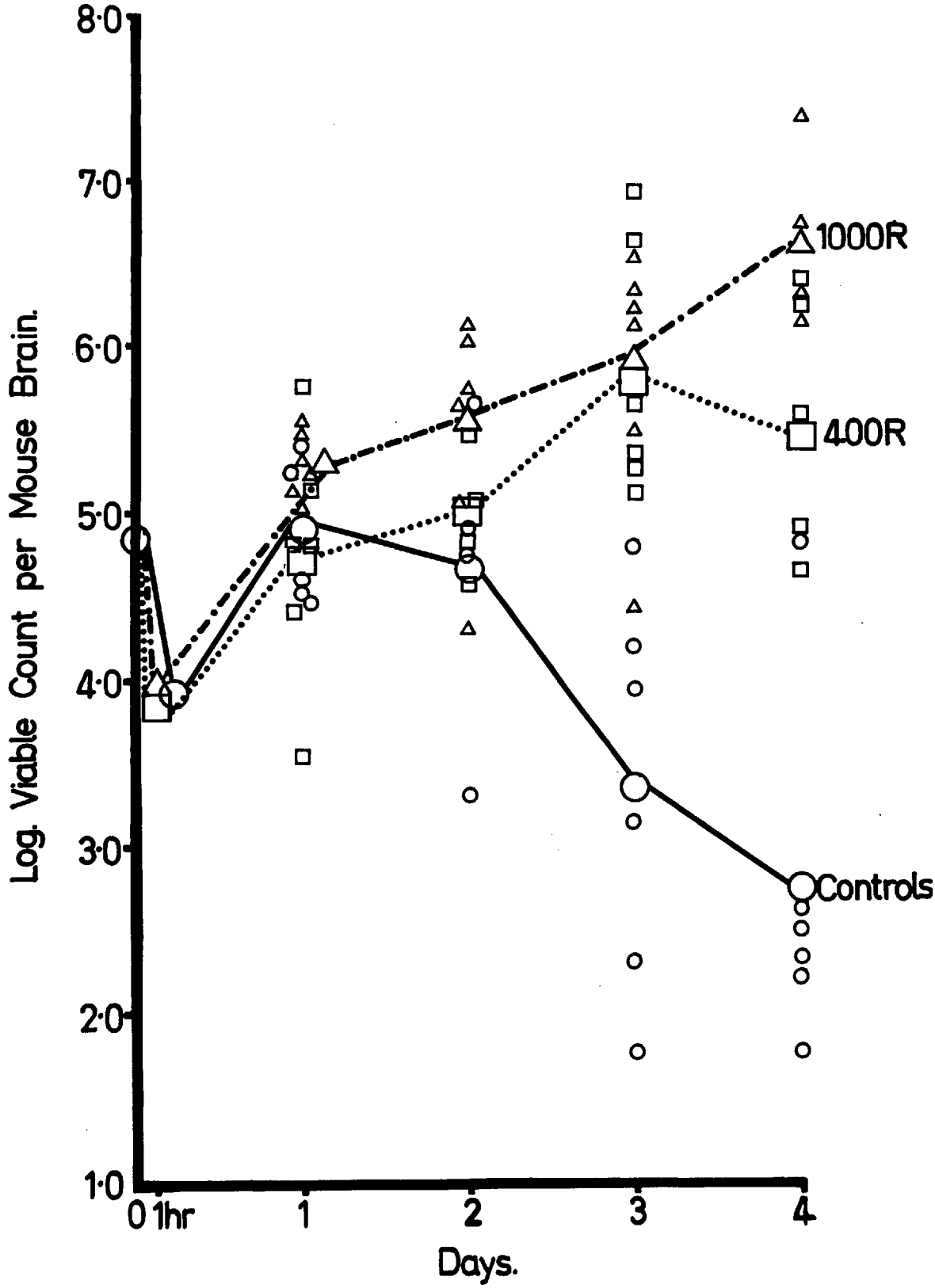
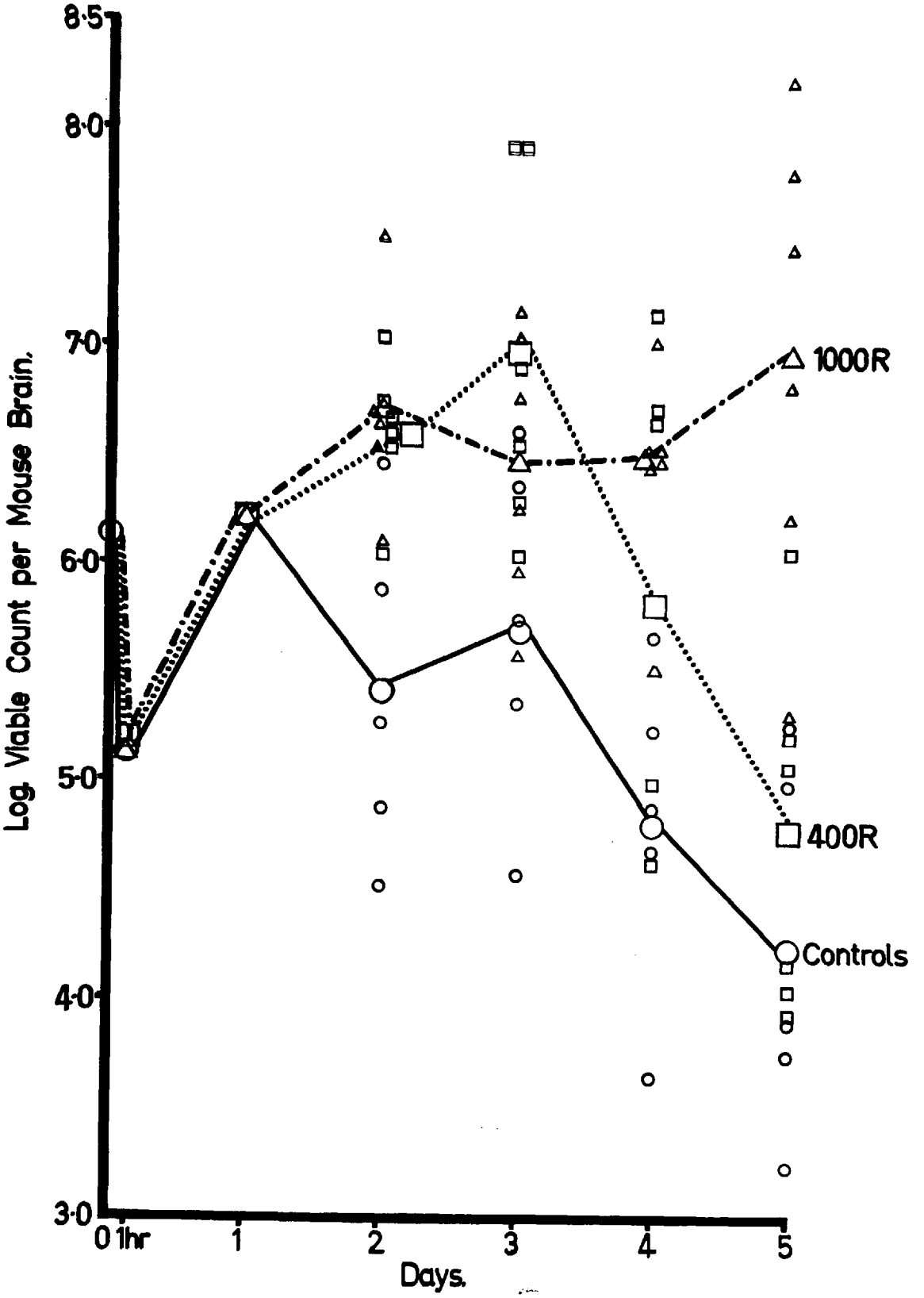

8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
Days.
1000R
400R
Controls

Figure 26. Intracerebral infection with B. pertussis B1772 in normal mice and in mice irradiated with 400 R or 1000 R to the head only (the body shielded) 3 months before challenge.


8·5
8·0
7·0
6·0
5·0
4·0
3·0
Log. Viable Count per Mouse Brain.
0 1hr
1
2
3
4
5
Days.
1000R
400R
Controls

5.5×10^2 organisms in the control mice.

There was a little evidence that at the fourth day the 400 R infected mice were showing signs of recovery from infection.

This observation was confirmed when mice were challenged with 1.3×10^6 B. pertussis 12 weeks after irradiation (Fig. 26).

Whereas from the first day onwards the G.M. counts in the 1000 R irradiated mice increased to reach 9×10^6 viable organisms, at the fourth day the G.M. counts in the 400 R irradiated mice reached a maximum at the second day and then rapidly declined to be little different from the control infection at the fourth day. It seems likely that there may be some recovery from irradiation damage to the brain with low doses of radiation.

7.3. The effects of irradiation on infections with high virulence strains of B. pertussis in immune mice

In the previous section it has been shown that some component of the brain is probably the most important system for the elimination of intracerebral infections with B. pertussis in non-immune mice. It was of interest to examine the fate of immune mice irradiated three days prior to challenge with both high or low virulence strains and to determine whether the brain performed the same role as in normal mice infected with B.pertussis.

7.3.1. Experimental

Mice were irradiated either to the head or body three days prior to challenge with the high virulence strain of B. pertussis 18/323. Two days after challenge certain of these mice were injected intraperitoneally with 0.1 ml. of complement inactivated immune rabbit serum (IRS).

In a further experiment mice were vaccinated intraperitoneally with 2×10^9 heat killed G353 vaccine 14 days before 400 R irradiation to either the head or the body. Mice were challenged intracerebrally three days after irradiation with either 5.6×10^3 or 1.1×10^4 B. pertussis G353, a high virulence strain. The number of survivors was compared with those in the vaccinated unirradiated groups of mice.

7.3.2. Results

In both sets of experiments the number of survivors in the groups of mice which had body irradiation was less than the number surviving either head irradiation or no irradiation (Table 17 and 18). This was in contrast to the results obtained with low virulence strains in irradiated non-immune mice where only prior irradiation of the head enhanced the infections.

Table 17 shows the results obtained with mice passively immunised with previously prepared rabbit antiserum (IRS). The number of survivors in the unirradiated group receiving IRS

was small but it was interesting that the same proportion of mice survived in this group as in the group which had received irradiation to the head and immune serum. This suggested that brain components were of little importance in protection against B. pertussis in the immune mouse. When only the bodies were irradiated, no survivors were recorded in the passively immunised mice. Thus, there were $^{6}/_{15}$ survivors among the head-irradiated mice and no survivors in those irradiated to the body. An χ^2 test comparing the effect of head and body irradiation gave a value of P of 0.022.

In view of the poor number of survivors in the control mice this experiment was repeated with mice that had been vaccinated with 2×10^9 heat killed B. pertussis G353. This dose of vaccine has been shown to protect nearly 100% of mice against subsequent challenge.

Mice were irradiated 14 days after vaccination and challenged 3 days after irradiation. The results (Table 18) confirmed the previous findings that irradiation of the head did not reduce the number of survivors in immune mice whereas irradiation of the body significantly reduced the number of survivors. An χ^2 analysis of the results gave a value for P of 0.026 (between head and body irradiated mice). A survival rate of 92% was found in immune mice challenged after head irradiation against a survival rate of only 55% in the body irradiated group. The difference, 37%, was similar to

the 40% difference found in the passively immunised mouse experiments.

This experiment confirmed the suggestion made earlier that brain components were of little importance in controlling infection in actively or passively immunised mice.

One of the main effects of body irradiation is to reduce the circulating white cells greatly (Section 7.1.) and a radiation dose of 400 R greatly reduced the number of circulating white cells but never reduced the number to zero, and, in fact, by the fourth day after irradiation the number was beginning to increase.

These observations indicated an important role for the leucocytes in immunity against intracerebral B. pertussis infections.

To obtain a larger reduction in the number of circulating leucocytes and thus a larger number of deaths from infection in the immune mouse would mean increasing the radiation dose and this would result in the death of some mice from radiation. By the fifth to sixth days when the first mice in the control groups were dying of infection it was 8 to 9 days after irradiation and the leucocyte count would have been increasing rapidly. Hence the small but significant difference in the survival rates of head or body irradiated immune mice.

TABLE 17. Number of Passively Immunised Mice surviving challenge after head or body irradiation.

Radiation Dose	Challenge Strain	Volume of I.R.S. injected	S/T
None	18/323	0.1 ml.	4/14
None	18/323	None	0/15
400 R heads	18/323	0.1 ml.	6/15
400 R heads	18/323	None	0/14
400 R bodies	18/323	0.1 ml.	0/15
400 R bodies	18/323	None	0/14

R = Roentgen

I.R.S. = Immune Rabbit Serum

S/T = $\frac{\text{Number of mice surviving challenge}}{\text{Number of mice challenged}}$

TABLE 18. Number of actively immunised mice surviving challenge after head or body irradiation.

Radiation Dose	Dose of Challenge Strain G 353	Amount of Vaccine	S/T	Total S/T
None	5.6×10^3	2×10^9	12/12	23/23
None	1.1×10^4	"	11/11	
400 R heads	5.6×10^3	"	11/11	20/22
400 R heads	1.1×10^4	"	9/11	
400 R bodies	5.6×10^3	"	5/10	11/20
400 R bodies	1.1×10^4	"	6/10	
None	5.6×10^3	None	0/12	0/12

R = Roentgen.

7.3.3. Experimental

In a confirmatory experiment, two groups of 11 and 12 mice were vaccinated intraperitoneally with 2 x 10^9 heat killed B. pertussis G353. Fourteen days later, these mice and two groups of 12 control mice were irradiated with 1000 R to the heads only. Three days after irradiation the mice were challenged with either 3 x 10^5 or 2.4 x 10^5 B. pertussis B1772, a low virulence strain.

7.3.4. Results

As shown in Table 19, prior irradiation with 1000 R to the heads only resulted in a challenge dose of 2.4 x 10^5 viable B1772 or 3 x 10^5 viable B1772 killing 10 of 12 and 11 of 12 mice respectively, compared with only one death with this dose in normal mice.

However, this was in sharp contrast to the effects of head irradiation when the mice had been previously immunised. In both experiments the number of deaths was significantly reduced, only 2 of 12 and 0 of 11 mice dying of infection, although the mice had been previously head irradiated. An χ^2 test on the immune and non-immune head irradiated mice gave values of P 0.001 in both experiments.

It was clear that head irradiation had greatly enhanced the infection of the low virulence strain in the non-immune mice as before, but there were few deaths in the immune head

irradiated mice. This was further evidence suggesting that brain cells played little part in protection in immune mice but were very important in deciding the fate of non-immune mice.

TABLE 19. Number of mice surviving challenge with a low virulence strain of B. pertussis after head irradiation and active immunisation intraperitoneally.

Head Irradiation Dose	Dose of Challenge Strain B 1772	Amount of Vaccine	S/T
1000 R	3×10^5	2×10^9 orgs.	10/12
1000 R	"	None	1/12
1000 R	2.4×10^5	2×10^9 orgs.	11/11
1000 R	"	None	2/12
Nil	3×10^5	None	11/12

R = Roentgen

S/T = $\frac{\text{Number of mice surviving challenge}}{\text{Number of mice challenged}}$

8. Experiments with Bordetella pertussis antiserum

8.1. Intracerebral injection of antiserum

The course of infection with high virulence strains of B. pertussis in actively and passively immunised mice has been well documented (Blyth, 1955; Brown, 1958; Berenbaum et al., 1959; Holt et al., 1961; Dolby and Standfast, 1961). All workers were agreed that infection proceeded as normal until the bacterial count reached 10^5 - 10^6 viable organisms. At this count damage to the blood brain barrier was sufficient to allow entry of antibody and from this time the viable count declined, the brain being found sterile from the fifth day onwards.

Spasojevic (1962) demonstrated that the intracerebral injection of a mixture of B. pertussis and specific antiserum incubated at 37°C for 60 minutes resulted in sterilisation of the brain 24 hours after challenge; the organisms never showed a multiplication phase.

Iida et al. (1963, 1966), during investigations on the effect of cortisone on immunity, found that both active and passive immunity were neutralised by subcutaneous injection of cortisone 2 hours before challenge with B. pertussis 18/323. I have examined the effect of cortisone on infection with mixtures of antiserum and organisms and confirmed that the effect

of the antiserum was neutralised, though the mechanisms of action of cortisone need not be identical in both cases.

Eldering et al. (1966) reported that the mouse high virulence strain G353 gave lower values for the 50% protection dose (PD_{50}) of antiserum than did another mouse high virulence strain 18/323. Various high and low virulence strains have been tested against the same antiserum using the method described by Spasojevic.

In passive and active immunity, breakdown of the blood brain barrier is necessary for passage of antiserum into the brain. With mixtures of antiserum and organisms no breakdown of the blood brain barrier was recorded. It was possible that antiserum was the only external factor required for killing and the blood brain barrier was the limiting factor preventing entry. This hypothesis forms the basis of the first experiment described below in which antiserum was injected intracerebrally before breakdown of the blood brain barrier.

8.1.1. Experimental

Groups of mice were challenged intracerebrally with 2×10^3 B. pertussis 18/323, a high virulence strain. Mice were injected intracerebrally with 0.05 ml. of an antipertussis serum at either 1, 2 or 4 days after challenge and a control group was injected with normal saline. The number of survivors was recorded daily.

Secondly mice challenged with the same dose of B. pertussis and given antiserum one day after challenge were sacrificed daily and bacterial counts determined.

8.1.2. Results

A significant protection was obtained by injecting the immune serum intracerebrally immediately after challenge. The amount of serum injected, 0.05 ml., was equivalent to 0.75 PD_{50} calculated by intraperitoneal/intracerebral method.

All control mice died of infection, the average death being 7.5 days, while $^{6}/_{10}$, $^{7}/_{10}$ and $^{2}/_{9}$ mice survived after injection of antiserum at either the first, second or fourth days, the average time to death being extended in the dying mice to 10.6, 9.6 and 9.0 days respectively (Table 20).

Examination of the viable counts (Fig. 27) showed that intracerebral injection of antiserum on the first day of infection caused an immediate decline in the number of organisms. Four of ten brains were sterile one day after injection of antiserum and the proportion remained fairly constant throughout. The increase in time to death in serum treated animals was confirmed by the lag in increase of viable count between days 1 and 2. The bacterial counts in these mice lagged behind the control mice for all five days of the experiment. It seemed that damage to the blood brain barrier was not a requirement for elimination of organisms if antiserum can reach the infected site before such a time.

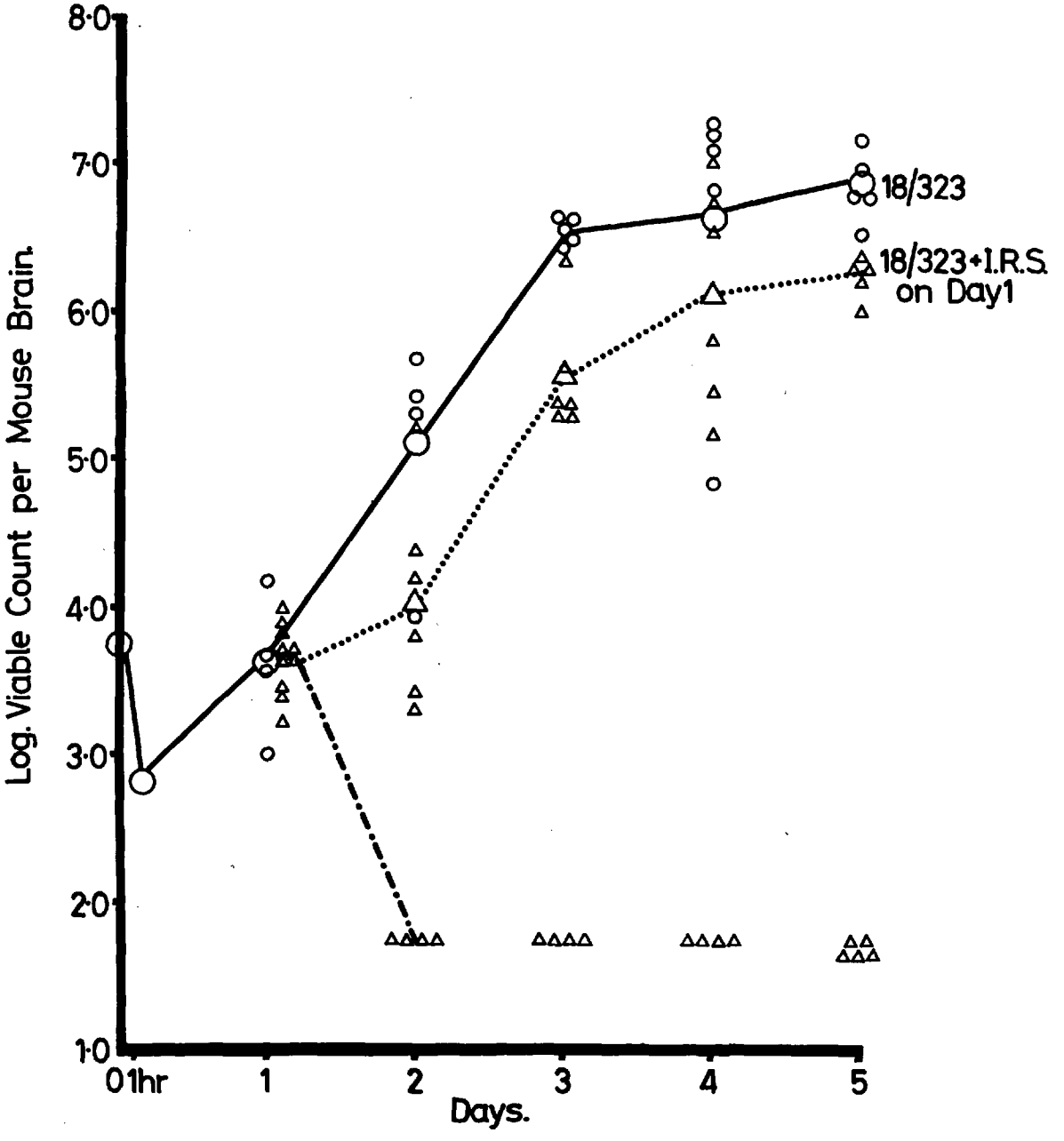
TABLE 20. Number of mice surviving challenge following injection of antiserum intracerebrally on various days after challenge.

Challenge inoculum of 18/323	I.R.S. injected on day:-	Time of Death	Average time to Death	S/T
2×10^3	1	8,9,12,14	10.6	6/10
"	2	6,9,14	9.6	7/10
"	4	8,8,8,9,9,10,11	9.0	2/9
"	None injected	5,6,7,7,7,8,8,8,9,10	7.5	0/10

I.R.S. = Immune Rabbit Serum

S/T = $\frac{\text{Number of mice surviving challenge}}{\text{Number of mice challenged}}$

Figure 27. The effect of intracerebral injection of *B. pertussis* antiserum 1 day after challenge on the course of infection with *B. pertussis* 18/323.


8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0 1hr
1
2
3
4
5
Days.
18/323
18/323+I.R.S.
on Day1

8.2. Intracerebral infections with incubated mixtures of immune serum and B. pertussis in normal and cortisone treated mice

The method described by Spasojevic (1962) for the incubation of antiserum-organism mixtures was used in the experiments reported in this section to investigate the claim of Eldering et al. (1966) that vaccines, regardless of serotype, protected mice more readily against the challenge strain G353 than against 18/323, although these strains were of approximately equal mouse virulence. The effect of antiserum on infections with high and low virulence strains in antiserum-organism mixtures has been explored.

These mixtures have also been injected into mice pretreated with cortisone.

8.2.1. Experimental

The course of infection with high and low virulence strains incubated with immune rabbit serum was investigated.

Serum was inactivated by heating at 56°C for 30 minutes before use. Equal volumes of serum and organisms were incubated at 37°C for 60 minutes. The mice were challenged with 5.5×10^3 B. pertussis B2288, a low virulence strain, contained in 0.025 ml. of the mixture and with 3.9×10^3 B. pertussis 18/323, a high virulence strain, contained in

0.025 ml. of another mixture.

This type of experiment was repeated on a number of occasions with high and low virulence strains, the challenge inocula on each occasion are indicated in the results (Figs. 28, 29; Table 21).

Finally, the effect of pretreatment of the mice with cortisone on the intracerebral infection with these immune sera-organism mixtures was investigated. Mice were injected subcutaneously with 2.5 mgm. of cortisone 6 hours prior and 1 and 2 days after challenge with the mixture. In all experiments the viable counts of serum-organism mixtures were made before and after incubation to check that inactivation of the serum had been successful.

8.2.2. Results

The courses of infection of B. pertussis 18/323 preincubated with immune serum shown in Fig. 28 and of B. pertussis B2288 preincubated with immune serum in Fig. 29.

In the first hour after challenge the G.M. viable counts in the brains injected with immune serum treated organisms were lower than those in the mice challenged with the control inoculum.

In the experiment with the high virulence strain, 4 of 8 brains contained less than 50 organisms by the first day and this proportion remained approximately the same throughout the

Figure 28. Course of infection following an intracerebral injection of an incubated mixture of immune serum and the high virulence strain 18/323.

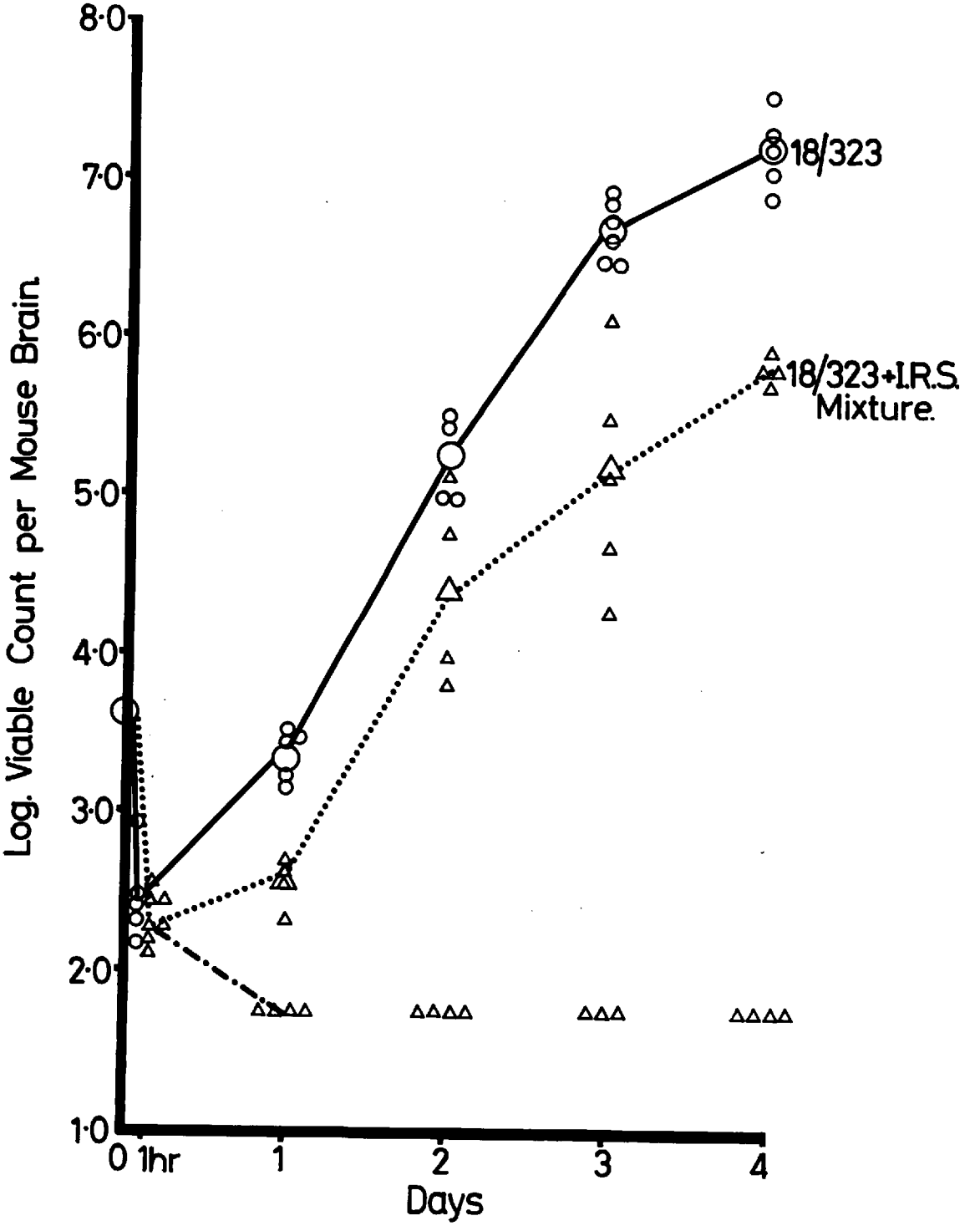
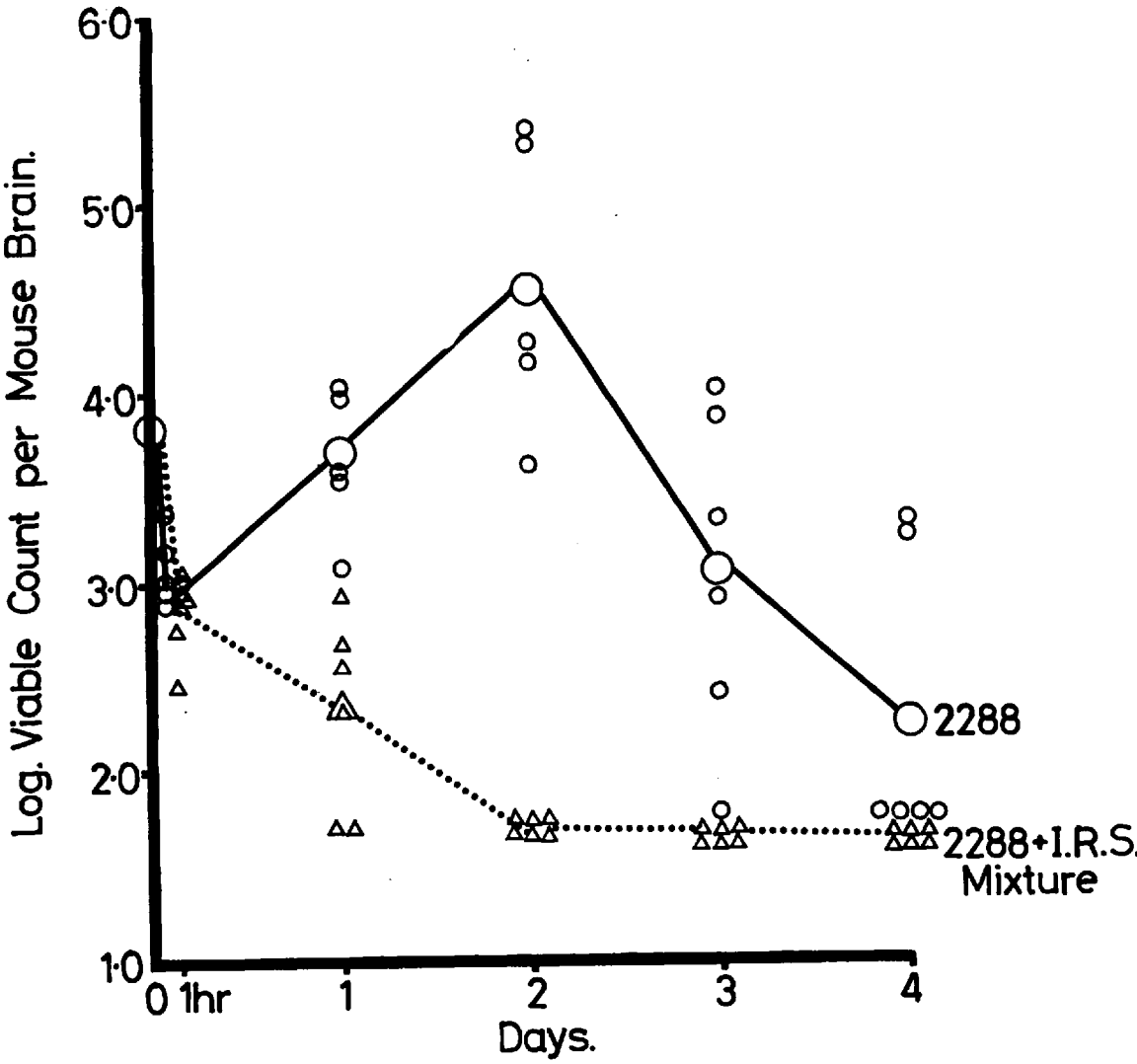

8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
Days
18/323
18/323+I.R.S.
Mixture.

Figure 29. Course of infection following an intracerebral injection of an incubated mixture of immune serum and the low virulence strain B2288.


6·0
5·0
4·0
3·0
2·0
1·0
Log. Viable Count per Mouse Brain.
0
1hr
1
2
3
4
Days.
2288
2288+I.R.S.
Mixture

experiment; the viable counts in the brains that were not sterile were lower than the viable counts in the control mice.

When 5.5×10^3 B. pertussis B2288 were injected after incubation with immune serum, 2 of 6 brains were sterile at the first day but the counts in the other 4 brains showed no evidence of a multiplication phase. From the second day onwards all brains were sterile.

It seemed that the same quantity of serum was more effective against the low virulence strain B2288 than it was against the high virulence strain 18/323.

Brain counts were determined at 1 hour, 1 day and 2 days with mixtures of low virulence strains and immune serum and the number of sterile brains has been recorded in Table 21.

When the larger dose of each strain was injected, the number of sterile brains increased considerably from the first to second days. With the smaller doses, however, the majority of brains were sterile 24 hours after challenge. At no time was there any evidence of a multiplication phase in the brains that did not become sterile until the second day. This seems to be unlike infections with the high virulence strains and immune serum mixtures in which case only the mice which have sterile brains within 24 hours of challenge appeared to survive.

Each of the three high virulence strains, 18/323, G353 and 2-Atox, was tested on three occasions using the same antiserum prepared in rabbits and inactivated at 56°C for

TABLE 21. Number of Sterile Brains detected on the first and second days after challenge with incubated mixtures of organisms and antiserum.

Strain	Number of mice	Viable Organisms in inoculum mixture	Number of Sterile Brains at:	
			Day 1	Day 2
B 124	8	4.4×10^3	3	7
B 124	8	3.2×10^3	6	8
B 1772	8	6.0×10^3	4	7
B 1772	8	2.3×10^3	6	7

No sterile brains were found in the control mice until the third day.

30 minutes. Each strain has been tested on three occasions and the total viable inocula for the three strains 6.4×10^3 18/323, 6.35×10^3 G353, and 7.1×10^3 2-Atox were spread over a very small range.

The daily deaths were recorded and totals showed that a greater proportion of mice survived when challenged with either G353 or 2-Atox mixtures than when challenged with 18/323 immune serum mixtures (Table 22).

An X^2 analysis of the results gave a value of P of 0.033 for 2-Atox against 18/323 and 0.003 for G353 against 18/323. These experiments confirm the work of Eldering et al. (1966) who showed that the challenge strain G353 gave smaller values for the PD_{50} of immune serum than did the strain 18/323 when the serum was injected intraperitoneally.

In these experiments, viable counts on B.G. medium have been taken as important in immune serum/organism mixtures. 18/323 was only 10% viable on B.G. medium compared with 35% for G353 and 2-Atox and this may have some bearing on the number of surviving mice.

Pretreatment of the mice with 2.5 mgm. cortisone acetate subcutaneously 6 hours before and 1 and 2 days after challenge with the immune serum/organisms mixtures greatly reduced the number of surviving mice (Table 23). The experiment was repeated on four occasions with various strains and all the results were significant. Iida et al. (1963, 1966) showed

TABLE 22. Number of mice surviving challenge after injection of incubated mixtures of immune serum and high virulence strains.

Strain	Inoculum	Immune serum in mixture	Number of mice	Day of Death	S/T	Total S/T	X^2	P
18/323	2200	Yes	15	7,8,8,9,9,11,11,12	7/15			
	1900	Yes	15	8,8,8,8,12,14	9/15	20/45	-	-
	2300	Yes	15	7,7,8,8,8,9,10,10,10,10,10	4/15			
	1900	No	4	8,8,8,9	0/4			
G353	2900	Yes	14	12,14,14,14	10/14			
	1800	Yes	15	9,12,12	12/15	34/44	8.72	.003
	1650	Yes	15	10,11,12	12/15			
	1650	No	5	7,8,9,9,9	0/5			
2-Atox	4600	Yes	15	7,9,10,12,13	10/15			
	1500	Yes	15	6,7,8,8,11,12	9/15	31/45	4.52	.033
	1000	Yes	15	9,9,13	12/15			
	1000	No	5	7,7,7,7,9	0/5			

TABLE 23. Number of mice surviving challenge after injection of incubated mixtures of immune serum and organisms into cortisone treated and normal mice.

Strain	Viable inoculum	Incubated with Immune serum	2.5 mgm. Cortisone S.C. 6 hours before and 1 and 2 days after infection	S/T	χ^2	P
18/323	700	Yes	No	9/12	6.04	.014
	"	Yes	Yes	2/12		
	"	No	No	1/12		
18/323	2.2×10^3	Yes	No	7/15	4.26	0.038
	"	Yes	Yes	1/15		
	"	No	No	0/5		
G353	1.6×10^3	Yes	No	12/15	12.73	<0.001
	"	Yes	Yes	1/14		
	"	No	No	0/5		
2-Atox	1.0×10^3	Yes	No	12/15	6.56	0.01
	"	Yes	Yes	4/15		
	"	No	No	0/5		

that cortisone injected prior to challenge of immune mice neutralised the immunity whether it was active or passive. My experiments with the Spasojevic technique confirmed the results of Iida and his co-workers, though the mechanism need not be the same in this case as in their immunity tests.

8.3. B. pertussis immunity in C'_5 complement deficient mice

From earlier experiments with mice deficient in the C'_5 component of complement it was suggested that bactericidal complement was of little importance in protection of the mouse against intracerebral infection with B. pertussis.

The experiments described here were designed to ascertain whether the bactericidal power of complement was a major factor in protecting immune mice against B. pertussis infections.

8.3.1. Experimental

Three strains of mice were used, B10.D2 "New Line" mice possessing all known components of complement, B10.D2 "Old Line" mice which lack the C'_5 component of complement and I.C.I. mice which are one of the strains used throughout this work.

The mice were injected intraperitoneally with a B. pertussis G353 heat killed vaccine 14 days before intracerebral challenge with B. pertussis 18/323. The number of mice surviving 14 days after challenge was recorded (Table 24).

8.3.2. Results

All non-immunised mice, regardless of the strain, were killed by the challenge doses of B. pertussis indicated in Table 24.

Although the doses of vaccine were large, 2×10^8 and 2×10^9, and it was not possible to obtain a dose response regression line, the fact that there was no significant difference in the number of survivors even in mice lacking the C'_5 component of complement strongly suggested that bactericidal killing by complement was not important in B. pertussis infections in immune mice.

The absence of C'_5, however, does not interfere with phagocytosis, and phagocytosis followed by intracellular killing probably by polymorphonuclear leucocytes could possibly be the mechanism by which immune mice deal with the bacteria.

TABLE 24. The effect of the C'_5 component of serum on the number of actively immunised mice surviving challenge with B. pertussis 18/323.

Strain of Mouse	Dose of Vaccine	Challenge Dose	S/T
B.10.D2 New Line	2×10^9	8×10^3	8/8
B.10.D2 New Line	Nil	"	0/4
B.10.D2 Old Line	2×10^9	7.2×10^3	8/8
" " " "	2×10^8	"	8/8
" " " "	Nil	"	0/8
I.C.I.	2×10^9	5×10^3	7/8
"	Nil	"	0/8

9. Histopathology of Bordetella pertussis Infections

In the previous sections bacteriological evidence of the courses of infection in the mouse brain under various conditions was presented.

Irradiation of the mouse brain prior to infection suggested that the local brain macrophages, the neuroglial cells, were involved in the response of the brain to B. pertussis infections.

Here histological evidence is provided in support of this thesis in sections taken from infected non-irradiated and infected irradiated brains.

All animals were injected with colchicine, an anti-mitotic agent, 5 mgm./kg. (Bryans 1959) 4 hours before being killed by decapitation. The sections were stained with haematoxylin and eosin.

In Photograph 2 a lateral ventricle of a normal mouse brain containing some choroid plexus is seen. This was typical of the ventricles found in the mid-brain. Under oil-immersion a ventricle with typical ciliated epithelial cells was seen (Photograph 3). This section was taken from a mouse which had been infected with influenza virus and the cells were still highly ciliated. This suggested that destruction of the ciliated epithelium was not the reason for

this virus giving rise to "interference" immunity against B. pertussis.

As early as 6 hours after infection with the low virulence strain B1772 many cell divisions were detected (Photograph 4). The four mitotic figures occurring in this section were all in the prophase. One was found in the choroid plexus, two between the ependymal cells and the fourth division in the brain substance a short distance from the ventricle. Apart from these divisions there were also normal microglial cells in the ventricular region at this time.

In another section (Photograph 5) taken at this time with the same low virulence infection many mononuclear cells, probably microglia, were located in the lateral ventricle. There was evidence of a cell division, prophase, in the centre of this cluster.

At 1 day after challenge with a low virulence strain microglial cells were very evident in the ventricles, also an occasional polymorph was found. The ependyma was still generally intact and seemed to retain its cilia (Photograph 6).

The above picture was very similar to that found with a high virulence strain infection 1 day after challenge (Photograph 7). A clump of mononuclear cells, again probably microglial cells, was found in the ventricles. The ependyma was still intact, although it had pulled away from the rest of the brain, probably in sectioning, and the cells still

appeared to be ciliated.

However, by the seventh day a dramatic change had taken place in the structure of the ventricle and surrounding brain (Photograph 8). The ependymal lining had been completely destroyed and the brain tissues invaded. There was an intense polymorphonuclear leucocyte infiltration.

After irradiation and infection with the low virulence strain B1772 a number of mitotic figure abnormalities occurred. The mice were challenged 3 days after irradiation and in Photograph 9 an abnormality was found 2 days after challenge. Here stored up irradiation had caused the formation of micronuclei when the cell was stimulated to divide by the pertussis infection.

Similarly 3 days after challenge other abnormalities were found. An anaphase bridge had formed where the chromosomes failed to divide completely and another micronucleated cell and abnormal division were evident in the choroid plexus (Photograph 10). In the following Photograph (11) details of the chromosome linkage of the anaphase bridge were seen under oil-immersion.

On the same day microglial divisions were still occurring quite a distance from the ventricle. Microglial divisions in the telophase and metaphase were seen deep into the brain, while a second prophase was evident a short distance from the ventricle (Photograph 12).

In Photograph 13 taken of a section from an irradiated brain 3 days after challenge an abnormal astrocyte division was seen in the ventricle. One chromosome had been left out of the division. There also appeared to be a certain amount of disorganisation to the ependyma.

In a section taken from another irradiated brain at the fourth day were seen a prophase in the ventricle and a metaphase plate between the ependyma cells (Photograph 14).

Finally by the seventh day of infection with a low virulence strain in an irradiated mouse an intense leucocytic infiltration was observed with severe damage to the ependyma (Photograph 15). The results of this type of infection were very similar to the damage that was found with a high virulence strain in normal mice (Cf. Photograph 8).

Further histological evidence on the brain's reaction to intracerebral infection was provided by autoradiographic studies. As described in detail earlier, mice were injected with tritiated thymidine 6 hours before killing by decapitation, fixed, sectioned and stained with haemalum. Synthesis of DNA and thus the probability that the cell will divide was indicated by the appearance of black dots over the nucleus.

Photographs 16 and 17 show the appearance of a lateral ventricle in a normal and irradiated uninfected brain. There was little evidence of cell labelling in these sections taken

from the mid-brain and any labelling that did occur was restricted to the upper and lower points of the ventricles and even then only a few labelled cells were observed.

As described earlier for the haematoxylin and eosin stained sections there was evidence of mitotic figures as early as 6 hours after challenge. In an irradiated brain (Photograph 18) labelled cells were seen a distance from the ventricle. Here there was evidence of astrocyte and microglial cell labelling. A higher power of Photograph 18 (Photograph 19) showed clearly the labelling on a microglial cell and a group of 3 cells, probably astrocytes.

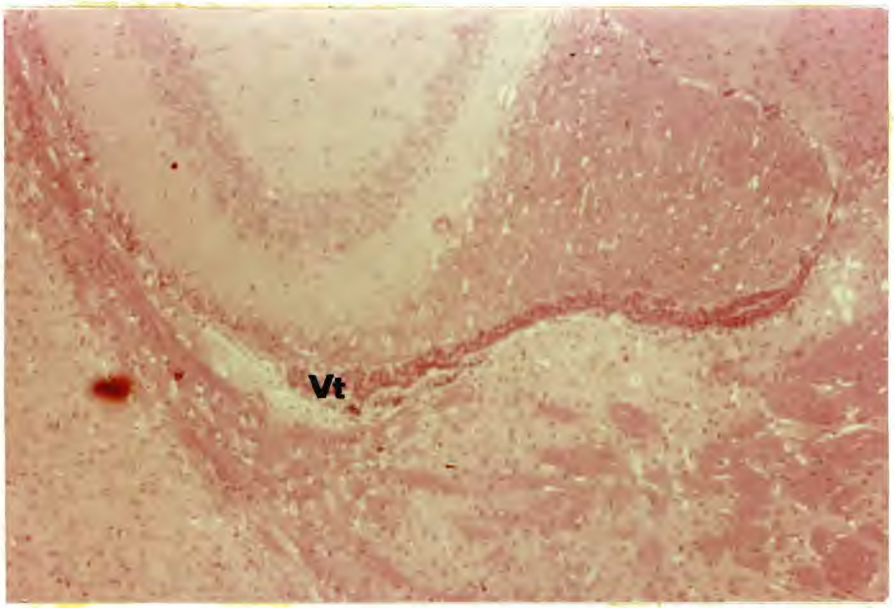
Previously many microglial cells were seen in the lateral ventricles as early as 6 hours after challenge. This was confirmed by autoradiographic experiments which showed many labelled microglial cells and an occasional astrocyte in the ventricle (Photograph 20). Even at 2 days after challenge labelled cells were still apparent a distance from the ventricle (Photograph 21).

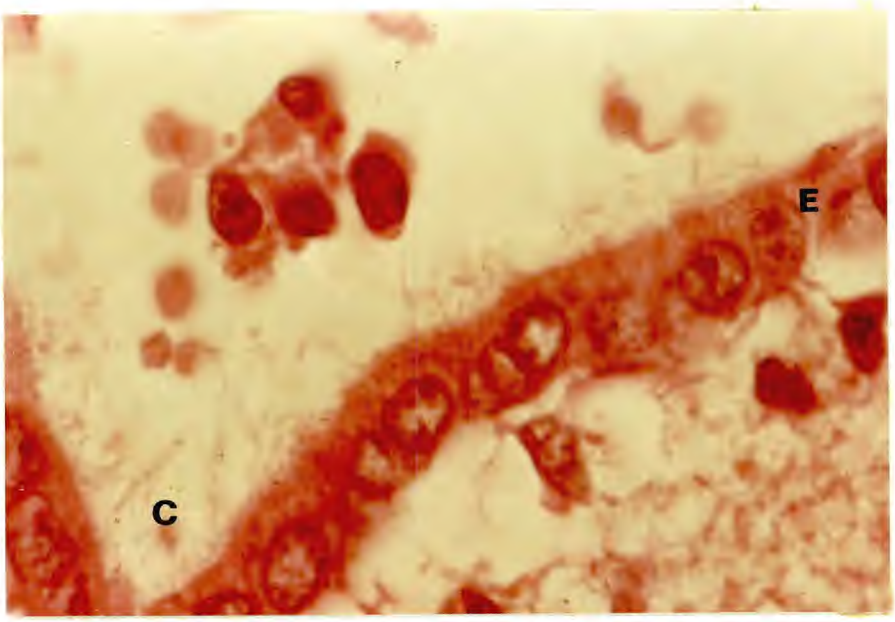
The three final photographs show the ependyma to be the probable site of multiplication of Bordetella pertussis in the mouse brain. Photograph 22 shows colonies of the high virulence strain 18/323 on the ependyma of the lateral ventricle 4 days after challenge. There was evidence of some ependymal damage and an intense leucocytic infiltration. In Photographs 23 and 24 the ependyma was again seen as the

site of multiplication of the low virulence strain B1772 in an irradiated brain 4 days after challenge. There was leucocytic infiltration but only an occasional microglial cell was seen at this time.

Photograph 2. The lateral ventricle of a normal mouse brain. Haematoxylin and Eosin Stain. X230.
Vt = Ventricle.

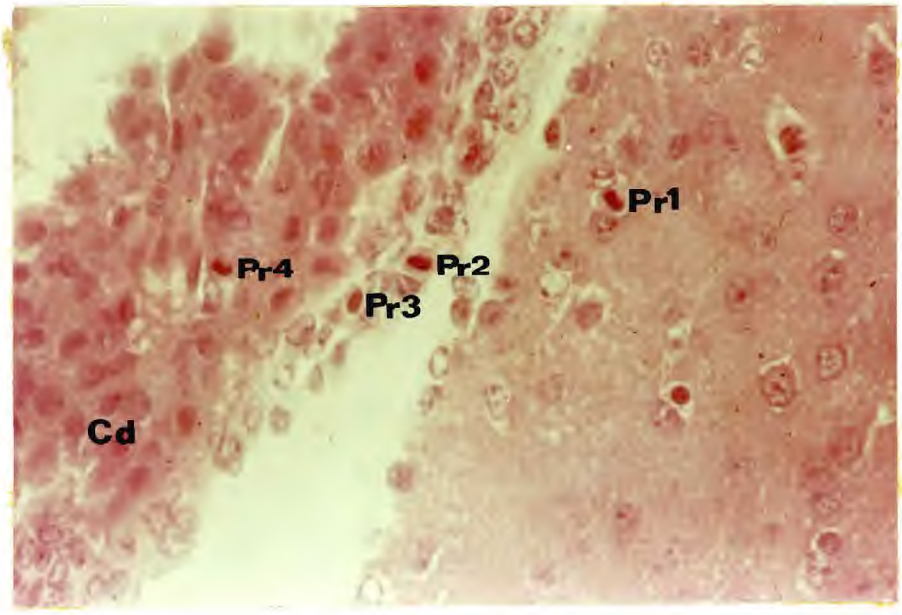
Photograph 3. The ciliated epithelial cells of the lateral ventricle from a mouse challenged 3 days earlier with influenza virus. Haematoxylin and Eosin Stain. X1400.
C = Cilia, E = Ependyma.

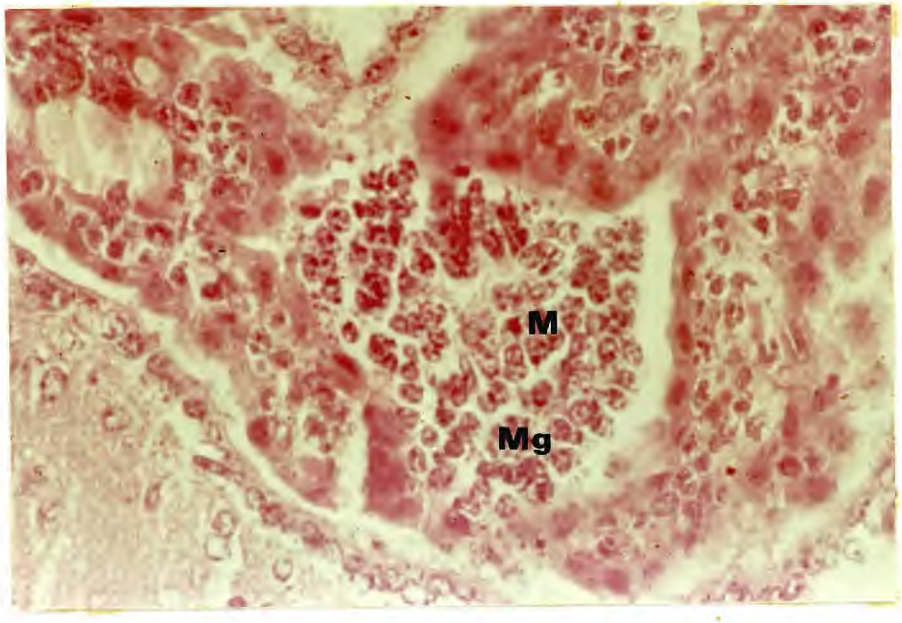

Vt


E
C

Photograph 4. Mitotic activity 6 hours after challenge with the low virulence strain B1772 in a normal brain. Cells in the prophase stage of division were found in the brain substance, Pr_1, 2 were found between ependymal cells (Pr_2, Pr_3) and another (Pr_4) in the choroid plexus (C.P.) Haematoxylin and Eosin Stain. X560.

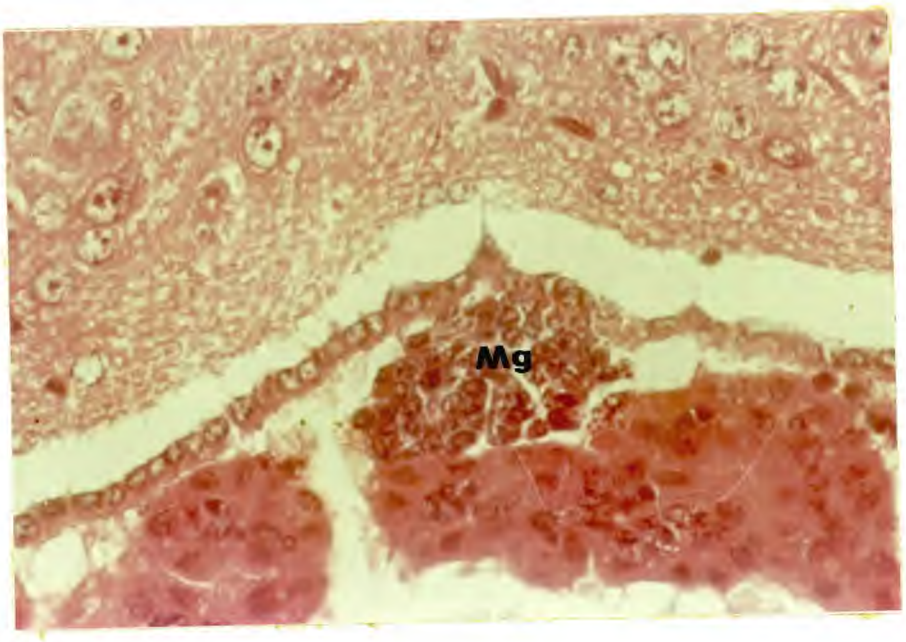
Photograph 5. Many mononuclear cells, probably microglial cells, located in the lateral ventricle 6 hours after challenge with the low virulence strain B1772. There was evidence of mitotic activity, probably in prophase, in the centre of this group. Haemotoxylin and Eosin Stain. X560. Mg = Microglial cells. M = Mitotic Activity.

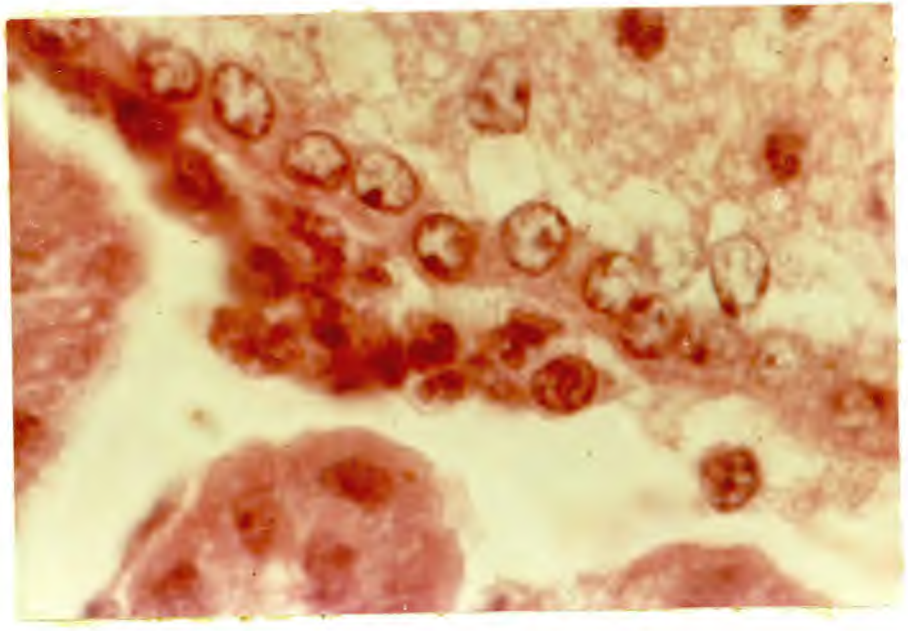

Pr1
Pr4
Pr2
Pr3
Cd


M
Mg

Photograph 6. A group of mononuclear cells was found 1 day after challenge with the low virulence strain B1772. Haematoxylin and Eosin Stain. X1400.
Mg = Microglial cells.

Photograph 7. A similar group of mononuclear cells found in the ventricle 1 day after challenge with the high virulence strain 18/323. Haematoxylin and Eosin Stain. X560.

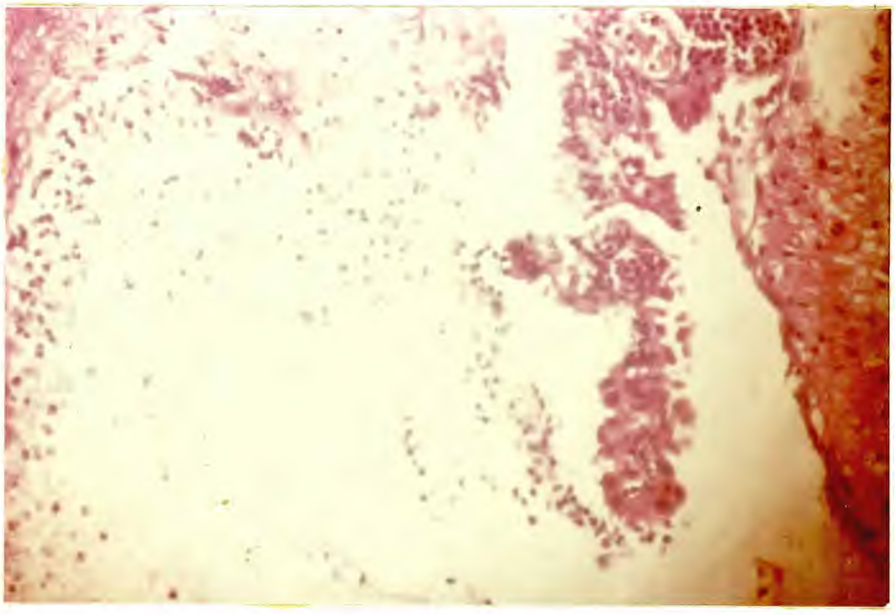

Mg

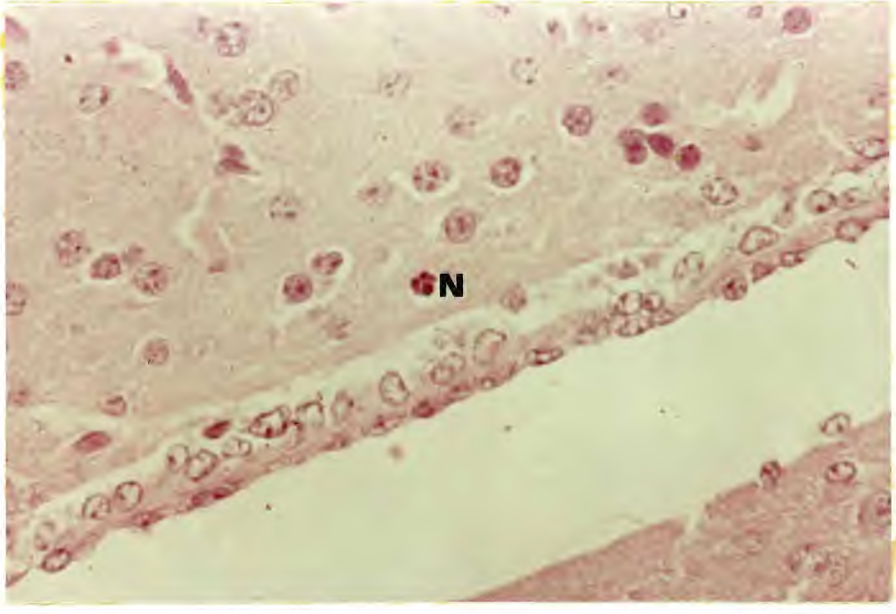


Photograph 8. Almost complete destruction of brain substance, severe hydrocephalus and infiltration of leucocytes into the ventricle 7 days after challenge with the high virulence strain 18/323. Haematoxylin and Eosin Stain. X230.

Photograph 9. A micronucleated cell in an irradiated brain 2 days after challenge with B. pertussis B1772. Haematoxylin and Eosin Stain. X560.

N = Micronucleated Cell.



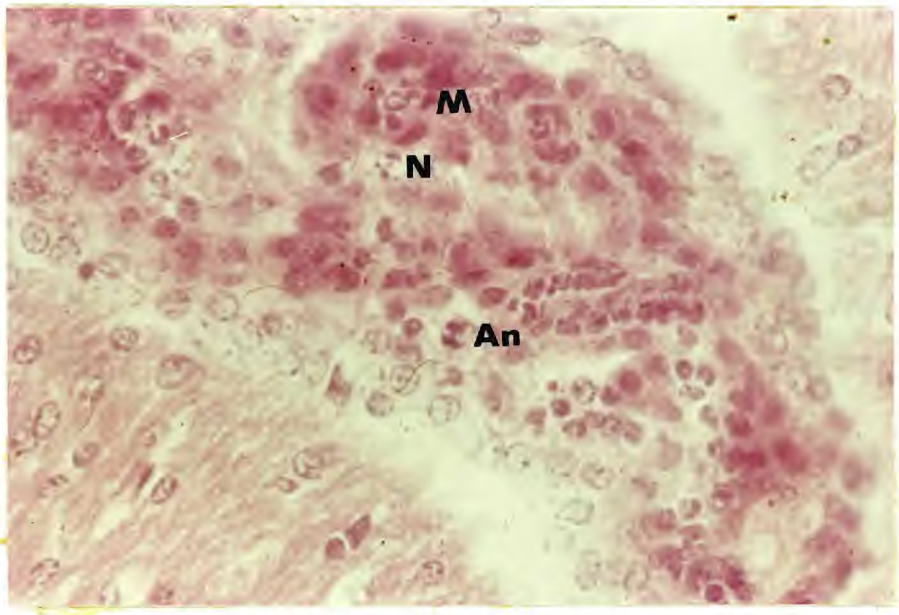

N

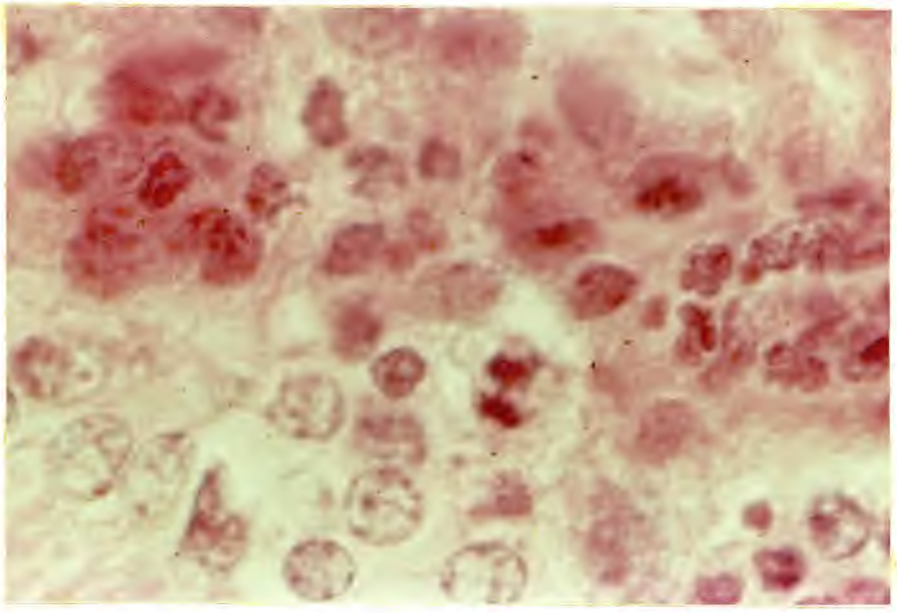
Photograph 10. Evidence of radiation damage detected in the ventricles. Radiation and stimulation by B. pertussis infection has caused the formation of an anaphase bridge and micronucleated cells. Haematoxylin and Eosin Stain. X560.

M = Mitotic Activity N = Micronucleated Cell

An = Anaphase Bridge.

Photograph 11. A high power view of the anaphase bridge shows the details of the chromosome linkage. Haematoxylin and Eosin Stain. X1400.


M
N
An

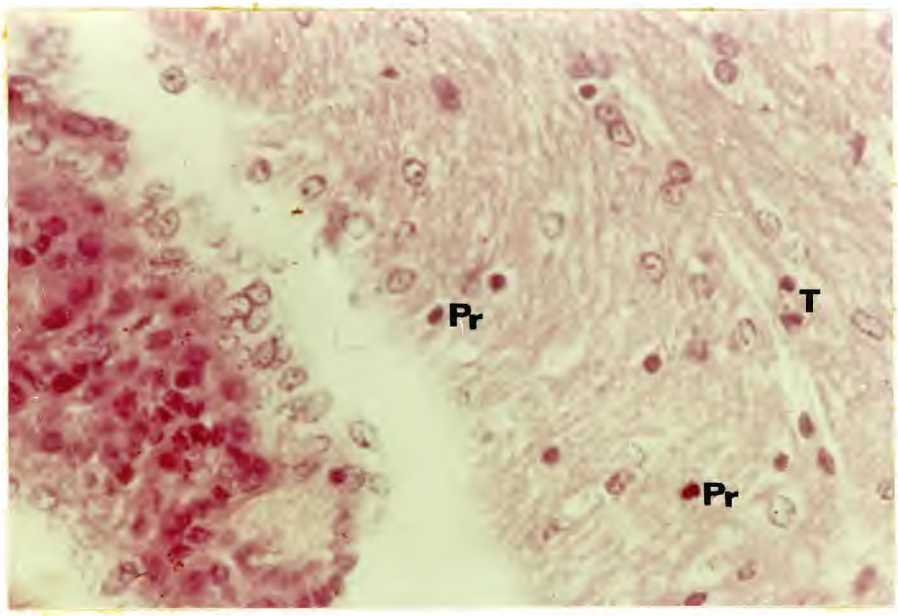


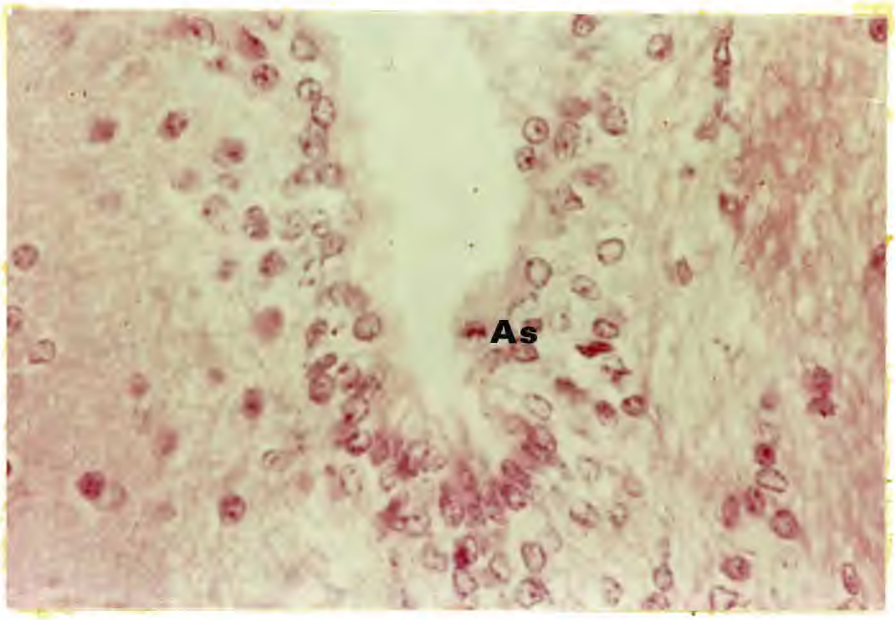
Photograph 12. Many mitotic figures were still evident 3 days after challenge in an irradiated brain. Haematoxylin and Eosin Stain. X560.

T = Teleophase Pr = prophase.

Photograph 13. An abnormal astrocyte division in the ventricle of an irradiated brain 3 days after challenge with B. pertussis B1772. Haematoxylin and Eosin Stain. X560.

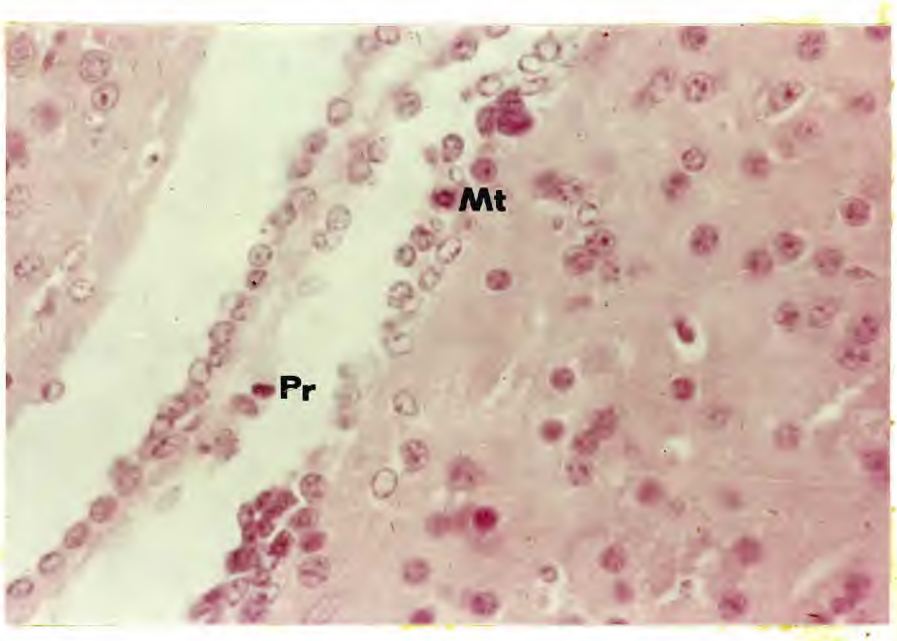
As = Astrocyte division.

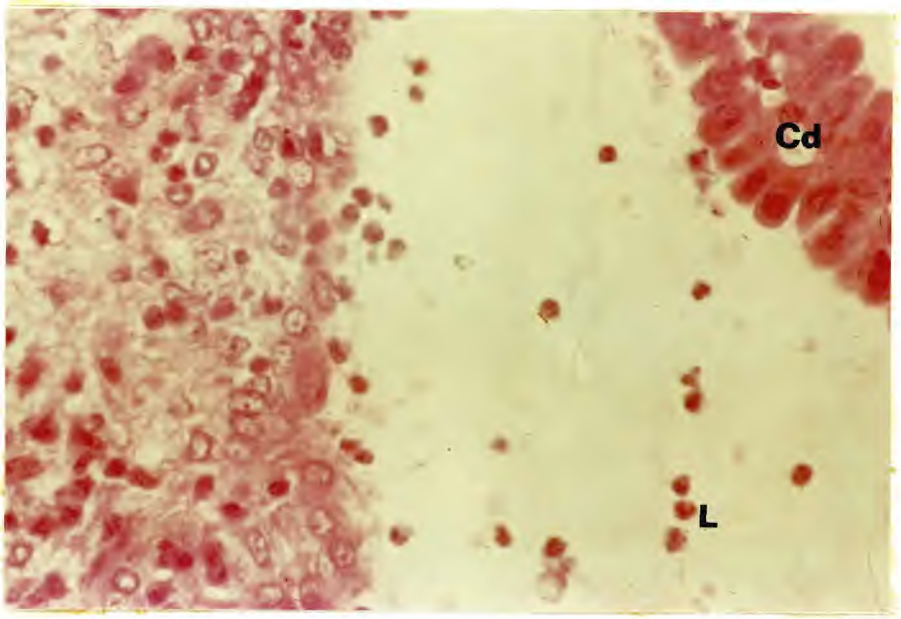

Pr
T
Pr


As

Photograph 14. A cell in the prophase stage and another in the metaphase stage of division in an irradiated brain 4 days after infection with B.pertussis B1772. Haematoxylin and Eosin Stain. X560.
Mt = Metaphase Plate Pr = Prophase.

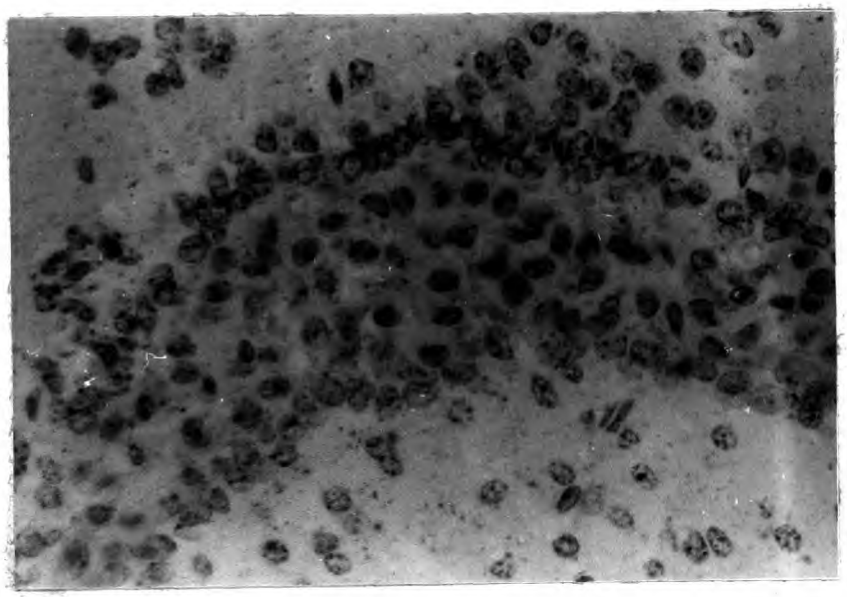
Photograph 15. Severe hydrocephalus and infiltration of leucocytes 7 days after infection with the low virulence strain B1772 in an irradiated brain. Haematoxylin and Eosin Stain. X1400.
Cd = Choroid Plexus L = Polymorphonuclear Leucocytes.

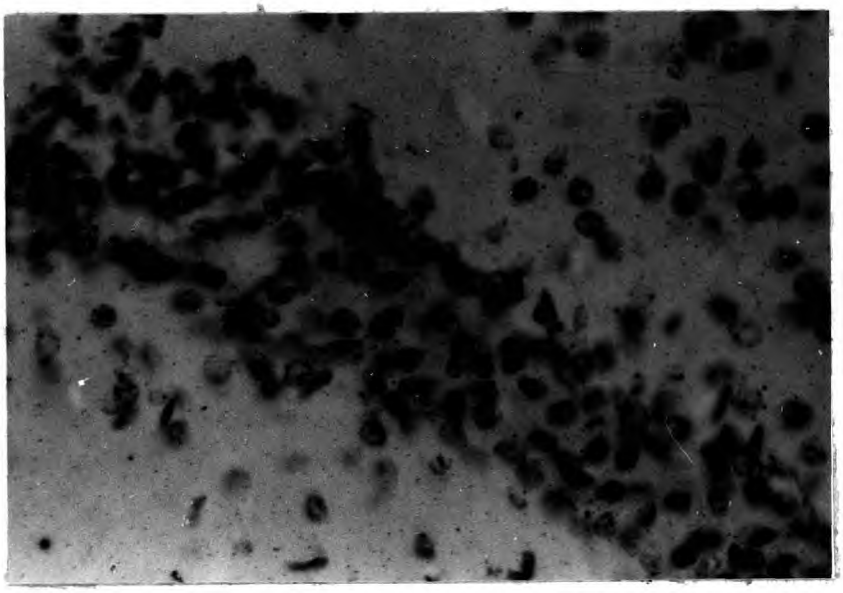

Mt
Pr


Cd
L

Photograph 16. The cells in the region of a lateral ventricle of a normal brain show no evidence of labelling with tritiated thymidine. Haemalum Stain. X230.

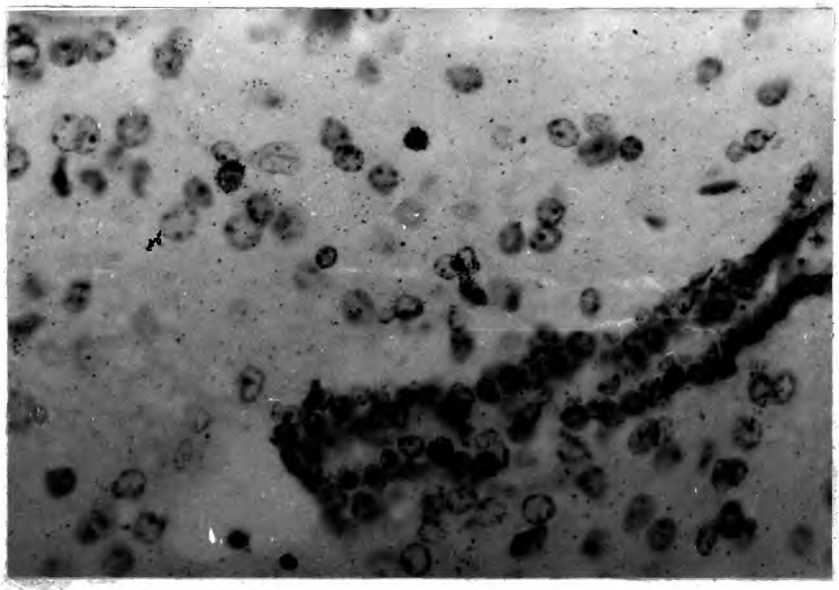
Photograph 17. The cells in the region of a lateral ventricle of an irradiated brain show no evidence of labelling with tritiated thymidine. Haemalum Stain. X230.

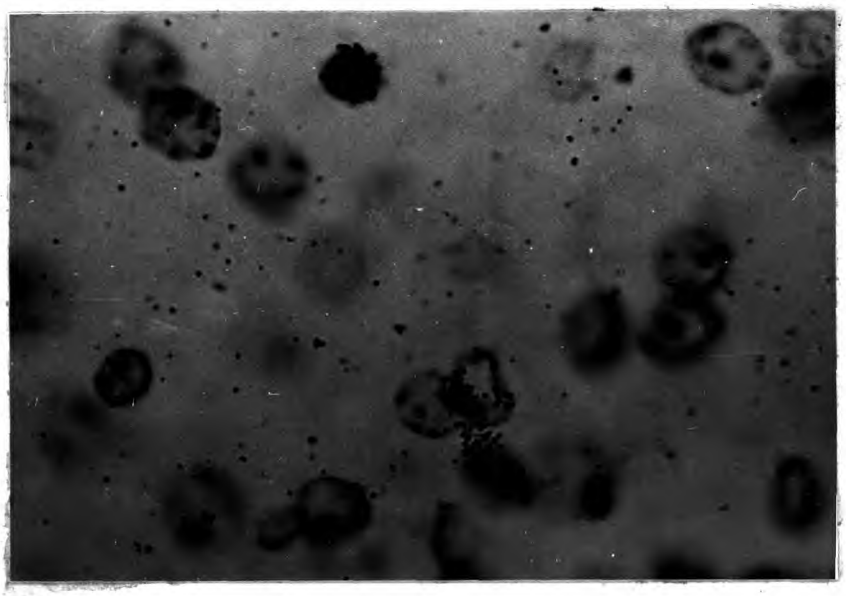




Photograph 18. Many labelled cells in an irradiated brain 6 hours after challenge with a low virulence strain of B. pertussis B1772. Haemalum Stain. X560.

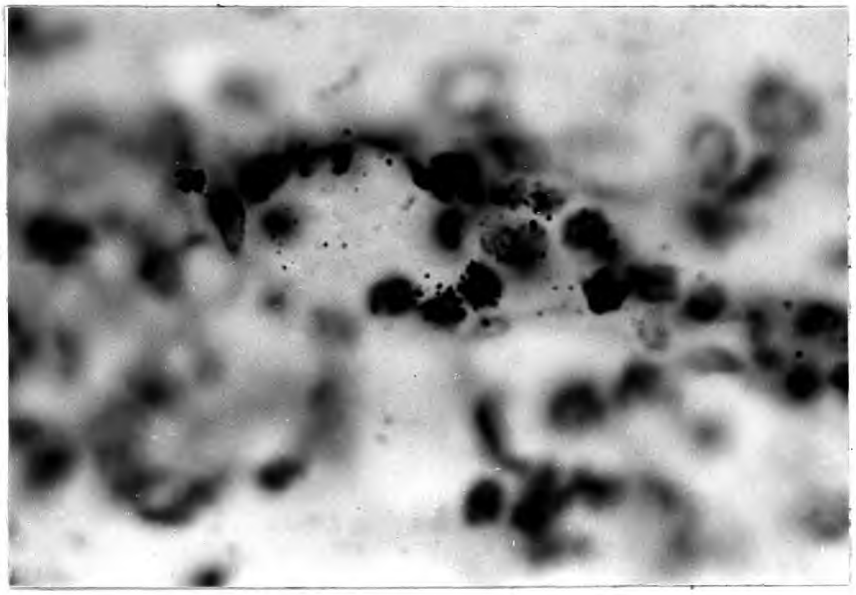
Photograph 19. A high power view of labelled cells in photograph 18. Two of the labelled cells are probably astrocytes and another 2 are probably microglial cells. Haemalum Stain. X1400.

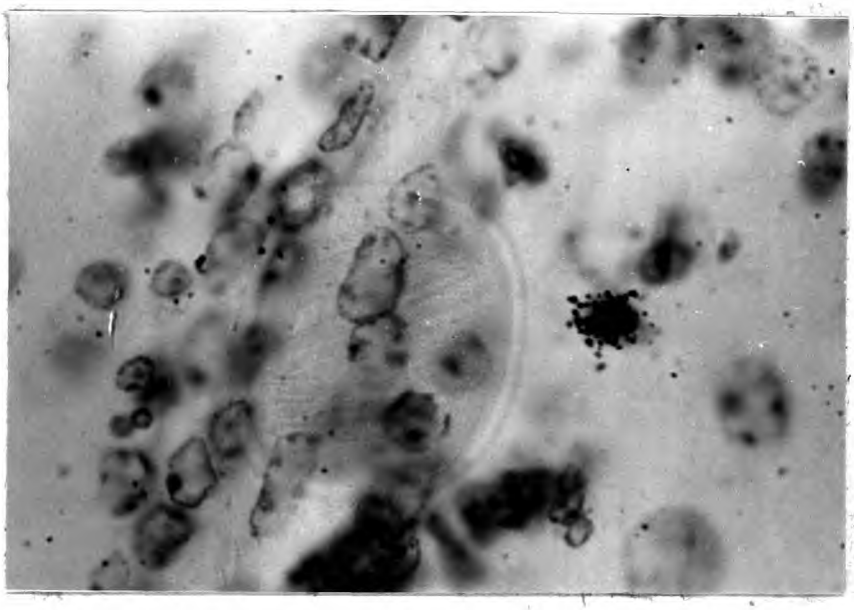




Photograph 20. In a control infection 1 day after challenge with a low virulence strain and a non-irradiated brain many labelled cells are seen within the ventricle. Haemalum Stain. X1400.

Photograph 21. A heavily labelled cell some distance from the ventricle in a control infection 2 days after challenge. Haemalum Stain. X1400.





Photograph 22. Many micro colonies of B. pertussis 18/323 adhering to the ependyma on the seventh day of infection. There is an intense polymorphonuclear leucocyte infiltration. Haemalum Stain. X1400.

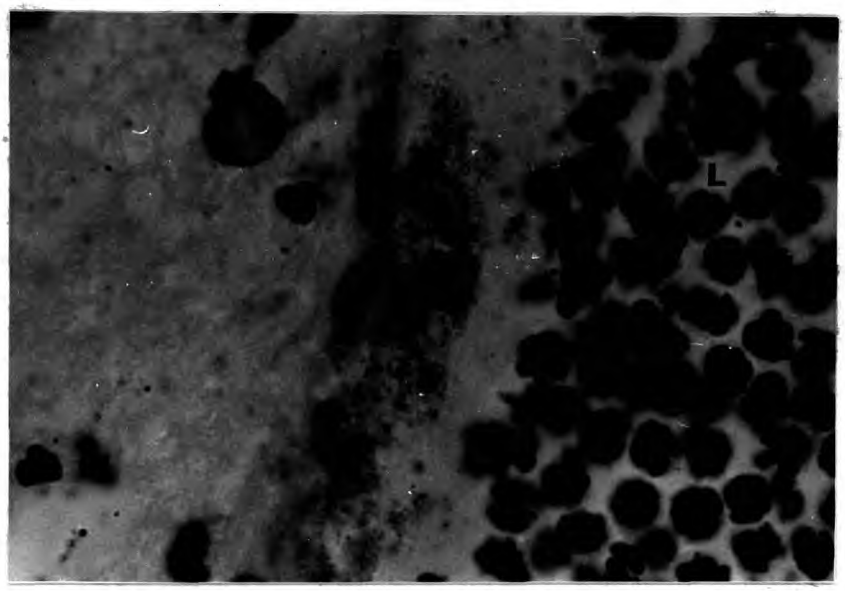
E = Ependyma Bt = Bacteria

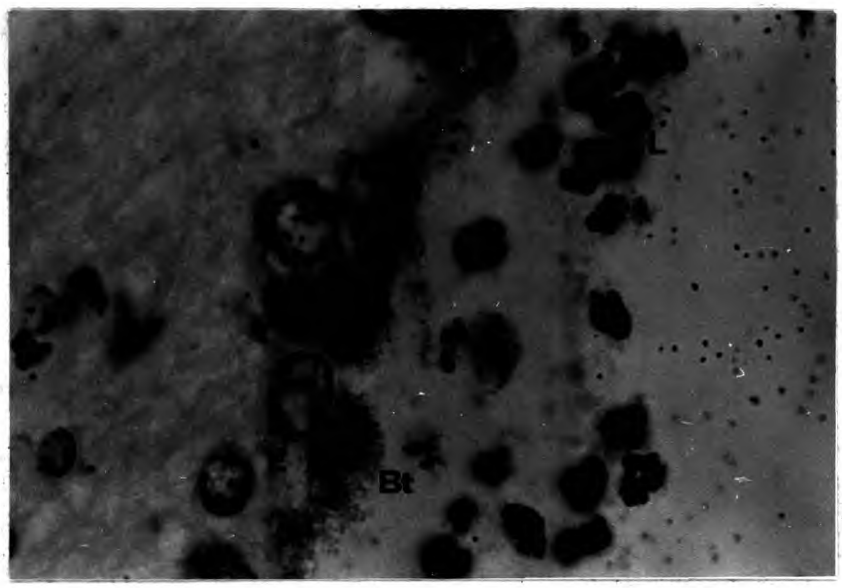
L = Polymorphonuclear Leucocytes.

Photograph 23. Many organisms of the low virulence strain of B. pertussis B1772 adhering to the ependyma on the fourth day of infection in an irradiated brain. There is some polymorphonuclear leucocyte infiltration. Haemalum Stain. X1400.

E = Ependyma Bt = Bacteria

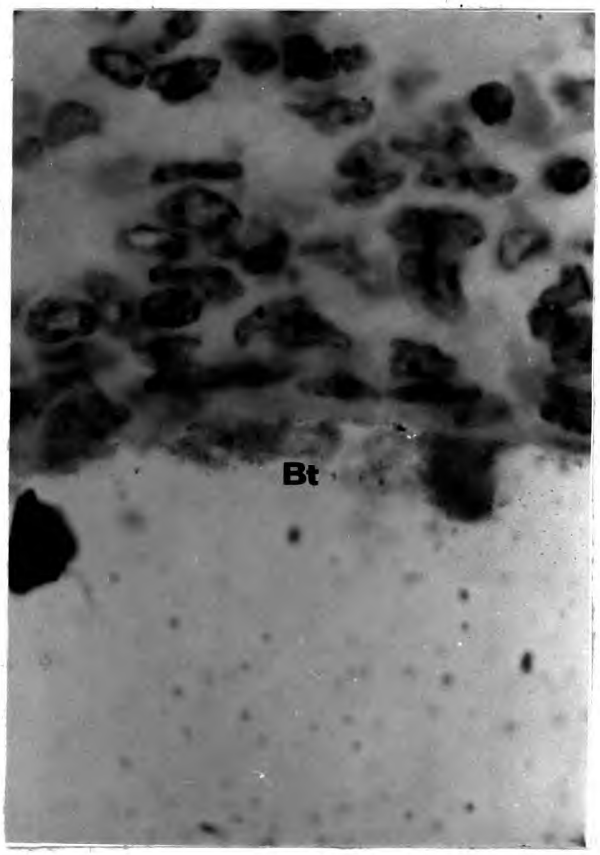
L = Polymorphonuclear Leucocytes.


L


Bt

Photograph 24. Many organisms of the low virulence strain of B. pertussis B1772 adhering to the ependyma on the fourth day of infection in an irradiated brain. Haemalum Stain. X1400.

Bt = Bacteria.


Bt

10. Discussion

Two distinct courses of infection were found with the high and low virulence strains of B. pertussis.

The courses with the two high virulence strains 18/323 and 2-Atox were very similar to each other, but that of G353 varied slightly from these two. With the former pair, the viable count increased by approximately 10-fold daily for 3 or 4 days after intracerebral challenge. After this time the rate of increase decreased slightly to 5- to 8-fold daily. This was almost identical to the results described by Dolby and Standfast (1961), Berenbaum et al. (1960) and Iida et al. (1962) for the strain 18/323 and by Andersen (1952b, 1957a) for the strain 2-Atox. However, with the high virulence strain G353 the viable count increased by 10-fold daily until the count reached approximately 10^6 viable organisms. At this point the viable count remained stationary for a further 24 hours after which time the rate of increase seemed to compensate for this lag phase (Fig. 6). This lag phase with G353 infections occurred when the viable count had reached 10^6 organisms which was the count indicated by Holt et al. (1961) necessary for breakdown of the blood brain barrier. In studies which were carried out in this laboratory the most potent vaccine, i.e. the vaccine with the lowest ED_{50} value, was produced from a culture of G353 (Holt - unpublished observations). Continuous

spill over of the organisms from the brain to the meninges may lead to the formation of small amounts of specific antibody and breakdown of the blood brain barrier at the count of 10^6 may lead to a transitional lag phase in the increase in viable count. This may also explain the decrease in multiplication rate of 18/323 and 2-Atox in the terminal stages of infection. Eldering et al. (1966) using active immunisation, and later Eldering et al. (1967) and Preston (1967) using passive immunisation reported that vaccines or antiserum regardless of serotype, gave much higher ED_{50} values when the mice were challenged with the strain 18/323. Both groups of workers also reported that the time taken to death for similar doses of 18/323 and G353 cultures was longer for G353 than 18/323, although the LD_{50}s of both cultures were very similar.

These results may be explained on the observation of a stationary phase in G353 multiplication (Fig. 6). If the formation of small amounts of antibody, as suggested, was correct then this would be additive with the antibody formed as a result of active or passive immunisation and give a higher survival rate of mice. This lag may also explain the longer time to death of mice challenged with G353. There is no evidence that these mice die with a higher bacterial count which was found to be between 10^7 and 10^8 viable organisms for all three high virulence strains.

Three low virulence strains were also studied, B124,

B1772 and B2288. Up to 24 hours after challenge with doses less than 10^4 organisms there was little distinction between these strains and the three high virulence strains. There was the same approximate 90% loss of the challenge inoculum on injection and the course of infection which was examined at short time intervals for 24 hours was little different from that for a high virulence strain infection. The individual bacterial counts at 1 day were closely grouped, the range often being less than 1 on a log scale, as they were throughout infection with the high virulence strains. Between days 1 and 2, whereas the count with the high virulence strain infections increased by at least 10-fold, the count with the low virulence strain infections decreased slightly, the average of 24 experiments was $\log_{10}$ 0.1. After the second day, the geometric mean viable count declined rapidly and brains containing less than 50 organisms were found from the third day onwards. The range of counts increased significantly after the first day, there often being over 500-fold difference between the highest and lowest counts. This indicated a large variance in the response of the mouse to infection by the low virulence strains compared with a small variance in the response to high virulence strain infections.

With higher doses of the low virulence strains the course of infection was extended and the brains of mice were still highly infected after 5 days. With very high doses,

i.e. greater than the LD_{50}, the mice died between days 2 and 3 with terminal viable counts of between 10^7 and 10^8 bacteria which were very similar to the terminal count with high virulence strains.

Examination of the 1 hour viable counts confirmed the work of Dolby and Standfast (1961) that there was an approximate 90% loss of organisms within minutes of injection. The loss was found to be independent of strain (Table 2), challenge size (Table 3) and a decrease in challenge volume to 0.0025 ml. resulted in only a slight increase in the number of organisms retained (Table 4).

Similar observations were made by Schlesinger (1949) who could only recover 3 - 10% of a virus inoculum 1 hour after injection. Similarly Cairns (1950) could find no significant increase in the percentage of a bacteriophage suspension retained when only 0.005 ml. was injected compared with 0.3 ml. in the controls.

When 0.025 ml. of a 1% solution of Pontamine Sky Blue was injected and the brains exposed 1 hour later the dye was concentrated in the ventricular region and the blue area on the reflected skin (Photograph 1) indicated that a considerable washback along the needle track had occurred. It was probable, therefore, that this loss of organisms was mechanical. This was further confirmed by other experiments when procedures taken to enhance infection did not result in a larger

percentage of organisms being retained at 1 hour.

Modulation was considered as a possible reason for the decline in infection with low virulence strains. Lacey (1960, 1961) showed that organisms, when grown in media where the sodium chloride content was replaced with the equivalent amounts of certain salts of the alkaline earth metals, were converted from the X-mode to the C-mode. He showed that the intranasal LD_{50} of a strain H5 was increased from 10^7 to 10^{10} organisms when grown on a medium containing magnesium sulphate instead of sodium chloride.

In these experiments growing both G353 and 2-Atox on either Cohen and Wheeler medium containing magnesium sulphate at 34°C or on Bordet-Gengou medium at 25°C increased the LD_{50} of these strains from approximately 50 viable organisms to greater than 2000 viable organisms. The course of infection (Fig. 14) showed that on injection the brains of surviving mice were sterile within 24 hours. In the mice that died there was a stationary phase between 1 and 24 hours, during which time the organisms were probably remodulating to the original form. The strain 18/323 proved to be a strain particularly resistant to modulation and none of the treatments tested increased the LD_{50}. Although it has been shown that the degree of agglutination of G353 and 2-Atox was considerably reduced by growth on the various media, it was not thought that this played any role in virulence. Other properties were also changed, such as toxicity and efficiency as a vaccine

(Holt - unpublished data).

Having shown <u>in vitro</u> that modulation of high virulence strains altered their virulence when tested by the intracerebral route, the brains of mice were removed when the bacterial count of the low virulence strains was decreasing. If modulation was the explanation of this decrease, these organisms, when reinjected into fresh brains, should continue to decrease in numbers. However, when these organisms were reinjected a normal course of infection emerged (Fig. 7) and it is therefore suggested that modulation of the low virulence strains is not the cause of the decrease in count.

Having concluded that the organisms have not changed, it is suggested that the mouse had changed in some way.

The experiments were designed to indicate whether a previous infection with a low virulence strain altered the response of the mouse to a second infection with the same strain or with a high virulence strain. The time interval between infections, $4\frac{1}{2}$ days, had been shown previously to result in the majority of brains being sterile after challenge doses of 10^4 viable organisms or less. The necessity of having near sterile brains for this experiment prevented any wrong interpretation of results due to interference immunity.

When the preinfected brains were rechallenged with the same low virulence strain, no increase in viable count was detected and in the two experiments recorded a high proportion

of brains containing less than 50 organisms was recorded two days after rechallenge. However, when the mice were rechallenged with a high virulence strain, no brains containing less than 50 organisms were detected and the course of infection was little different from that in the control mice. Thus it seems that the low virulence infection was able to stimulate a response, which was still evident to some extent $4\frac{1}{2}$ days after challenge, to another low virulence infection but did not influence a high virulence infection. The type of response was not clear from this experiment and further experimental work was necessary. The importance of having the brain sterile or only slightly infected for the above experiment was made evident by reference to Fig. 9. Here mice infected with a low virulence strain were rechallenged at the peak of infection with the high virulence strain 18/323. A high proportion of brains containing less than 50 18/323 organisms were detected on the first day after rechallenge and this number remained fairly constant on each subsequent day. This result was analogous with "interference" phenomenon described by Evans and Perkins (1954a), who used heat killed vaccines which, when injected intracerebrally, gave rise to "interference" to infections with the high virulence strain 18/32 Later Blyth (1955) showed that the brains of surviving mice were sterile 24 hours after challenge and the proportion of sterile brain did not increase after this time. It is suggested

that the low virulence strain is acting in the same way, giving rise to "interference immunity" and thus mice are surviving when rechallenged with a high virulence strain. It has been seen that once the brains were sterile there was no "interference immunity" to the high virulence strain. It seems that the most likely explanation of this "interference" phenomenon is that all strains have the same "fixation sites" on the cilia of the ependyma and that prior injection of either a low virulence strain or a killed suspension of organisms takes up a majority of the "fixation sites", thus decreasing the probability of the high virulence strain finding free "sites".

Evans and Perkins (1954b) suggested that this "interference" phenomenon may be similar to the "interference" met with in the field of virology. Dalldorf et al. (1947) showed that vaccinia virus induced resistance in mice to intracerebral infection with B. pertussis and Henle and Henle (1946) and Mims (1960b) showed in mice surviving the neurotoxic action of influenza virus that the ependymal lining was destroyed.

Intracerebral infection with West Nile virus was shown by Finter (1964) to produce large amounts of interferon but no interferon could be detected in mice infected with B. pertussis (Finter - personal communication).

Although it was shown by Henle and Henle and Mims that the ependyma of mice infected with a mouse virulent strain of

influenza virus was severely damaged, no damage was detected in my experiments when mice were infected with a strain of influenza virus of low mouse virulence (Photograph 3) and challenged with either a high or low virulence strain of B. pertussis.

It is suggested that the mode of action of the "interference effect" of influenza and vaccinia viruses is not the formation of interferon or necessarily the destruction of the ependyma but the occupation by the virus of the same "fixation sites" required by the infecting strains of B. pertussis. Further evidence for this hypothesis is provided in some work not recorded here which showed that both high and low virulence strains of B. pertussis haemagglutinated fowl red blood cells as do both influenza and vaccinia viruses.

Earlier it was shown that the fall in intracerebral count with low virulence strains was not due to modulation and that previous infection with a low virulence strain increased the resistance to reinfection with the same strain. The possible role of bactericidal complement in these phenomena was investigated.

Normal guinea pig serum was highly bactericidal for B. pertussis, more so against the high virulence strains than against the low virulence strains (Table 6) but normal mouse serum was not bactericidal against B. pertussis. The lack

of bactericidal action of normal mouse serum has been demonstrated against a variety of organisms (Marcus et al., 1954, Muschel and Muto, 1956). It was also found that the addition of normal mouse serum to normal guinea pig serum completely inhibited the bactericidal power of the latter to B. pertussis. This was similar to the findings of Rice and Crowson (1950) who showed that mouse serum was anti-complementary to guinea pig serum.

Glynn and Medhurst (1967) were able to demonstrate the bactericidal action of mouse serum in vivo against E. coli injected into two strains of mice, one of which possessed all the components of complement while the other was deficient in the C'_5 component (Terry et al., 1964).

When a low virulence strain of B. pertussis was injected into these two strains of mice, no enhancement of infection was found in the mice deficient in the C'_5 component. From these experiments it was concluded that bactericidal complement played little or no role in determining the course of B. pertussis infections.

Having succeeded in reducing the level of infection of a low virulence strain by preinfection of the mice with the same strain, methods for the enhancement of infection were studied. Iida et al. (1963) were able to enhance the infection of a low virulence strain but not a high virulence strain by pretreatment of the mice with cortisone.

When mice were pretreated with cortisone, infections with all three low virulence strains, B124, B1772 and B2288, were highly enhanced (Fig. 17; Tables 8, 9). Little enhancement of infection could be detected on the first day after challenge, but after this time the viable count in the treated mice increased rapidly until mice were dying from the fifth day onwards with counts in excess of 10^7 organisms. The infection with a low virulence strain in cortisone treated mice mimicked very closely the infection with a high virulence strain in normal mice.

Contrary to the reports of Iida et al. (1963), it was found that cortisone enhanced infections with high virulence strains (Figs. 18, 19; Table 9). Enhancement was found from the first day onwards and treated mice died with slightly higher viable counts than untreated mice and at an earlier time. The individual counts were very closely grouped at each time interval studied, which probably indicates that any response of the mouse to infection with a high virulence strain has been largely inhibited.

Baron and Abelson (1954) showed that cortisone was a normal constituent of the cerebrospinal fluid (c.s.f.) of humans and Abelson et al. (1955) and Christy and Fishman (1961) found that the concentration of cortisone in the c.s.f. had increased by 10 times 4 hours after the oral administration of cortisone acetate.

De Duve *et al.* (1953) first described lysosomes and many workers (Wolf *et al.*, 1943; Naidoo and Pratt, 1951; Becker *et al.*, 1960) showed that ependymal cells contained a great many lysosomes which tended to be more numerous in the apical halves of the cells. Although most of these studies were made on the rat brain, a similar situation was shown to exist in the mouse brain (Wolf *et al.*, 1943).

Fell (1962) and Weissman (1964) both showed that hydrocortisone stabilised the lysosome membrane preventing release of the enzymes. Weissman and Thomas (1963) and Weissman *et al.* (1963) demonstrated the labilising action of vitamin A and streptolysins O and S on lysosome membranes. Similarly, Becker and Sutton (1963) and Sutton (1965) found that vitamin A increased the number and polarisation of lysosomes in the choroid plexus and suggested that lysosomes probably participated in the formation of the c.s.f.

Weissman and Thomas (1962) demonstrated that endotoxin increased the labilisation of lysosomes and that cortisone prevented subsequent release of enzymes. If the enzymes were bactericidal for *B. pertussis* release would lead to an eradication of infection.

It has been shown that small challenge doses of the low virulence strains multiply in the mouse for only 24 to 48 hours and it was suggested that the reason for this was that the release of enzymes had been stimulated by endotoxin and had

killed the low virulence strains. High virulence strains either did not stimulate release of enzymes or were not susceptible to their bactericidal action. Cortisone enhanced infection by preventing the release of the enzymes.

If vitamin A and streptolysin O stimulated the release of enzymes, pretreatment of the brains with these compounds should make the brains uninfectable with the low virulence strains. When these experiments were attempted a stimulation and not an early eradication of infection was found, contrary to expectation (Figs. 20, 21, 22).

In view of this, it was decided to measure the free and total amounts of four enzymes in the mouse brain during infection (Tables 10, 11, 12). No significant increase in the amounts of free or total enzymes was found for any strain tested.

No increase in the amount of free enzymes was found on treatment with vitamin A or streptolysin O and cortisone did not seem to reduce the free amount (Table 13). Berenbaum et al. (1960), Iida et al. and I have found in the mouse brain that bacterial multiplication was restricted to the surface of the ependyma but the enzyme measurements were made on whole brains. If there was a release of a quantity of enzymes into the c.s.f. from the ependyma only, this may not be detected in these determinations due to dilution by the whole brain.

Another action of cortisone is that it reduces the amount

of haemolytic complement in male and female mice (Caren and Rosenberg, 1966). In view of the earlier results with bactericidal complement, this action of cortisone was not thought to be important here. The enhancing effects on infection of cortisone, vitamin A and streptolysin O were not yet clear but Iida et al. (1963) showed that cortisone reduced the amount of circulating leucocytes for two or three days after treatment and streptolysin O is known to be lethal for polymorphonuclear leucocytes.

Another method known to reduce the number of circulating leucocytes is sublethal doses of X-irradiation (Bacq and Alexander, 1961).

In my experiments a dose of 400 R was found not to be lethal for mice and it reduced the number of circulating leucocytes to approximately one-tenth of the normal value (Table 14). When only the body was irradiated, with head protected, there was no enhancement of infection (Fig. 23). Both Berenbaum et al. (1960) and Iida et al. (1962) failed to find leucocytes in the brains of mice infected with a high virulence strain until the viable count was in excess of 10^6 organisms. Similarly, only an occasional polymorphonuclear leucocyte was found in sections taken from brains infected with low virulence strains.

From this evidence it was concluded that polymorphonuclear leucocytes were not important in determining the course of

infection of low virulence strains of B. pertussis.

However, when the heads and bodies, or heads alone, were exposed to irradiation doses as low as 400 R significantly enhanced infection.

When heads were irradiated three days prior to challenge, the infection was enhanced by irradiation doses of 400 R and 1000 R (Fig. 24). 1000 R irradiation was slightly more effective than 400 R irradiation and this was confirmed in an experiment when the number of deaths was recorded (Table 15). 1000 R irradiation resulted in approximately 25 - 30% more deaths from infection than did 400 R irradiation.

When the time interval between irradiation and challenge was extended from 3 days to 3 weeks infection after 1000 R irradiation was again enhanced more than the infection after 400 R irradiation. There were some signs of recovery in the terminal stages of infection after 400 R irradiation (Fig. 25).

This was confirmed when the time interval between irradiation and challenge was extended to 12 weeks. Again, the infection in 1000 R irradiated mice was markedly enhanced while the infection in the 400 R irradiated mice was only transiently enhanced and all these mice recovered from infection (Fig. 26).

Thus it seemed that irradiation of the head alone was sufficient to enhance infection of low virulence strains of B. pertussis.

Hopewell and Wright (1967) showed that with doses of 200 - 400 R the cerebral glial reaction to injury was diminished and

seemed to be independent of the time between irradiation and injury. They suggested that radiation damage was "stored up" and when the cells were stimulated to divide they either failed to divide or died. It was suggested that the majority of microglial cells were damaged with doses as low as 600 R but doses greater than this were needed to damage other cells e.g. astrocytes and oligodendrocytes (Hopewell - personal communication).

Examination of haematoxylin and eosin stained sections showed that infections with low virulence strains of B. pertussis in normal brains stimulated the division of many cells indicated by the number of mitotic figures seen. As early as 6 hours after challenge many mitotic figures were seen (Photograph 4) and clumps of cells, probably microglial cells, were found in the ventricles (Photograph 5). A similar reaction was found in response to a high virulence strain (Photograph 7). In the irradiated group of mice challenged with a low virulence strain mitotic figures were present, some of which showed abnormalities typical of radiation damage e.g. micronucleated cells (Photographs 9, 10), anaphase bridge (Photograph 11) and chromosome material left out of the division (Photograph 13). Mitotic figures seen in the ventricles suggested that these cells were mobile.

Konigsmark and Sidman (1963) suggested that brain macrophages were haematogenous in origin but autoradiographic

studies confirmed the above thesis that they were of local origin.

In the autoradiographic studies cells were labelled quite a distance from the ventricles (Photograph 18) whilst other cells which appeared to be in the ventricle were highly labelled (Photograph 20).

Some workers (Messier et al. 1958, Bryans, 1959, Altman, 1963) have suggested that the subependymal layer, a source of newcells, exists well into the adult life of rats and mice and that mitotic figures are evident under apparently normal conditions. However, a majority of these divisions occur in the ventricular region of the fore-brain and our sections were taken from the mid-brain where the subependymal layer is rare or absent.

It is my hypothesis that both high and low virulence strains of B. pertussis stimulate the division of neuroglial cells and the migration of these cells to the site of infection. These neuroglial cells, probably microglial cells, are able to phagocytose and kill the low virulence strains but not the high virulence strains. The high virulence strains owe their virulence to the property by which they are able to resist killing by neuroglial cells, in the 2 or 3 days after challenge, and by polymorphonuclear leucocytes in the terminal stages of infection.

The infection with low virulence strains is enhanced by

irradiation damage, cortisone, vitamin A and streptolysin-O because they inhibit normal cell division and migration of cells.

Evidence for this effect of cortisone was provided by several workers (Foley, et al., 1953; Field, 1957 and Clemente, 1958) who all showed that cortisone reduced phagocytosis and microglial cell migration around cerebral cortex brain lesions.

Similarly Langman and Welch (1966, 1967) showed that the treatment of mice and rats with large doses of vitamin A interfered with cell divisions resulting in the appearance of many abnormal cell types.

Streptolysin-O which destroys leucocytes may have the same effect on brain cells.

The site of multiplication of both high and low virulence strains seems to be identical, the ependyma (Photographs 22, 23 and 24). An intense leucocytic infiltration was seen in the terminal stages of infection with a high virulence strain (Photographs 8,22) and with a low virulence strain in irradiated brains (Photographs 15, 23).

The work on the response of the normal mouse to infections was extended to determine the response in passively and actively immunised mice and in mice challenged with immune serum organism mixtures as described by Dr. Spasojevic (Spasojevic, 1962).

The course of infection of high virulence strains in actively and passively immunised mice has been well documented as shown earlier. All were agreed that eradication of infection did not occur until damage to the blood brain barrier was obtained with an intracerebral viable count of approximately 10^6 organisms. The mode of eradication was not determined.

In the experiments described in this thesis mice were actively immunised, irradiated to either body alone, or head alone 14 days later and challenged 3 days after irradiation with a high virulence strain. When the number of survivors was compared with an unirradiated group of mice (Table 18) it was found that head irradiation made little difference to the number of surviving mice. This lack of effect on the number of mice surviving head irradiation in immune mice was confirmed when actively immunised mice and normal mice were irradiated and challenged with the same dose of a low virulence strain. Irradiation and challenge of the normal mice resulted in the death of most of the mice as expected from results described earlier but when these irradiated mice had been immunised only 2 of 23 mice died (Table 19). Thus although head irradiation had enhanced the low virulence strain infection, most immune mice survived which indicated that phagocytic cells of local brain origin were of little importance in active immunity, whereas cells of haematogenous origin were very important. Similarly when mice were passively immunised irradiation of

the body alone reduced the number of surviving mice (Table 17). Irradiation of the head did not alter the number of survivors.

Thus it seems that the important cell in the immune mouse is probably the polymorphonuclear leucocyte and that this cell in combination of antibody is capable of phagocytosing and killing high virulence strains of B. pertussis. Iida et al. (1963, 1966) showed that cortisone neutralised both active and passive immunity to B. pertussis but contrary to their expectations found a normal amount of leucocytes and antiserum in the ventricles though the organisms seemed to remain attached to the ependyma. A possible explanation of this action of cortisone is that it has depleted complement (Caren and Rosenberg, 1966) or prevented the release of small amounts of lysosomal enzymes which may be necessary for the detachment of organisms from the ependyma.

The main prerequisite for the elimination of organisms seemed to be the availability of specific antibody and this was not available until damage to the blood brain barrier occurred. However, when immune serum was suprainoculated intracerebrally one day after infection, a high proportion of survivors was recorded and it was found that the bacterial count decreased immediately after injection of the serum. It seemed, therefore, that although in the immune mouse both antibody and leucocytes were present when the count reached

10^6 organisms there might be another method for the elimination of organisms.

When the course of infection with immune serum-organism mixtures was determined it was found that the infection in the surviving mice was eradicated within 24 hours of challenge (Figs. 28, 29). It has been shown earlier that leucocytes were not present in the brain at this time and the results of experiments with mice deficient in the C'5 component of complement suggested that the bactericidal action of complement and antibody was not important in the immune mouse (Table 24). It was also found that when these immune serum mixtures were injected into cortisone treated mice (Table 23) the number of survivors was significantly lower than in the control groups.

From the earlier results it was found that these two types of treatment interfered with the division and migration of neuroglial cells. It seems probable therefore that in mice challenged with immune serum-organism mixtures eradication of organisms is by neuroglial cells and that pretreatment either by cortisone or irradiation prevents this eradication resulting in the death of the mice from infection.

This explanation of the action of cortisone will also account for the neutralisation by this drug of the modulating effect of growth on various media (Section 5.5).

The brain seems more able to deal with challenges of

immune serum and low virulence strains than it does with immune serum and high virulence strains.

There seemed to be a higher number of mice surviving challenge mixtures of immune serum with 2-Atox and G353 than with 18/323. This difference may be accounted for by the fact that only viable organisms were considered in the mixtures and as both G353 and 2-Atox are 40% viable whereas 18/323 is only 10% viable there are 4 times as many organisms, live and dead, in the 18/323 mixtures as in mixtures with G353 and 2-Atox. Thus there is more antiserum per organism with the latter strains which may give rise to a greater opsonization and thus a greater survival rate.

11. Conclusions

1. Strains of Bordetella pertussis were found to fall in one or other of 2 categories when tested by the intracerebral injection of mice. Some strains were of high virulence, some of low virulence.

2. The site of multiplication in the brain, with both types of strains, was shown to be the ciliated lining of the ventricles, the ependyma.

3. The courses of infection for doses of 5×10^3 viable organisms of the 3 high virulence strains tested were very similar to each other. There was a pause in the multiplication of strain G353 between the third and fourth days of infection not found with the virulent strains 18/323 and 2-Atox.

4. The courses of infection of the 3 low virulence strains tested were also very similar to each other. Challenge doses smaller than 10^5 viable organisms gave rise to a transient infection lasting 4 to 5 days. Doses between 10^5 and $10^{6.5}$ resulted in an infection of 8 or more days' duration but few mice died. Doses greater than $10^{6.5}$ resulted in the death of most mice in 2 to 3 days with terminal viable counts of $10^7 - 10^8$ organisms. This count was very similar to that found in the brains of mice dying from infection with a high virulence strain.

5. Approximately 90% of the challenge dose was lost within 1 hour regardless of strain, challenge size or challenge volume. This loss of organisms was probably due to washback along the needle track.

6. The decrease in number of organisms after the second day of infection with small doses of low virulence strains was not due to modulation of the organisms.

7. The low virulence strains are less sensitive to the bactericidal action of complement than high virulence strains. Infection with low virulence strains was, however, not enhanced in mice lacking the C'_5 component of complement.

8. The LD_{50} of the high virulence strains G353 and 2-Atox, but not of 18/323, could be increased by growth of these organisms either on a medium with high magnesium content at 34°C or on Bordet-Gengou medium at 25°C. When these organisms were injected intracerebrally into the mice, the brains of surviving animals were sterile 24 hours after challenge.

9. When mice were challenged with a low virulence strain and were rechallenged $4\frac{1}{2}$ days later with the same low virulence strain, no increase in the viable count was detected and many mice had sterile brains 2 days after rechallenge.

10. However, when the brains were reinfected with a high virulence strain the course of infection was little different from that in the control mice infected with the high virulence strain.

11. When mice infected with a low virulence strain were rechallenged at the peak of an infection by a high virulence strain many mice survived. This was thought to be due to interference immunity similar to that described by Evans and Perkins (1954b).

12. It is suggested that this type of interference immunity is due to competition for fixation sites on the ciliated lining of the ependyma.

13. It was also shown that preinfection with vaccinia virus or influenza virus increased the resistance of the brain to infection with high virulence strains of B. pertussis. Histological evidence suggests that this was not due to destruction of the ependymal cells. It is probable that this again is due to competition for fixation sites or destruction of these sites by these two viruses.

14. Treatment of the mice with cortisone acetate subcutaneously prior to challenge greatly enhanced the infection of low virulence strains, mice dying with terminal viable counts of 10^7 - 10^8 bacteria.

15. The same cortisone treatment also enhanced infection with high virulence strains, the mice dying at an earlier time with slightly higher terminal bacterial counts.

16. Pretreatment of the mice intracerebrally with either vitamin A or streptolysin-O also enhanced infection with low virulence strains.

17. Neither high nor low virulence strains seemed to stimulate the release of large amounts of lysosomal enzymes in the brain.

18. Prior irradiation of the head, with the body shielded, with doses as low as 400 R was sufficient to enhance greatly infections with low virulence strains. Enhancement of infection was still evident in mice receiving 1000 R to their heads 12 weeks before challenge.

19. Suppression of the circulating white blood cells by radiation did not enhance infection.

20. Histological evidence suggests that infections with B. pertussis stimulates neuroglial cells to divide but that after radiation they either fail to do so or die.

21. It is suggested that the neuroglial cells involved are probably the microglial cells and that these cells migrate to the infected sites, are able to phagocytose and kill the low virulence strains but not the high virulence strains. There is an intense leucocytic infiltration in the terminal stages of infection due to high virulence strains and in infection with low virulence strains in irradiated mice. It is probable therefore that both types of strain are able to resist killing by leucocytes.

22. The infection-enhancing effect of cortisone could be due to an ability to reduce phagocytosis and migration of neuroglial cells in brain lesions.

23. The similar effects of vitamin A are probably due to its ability to cause abnormal cell divisions in the brain. The reasons for the infection-enhancing effect of streptolysin-O is not clear though they may be similar to those for vitamin A.

24. Irradiation of the head did not affect the survival rate of actively and passively immunised mice but irradiation to the body, with the head shielded, significantly reduced the number of surviving mice. It is suggested that in the immune mouse bacteria are killed by polymorphonuclear leucocytes in combination with antibody and possibly complement.

25. When incubated mixtures of immune serum and organisms were injected intracerebrally, many of the mice yielded only a few bacteria from the brain 24 hours after challenge. The proportion with sterile brains did not increase after this time.

26. When these mixtures were injected into mice treated with cortisone acetate only a few mice survived. It is suggested that cortisone prevents the migration of the microglial cells which would otherwise have phagocytosed and killed the bacteria, and that the mice consequently die from infection.

27. Cortisone probably enhances infection with modulated organisms by the same mechanism.

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