

ANTIGENIC MODULATION IN BORDETELLA PERTUSSIS

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SUMMARY

SUMMARY

The sequence of loss of the immunological and pathophysiological activities of Bordetella pertussis during the process of antigenic modulation in liquid medium was investigated. Modified Hornibrook medium and Stainer and Scholte medium were used. Modulation was induced by growth of cells in the presence of high levels of magnesium sulphate and low levels of sodium chloride; cells grown in normal media containing high levels of sodium chloride and low levels of magnesium sulphate served as controls. The levels of the various activities present in the cultures and distributed between cells and culture supernatant were determined at intervals.

The leukocytosis-promoting activity was found to be lost during modulation along with other activities previously reported. An assay method for the quantitation of LPF potency was developed.

Modulation occurred within 10h of inoculation of X-mode cells into C-medium, and required about 1 to 2 cell divisions during which about 80% to 98% of each of the various activities of the X-mode was lost.

The various activities appeared to be lost at different rates. The mouse protective activity and the haemagglutinating activity were lost more rapidly than the histamine-sensitizing and leukocytosis-promoting activities; the heat-labile toxicity was lost at a slower rate. Loss of X-specific surface agglutinogens was shown but there appeared to be no

change in the lipopolysaccharide component of the cells.

Electrophoretic analysis of cell proteins revealed that 28k, 30k and 100k molecular weight polypeptide bands in the X-mode cells were absent or greatly reduced in the C-mode cells after 24h of growth. There was some evidence that no further synthesis of these polypeptides occurred after transfer of X-mode cells into C-medium.

The loss of activities in C-mode cells appeared to be due not only to the dilution of the activities among progeny cells and to liberation of cell components into the culture medium but also to the denaturation or degradation of these components.

Cells grown in media without added sodium chloride or magnesium sulphate also showed decrease in histamine-sensitizing activity but not to the same extent as cells grown in C-medium.

Results from this investigation support the view that at least four distinct cellular components are lost during modulation, namely, (1) a protective antigen/haemagglutinin component, (2) the histamine-sensitizing and leukocytosis-promoting component (3) the heat-labile toxin and (4) adenylate cyclase.

It is proposed that cyclic AMP may be involved in the control of the synthesis of components responsible for the various activities of B. pertussis. The level of cyclic AMP increased in parallel with cell numbers in X-mode cultures and was not detectable in C-mode cultures.

Modulation was shown to be a freely reversible process and when cyclic AMP formation was enhanced in reverting cells, the regeneration of the various activities also appeared to be enhanced.

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LIST OF ABBREVIATIONS

AMP	Adenosine-5'-Monophosphate
ATP	Adenosine-5'-Triphosphate
BG	Bordet-Gengou
cAMP	Cyclic Adenosine-3',5'-Monophosphate
C-mode	Cyanic mode: variant of <u>B. pertussis</u> produced during modulation
C→C	Growth of C-mode cells in C-medium
C→X	Growth of C-mode cells in X-medium
C.F.U.	Colony forming units
cpm	Counts per minute
DNA	Deoxyribonucleic acid
E.A.E.	Experimental allergic encephalomyelitis
HA	Haemagglutinin
HSE	Histamine-sensitizing factor
HSD ₅₀	Median histamine-sensitizing dose
HLT	Heat-labile toxin
IAP	Isletsactivating protein
I-mode	Intermediate mode between the X- and C-modes
IU	International Unit
k	Thousand
LD ₅₀	Median lethal dose
LPF	Lymphocytosis or Leukocytosis- promoting factor
LPS	Lipopolysaccharide
Mol. wt.	Molecular weight

MPT	Mouse protection test
M.R.C.	Medical Research Council
NAD	Nicotinamide adenine dinucleotide
No.	Number
o.u.	Opacity unit
PA	Protective antigen
P.H.L.S.	Public Health Laboratory Service
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
TEMED	N,N,N',N' tetramethylethylenediamine
Tris	Tris-(hydroxymethyl) aminomethane
WBC	White blood cells
W.H.O.	World Health Organisation
X-mode	Xanthic mode : normal staten of <u>Bordetella pertussis</u>
X→X	Growth of X-mode cells in X-medium
X→C	Growth of X-mode cells in C-medium
g	Gravitational force, unit of centrifugation

INTRODUCTION

SECTION 1 : TAXONOMY OF BORDETELLAPERTUSSIS

Bordetella pertussis belongs to the family Brucellaceae of the order Eubacteriales (Pittman, 1957).

The genus *Bordetella* is divided into three species : the non-motile *B. pertussis* and *B. parapertussis* and the motile *B. bronchiseptica*. These are minute Gram-negative coccobacilli; all are haemolytic; carbohydrates are not fermented; litmus milk is not made alkaline and dermonecrotic toxin is produced.

B. pertussis, the whooping-cough bacillus, was originally listed in the genus *Haemophilus* in an early generic arrangement of bacteria by the Committee of the Society of American Bacteriologists (Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith, 1920). At that time blood was considered necessary for the cultivation of the organism. The *parapertussis* bacillus was placed in the same genus (Bradford and Slavin, 1937; and Eldering and Kendrick, 1938). This organism causes about 5% and sometimes up to 20% to 30% of the reported cases of whooping-cough in the United States and Europe (Eldering and Kendrick, 1952; Lautrop, 1971; Borska and Simkovicova, 1972; and Linnemann and Perry, 1977).

Bordetella bronchiseptica which occurs frequently in the respiratory tract of rabbits, guinea-pigs, dogs and cats was isolated from a case of whooping-cough by Brown in 1926 (Medical Research Council, 1951), having previously been isolated from dogs (Ferry, 1911). Since then classification

of this organism has been in the genera Alcaligenes, Brucella and Haemophilus (Bergey's Manual of Determinative Bacteriology eighth edition, 1974).

Andersen (1953) showed that the three organisms, Haemophilus pertussis, Bacillus parapertussis and Brucella bronchiseptica had a number of features in common and believed that they represented closely related members of the same bacterial group. It was Moreno-Lopez (1952) who first proposed the generic term Bordetella for this group of organisms and re-named the organisms Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. This proposal was supported by the findings of later workers (Kendrick, Nadolski, Eldering and Baker, 1953; Proom, 1955; and Pittman, 1955). The proposal was accepted and the new genus, Bordetella, was included in the seventh and eighth editions of Bergey's Manual of Determinative Bacteriology.

Table 1 summarizes the most important differential characters of the three Bordetella species.

SECTION 2 : NATURAL HISTORY OF WHOOPING-COUGH

Whooping-cough, or pertussis, is an acute communicable disease, characterized by spasmodic attacks of coughing that are accompanied by an inspiratory whoop; there is lymphocytosis and sometimes a neurological complication. According to Lapin (1943), the disease was first described

Table 1: Differential characteristics of
the three Bordetella species

Characters**	<u>B. pertussis</u>	<u>B. parapertussis</u>	<u>B. bronchiseptica</u>
Flagella	-	-	+
Reduction of nitrates	-	-	+
Utilization of citrate	-	+	+
Urease	-	+	+(in 4h)
Oxidase	+	-	+
Catalase	+ or -*	+	+
Growth on peptone agar			
- growth	-	+	+
- browning of agar	-	+	-
Sensitization of mice to histamine	+	-	-
Specific heat-labile agglutinogens			
- factor 1	+	-	-
- factor 12	-	-	+
- factor 14	-	+	-
G + C content moles %	61	61	66

** Data selected from Lautrop (1960) and Pittman (1974).

* * 70% of freshly isolated strains are positive.

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in 1578 by Baillou and epidemics in England between 1670 and 1680 were described by Sydenham.

The organism causing pertussis was first observed and isolated in 1906 by Bordet and Gengou who named the Gram-negative coccobacillus "le microbe de la coqueluche". The aetiology of the disease was confirmed in 1933 by the MacDonalDs who inoculated their own children with a fresh isolate of the organism. (MacDonald and MacDonald, 1933).

Pertussis is non-invasive and in a typical case, there is an incubation period of seven to fourteen days, followed by three main stages : the catarrhal, the spasmodic and the convalescent (Bradford, 1959). Usually, the catarrhal stage lasts from one to two weeks and is accompanied by a low grade fever and a mild cough often starting at night and becoming progressively more intense and diurnal. The paroxysms may vary in number from a few to 20 or more per day. A severe paroxysm may last for 5-10 minutes, vomiting is frequent and convulsions and exhaustion may follow bouts of violent coughing. Occasionally, toxic encephalitis occurs, but the most frequent cause of death is secondary infection. At about the fourth week, the number and severity of the paroxysms decrease and the convalescent period begins. Full recovery from the infection can take up to several months (Bradford, 1957; Lorber, 1975).

Pertussis is widely distributed throughout the world, especially in highly populated and underdeveloped countries (Table 2). The infection can occur in any age group

Table 2: Incidence of pertussis in
selected countries

Country	No. of reported cases			
	1973*	1974*	1975 ⁺	1976**
Europe :				
United Kingdom	2398	15,998	9978	4369
Denmark	-	-	1457	16,385
France	560	326	367	353
Italy	12,084	8198	10,397	19,246
Americas :				
U.S.A.	-	1758	1583	927
Argentina	29,604	23,733	11,169	44,195
Venezuela	16,726	9562	9622	9204
Africa :				
Senegal	20,359	31,867	-	-
Algeria	-	-	1379	2862
Zaire	21,826	28,595	>19,000	18,975
Asia :				
Phillippines	20,570	-	-	-
Japan	-	-	1052	2471
Thailand	-	-	2846	1980
Oceania :				
Fiji	-	-	7	28
New Hebrides	508	154	52	30

Selected statistics from :

* World Health Statistics Reports (1975) 28

+ World Health Statistics Report (1976) 29, 416-419

** World Health Statistic Report (1977) 30, 360-364

(Bradford, 1959; Linnemann and Nasenbury, 1977) but is most common, most severe and has highest mortality rates in infants up to one year old or less (Bradford, 1957; Lorber, 1975; Kendrick, 1975). The disease occurs at all times of the year with a peak incidence during the winter months. Pertussis has no natural animal reservoir and no healthy carriers (Wilson and Miles, 1975). It is highly communicable, being transmitted directly by droplets from coughing or sneezing and the most infectious period is during the catarrhal or early paroxysmal stages. After household exposure practically all unimmunized contacts acquire the disease. Attack rates of 70% of all children in unvaccinated urban communities and 80% to 90% of children exposed to pertussis at home have been reported (Medical Research Council, 1951; Joint Committee on Vaccination and Immunization, 1977).

Little is known of the pathology of pertussis infection other than that the organisms localize and multiply among the cilia of the epithelium of the respiratory tract. The multiplying bacteria, without invasion of the underlying tissues, induce clinical symptoms and pathology of the disease which include lymphocyte infiltration of the peribronchial tissues and alveolar walls. (Pittman, 1970).

B. pertussis is sensitive in vitro to several antibiotics (Bass, Klenk, Kotheimer, Linnemann and Smith, 1969) but antibiotic treatment has been of little or no value in altering either the course or the morbidity of the disease

unless started before the paroxysmal stage (M.R.C. 1953; Pittman, 1970; Olson, 1975). However, some antibiotics notably erythromycin and co-trimoxazole may be prophylactically beneficial to children in a household where whooping-cough has been confirmed (Bass et al., 1969; Adcock et al., 1972; Alterier and Ayoub, 1977; Arneil and McAllister, 1977; Cullen and Cullen, 1978). Chloramphenicol, erythromycin, tetracycline or ampicillin can be used to control secondary infections during whooping-cough (Pittman, 1970; Lorber, 1975).

An attack of whooping-cough usually results in good immunity to the disease, although there have been a few reports of second attacks (Standfast, 1969). The basis of immunity in pertussis is not well understood. There is some evidence suggesting that immunity may not be mediated by serum antibodies (Martin, 1958; Butler, Wilson, Bensen, Dudgeon, Ungar and Beale, 1962; Brown, Volk, Gottshall, Kendrick and Andersen, 1964). There is also a suggestion that secretory antibodies may be involved (Geller and Pittman, 1973). Pertussis immune globulin (human) has been used for passive immunization of children exposed to pertussis infection but opinion varies as to its efficacy (Kabat, 1963; Balagtas, Nelson, Levine and Gotoff, 1971). Immunity to pertussis, either from infection or active immunization, lasts for only a few years (Pittman, 1970) and there is no passive transfer of immunity from mother to foetus (Lorber, 1975).

SECTION 3 : PERTUSSIS VACCINE
AND VACCINATION

Since an attack of whooping-cough usually results in a good immunity to the disease, it was reasonable therefore, to expect that some kind of bacterial vaccine might be effective in prophylaxis of the disease. Indeed many attempts dating back to 1914 have been made to achieve active immunization against pertussis (Ungar, 1963). Zachariassen quoted by Madsen, (1933) was the first to demonstrate a degree of protection from a plain vaccine given shortly before an epidemic of whooping-cough in the Faroe islands where only 67.5% of the vaccinated children contracted the disease compared with 98.2% in the unvaccinated group.

The first well controlled field trial in America was by Kendrick and Eldering (1939) who reported approximately 90% protection in school exposures and 60% in home exposure in vaccinated children compared with unvaccinated controls. Similar field trials were organised by the Medical Research Council in Britain from 1942-1944 but these were unsuccessful (McFarlan, Topley and Fisher, 1945). However, in a later series of well organized field trials in Britain, some vaccines were found to be highly effective and others of relatively low efficacy. Also there was a high degree of correlation between the results of mouse-protection tests and the protective power of vaccines in the field (M.R.C., 1951; 1956(a); 1956(b); 1959). From 1959, the intracerebral mouse-protection test of Kendrick et al., (1947) became a standard procedure in the control laboratory.

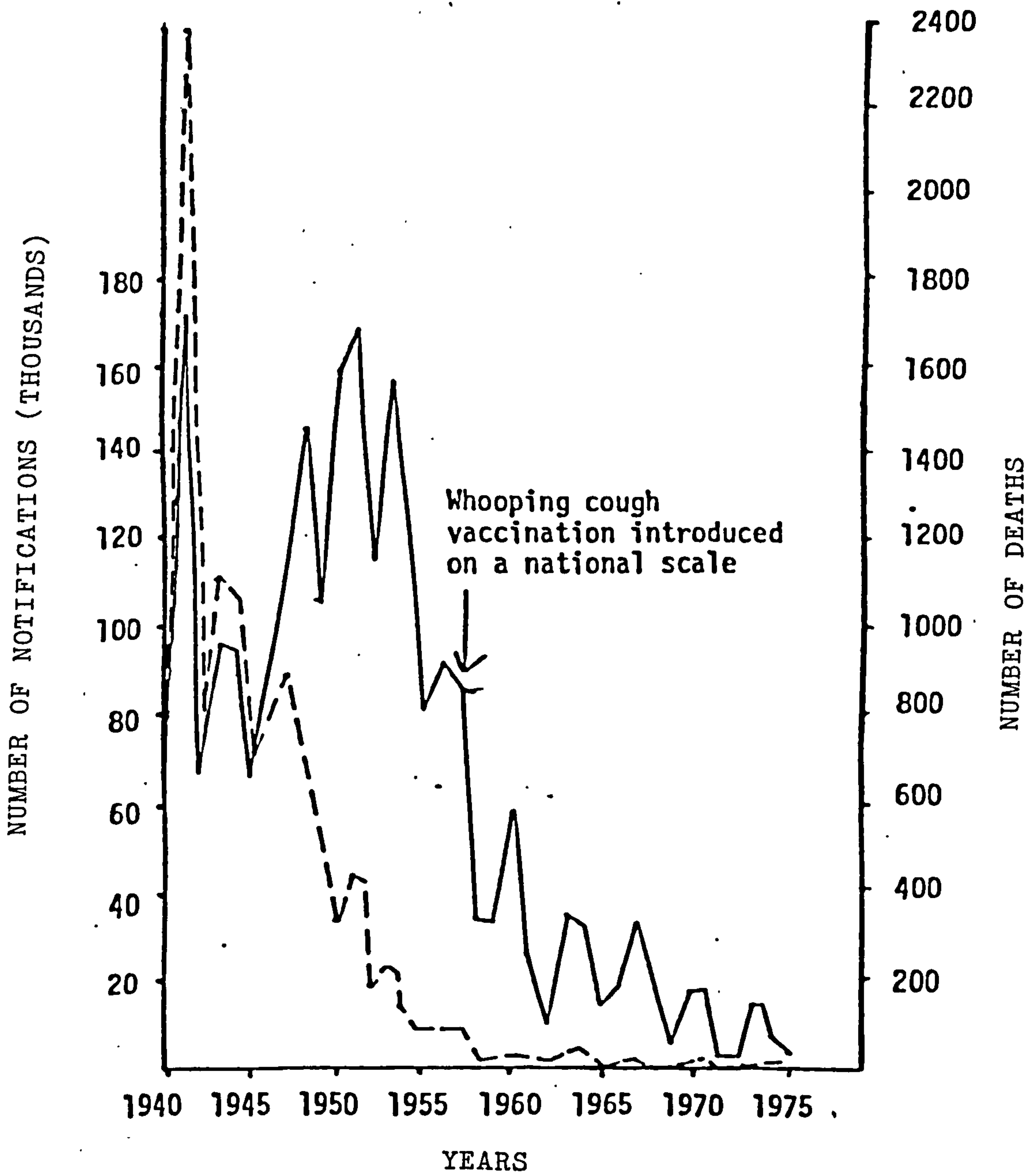
By 1964, this test was recommended by World Health Organization as the standard assay of vaccine protective potency prior to release (W.H.O., 1964). As a result of the success of the M.R.C. field trials, pertussis vaccine, consisting of inactivated whole cells of B. pertussis combined with diphtheria and later tetanus toxoids, was introduced on a national scale in Britain in 1957 (Joint Committee on Vaccination and Immunization, 1977). In Canada, the vaccine had been in general use since 1943 (Wilson, 1945).

After the introduction of a nation-wide immunization programme in Britain, there was a rapid drop in the incidence of pertussis (Cohen, 1963; Warin, 1968; Miller et al., 1974). The figures for the notifications and deaths due to pertussis in England and Wales from 1940-1975 are shown in Figure 1. In the 1960's however, the rate of drop in incidence of pertussis slackened and scattered outbreaks of the disease appeared in vaccinated children. Doubts about the efficacy of pertussis vaccine were raised when the Public Health Laboratory Service, (P.H.L.S.), reported that 56% of fully vaccinated children contracted pertussis following home exposure during the 1963-1964 epidemics. This report led Preston (1963; 1965) to examine freshly isolated strains of B. pertussis from cases of whooping-cough. He reported a change in the prevalent serotypes from types 1, 2 and 1, 2, 3 common before 1958 to type 1, 3 strains which were dominant in 1963-1964. Preston's work, later confirmed by the P.H.L.S.

Figure 1 : Whooping-cough. Notifications and deaths in England and Wales from 1940 - 1975.

————— notifications

- - - - - deaths



(PHLS, 1973), showed that the vaccines in use then lacked antigen 3 and thus the change in the serotypic composition of the prevalent bacteria might have accounted for the reduction in the efficacy of the vaccine from 1963-1968. Other reasons offered for the increase in the incidence of pertussis at that time were that some of the vaccines in distribution were prepared according to the British standard which contained a mouse protective potency of 2 International units per human dose as compared to the International standard with 4 International units per human dose. Also adjuvant was omitted from the British vaccines in the 1960's because of the danger of provoking poliomyelitis. Based on these observations, the following changes were recommended for the British vaccines in 1967:-

- 1) raising the mouse potency requirement from 2 I.U. to 4 I.U.
- 2) reintroduction of adjuvant and
- 3) inclusion of antigen 3.

Preston and Stanbridge (1972) analysed the efficacy of these new vaccines over the period 1969-1971 and concluded that a much better degree of protection was attained. Thus, despite minor setbacks, the incidence of the disease in Britain has fallen by about 98% since the nationwide campaign started, and at a rate that probably cannot be accounted for by natural trends e.g. changes in socio-economic conditions, general hygiene etc. (Perkins, 1970; Miller et al., 1974).

SECTION 4 : CULTURAL AND BIO-
CHEMICAL CHARACTERISTICS
OF BORDETELLA PERTUSSIS

Since B. pertussis was first described by Bordet in 1900, many attempts have been made to develop a suitable medium for the isolation and growth of the organism. The initial problem was that B. pertussis was originally thought to have complex growth requirements. However, later workers showed that the organism has simple growth requirements and that the initial problems were due to inhibitors in the media. These inhibitors can now be removed or inactivated in various ways. There was also the problem of some media not being able to support growth from small inocula. Most earlier media needed a large inoculum to initiate growth.

Bordet and Gengou (1906) isolated B. pertussis on a solid medium containing glycerinated-potato extract and 50% (v/v) whole human or rabbit blood. Despite many subsequent attempts to eliminate the blood (Krumwiede et al., 1923; Povitsky, 1923; Silverthorne and Cameron, 1942), it appeared to be essential for the growth of Phase I organisms, and seemed to indicate a complex set of growth requirements for B. pertussis.

Hornibrook (1939) was the first to grow the organism successfully in a liquid medium without blood. His medium consisted originally of hydrolysed casein, soluble starch, salts, cysteine and yeast extract. Later, in 1940 he modified the medium to a defined mixture of amino-acids in

place of casein, inorganic salts, cysteine, starch and nicotinic acid, the latter being essential for growth from small inocula. He noted that nicotinamide was also an effective growth factor for B. pertussis. Early trials showed that modifications were necessary in Hornibrook's medium because certain strains of B. pertussis grew poorly or not at all in the medium. Subsequent attempts at improvements, which mainly involved the alteration of growth factor source to liver extract, were not much better (Wilson, 1945; Farrell and Taylor, 1945).

Cohen and Wheeler (1946) described a modification of Hornibrook's medium with casamino acids, cysteine, inorganic salts, yeast dialysate and starch. They were the first and virtually the only workers to determine the optimum inorganic salt composition. However, their medium required a large inoculum of about 10^6 organisms ml^{-1} of medium. The medium gave a high level of protective antigen in the cells and was recommended by W.H.O. as the standard medium for vaccine production (W.H.O., 1953). With a few modifications, it is also the principle medium used in nutritional and metabolic studies on B. pertussis.

Taking into consideration all the problems encountered by earlier workers, Wilson, (1963) formulated a chemically defined liquid medium which was essentially a modification of Cohen and Wheeler's medium. It consisted of amino acids, glutamine, glutathione, inorganic salts, niacin, ascorbic acid, ATP, co-enzyme A, NAD, NADP and nucleic acid derivatives and tris (hydroxy-methyl) amino methane for

buffering actions. He also noted that the salts NaCl, KCl, MgCl₂ and CaCl₂ were required to keep the cells in suspension. However, the medium contained a large number of constituents (e.g. 20 amino acids and 24 other factors) and belies the simple nutritional requirements of B. pertussis.

Stainer and Scholte (1971) described the simplest chemically defined medium in which excellent cell yields were obtained. However, the medium was designed for vaccine production and requires large inocula; thus its suitability for metabolic studies is questionable.

Attempts to make solidified versions of early liquid media were unsuccessful unless blood or charcoal were added (Verwey et al., 1949; Ungar, James, Muggleton, Pegler and Tomich, 1950; Powell et al., 1951; Mazloun and Rowley, 1955; Jebb and Tomlinson, 1955 and Kuwajima et al., 1956; 1957). Sutherland and Wilkinson (1961) claimed to have developed a solid medium supporting the growth of low inocula. Their medium was semi-defined and contained an anion-exchange resin (Dowex-1, 0.1% (w/v)) to remove growth inhibitors. Parker (1976) described a solid medium which was a modification of Stainer and Sholte's medium but solidified with agarose. The medium supported the growth of small inocula and appeared to be very promising for future metabolic and genetic studies on B. pertussis.

While attempts were being made to formulate a suitable medium for the growth of B. pertussis, a number of growth inhibitors were identified by some workers. These included

unsaturated fatty acids (Pollock, 1947), colloidal sulphur (Proom, 1955; Rowatt, 1957(a); 1957(b)) organic peroxides (Rowatt, 1957(a)) and manganous ions (Parker, 1976). The fatty acids were neutralized by adding starch, charcoal or anionic resins to the medium (Rowatt, 1957(b); Kuwajima et al., 1957; Sutherland and Wilkinson, 1961). Rowatt (1957(a)) and Parker (1976), showed that the colloidal sulphur could be neutralized by albumin, activated charcoal or filter-sterilized cysteine, The effect of the organic peroxides was prevented by filtered (but not autoclaved) ferrous sulphate and haemin (Rowatt, 1957(a)), and the manganous ions were neutralized by the addition of red blood cells or haemin (Parker, 1976).

Hornibrook (1939; 1940) showed that nicotinic acid or nicotinamide was necessary for growth. Later workers (Farrell and Taylor, 1945; Ungar et al., 1950; Watanabe, Nishimura and Tonita, 1952; Imamura, 1952) confirmed the requirement for nicotinic acid. Farrell and Taylor (1945) and Watanabe et al., (1952) found thiamine to be essential to some strains and further reported stimulation of growth by riboflavin, p-aminobenzoic acid and pyridoxine.

Imamura (1952) showed that pertussis organisms would grow in a medium containing cystine and glutamic acid or proline as the only amino-acids. However, Proom (1955) stated that eight amino acids were needed for the growth of B. pertussis. A medium containing starch, glutamate, cystine, nicotinamide and salts was formulated by Jebb and Tomlinson (1957) but growth was slow and the yield of

bacteria was poor. Goldner, Jakus, Rhodes and Wilson (1966) and Vajdic, Goldner, and Wilson (1966) defined ten required amino-acids for growth of B. pertussis but showed that growth was limited by depletion of only three of these, namely glutamic acid, proline and glutamine. Some later workers grew B. pertussis with excellent yield and with good protective properties in a medium consisting only of glutamate, proline and cystine, with salts and growth factors (Stainer and Scholte, 1971). No starch or any other absorbent was necessary. It was earlier shown that B. pertussis uses glutamate for growth in preference to other amino-acids (Rowatt, 1955).

B. pertussis does not ferment or oxidise any of the common sugars (Jebb and Tomlinson, 1951). These workers showed the major metabolic activity of the organism to be oxidative deamination of amino acids with the formation of NH_3 and CO_2 . They further stated that the first step in the oxidation of glutamate was deamination with the production of α -ketoglutarate. Rowatt (1957 (a)) suggested that glutamate was the principal source of nitrogen for the cell, with only small amounts coming from other amino acids. Furthermore, only aspartic acid, proline and glutamine are thought to be able to replace glutamic acid as an energy and nitrogen source (Jebb and Tomlinson, 1955; Rowatt, 1957(a); Goldner et al., 1966) while other utilizable amino acids (serine, glycine and alanine) served only as sources of energy or possibly carbon. It was suggested that the oxidation of glutamic acid must be an efficient

process as 2mg of glutamic acid yielded 1mg of cells (Rowatt, 1957(a)). Goldner et al., (1966) and Rowatt (1957(a)) have proposed that a control mechanism must exist whereby, in a mixture of amino acids, glutamic acid is apparently utilized first. However, Lane (1970) was unable to confirm this.

The alteration of certain amino-acids levels in the culture medium may lead to changes in the antigenic characters of the pertussis cell. (Goldner et al., 1966; Stainer and Scholte, 1969 and Lane, 1970). This will be discussed later.

The pH requirement of the organism was also investigated by many workers. Hornibrook (1939) recommended pH 7.4 and Cohen and Wheeler (1946), pH 7.2-7.3.

The pH of the media were adjusted to these values before autoclaving. Ungar et al., (1950) found an optimal pH range of 7.6-7.85 but growth was very slow. Rowatt (1957(b)) found that the initial growth rate was the same from pH 6.9 to 7.7, although at pH 7.6-7.7 the growth rate became slower than at lower pH values. Consequently, Rowatt (1957) used the lowest possible value namely, pH 6.9-7.0 which gave a medium capable of taking up a larger quantity of alkali than when the initial pH was higher. Some ammonia in the casamino-acids was lost during autoclaving, so that to obtain a medium, containing 1% casamino-acids, at pH 6.9-7.0 it was necessary to adjust the pH to 7.2 before autoclaving.

SECTION 5 : PATHOPHYSIOLOGICAL AND
IMMUNOLOGICAL ACTIVITIES
OF BORDETELLA PERTUSSIS

Freshly isolated strains of B. pertussis elicit a variety of unique immunological and pathophysiological responses in man and certain experimental animals.

These responses include :-

- a) increased resistance of mice to experimental infection with B. pertussis,
- b) increased susceptibility to various forms of shock,
- c) increase in the number of circulating leukocytes, especially lymphocytes,
- d) increased susceptibility to autoimmune diseases,
- e) increased antibody production especially of the IgE class,
- f) many other responses usually associated with adjuvant action and with the enhanced susceptibility to shock,

A brief description of the various activities and the cell components thought to be responsible is given below.

i. Protective activity

From the point of view of preventive medicine the most important component of B. pertussis is the one responsible for immunizing children against pertussis infection (M.R.C., 1951; 1956). This component, called the protective antigen (PA), is probably the same as that responsible for

the protection of mice against intra-cerebral challenge with virulent pertussis organisms (Kendrick et al., 1947). Standfast (1951(b)), Dolby and Standfast (1958) demonstrated the presence of another antigen on the pertussis cell which protected mice against intranasal challenge with virulent pertussis organisms. Standfast (1958) showed that the intracerebral and intranasal protective antigens were not identical in that the former was heat-labile (100°C for 1h) while the latter was heat stable (100°C for 1h). Unless otherwise stated, it is the intracerebral PA that will be discussed here. The PA is believed to be a cell wall component (Munoz, Ribic and Larson, 1959; Billaudelle et al., 1960; Munoz, 1963; Wardlaw and Jakus, 1966; Munoz, 1971; and Zakharova 1978) but its precise nature remains unknown. Munoz (1963), Guerault (1962) and Olson (1975) showed that the protective activity of B. pertussis was destroyed by heat (80°C for 30 minutes), by proteolytic enzymes (trypsin, pronase) and by 45% (w/v) phenol indicating that the PA is protein in nature. This was later confirmed by the work of Jakus, McCluer and Wardlaw (1968) who also showed that the protective activity was lost at high pH. Munoz (1963) demonstrated the ability of the PA to withstand acetone indicating that lipid moieties were not essential for activity. Further evidence for the absence of carbohydrate in PA was shown by Wardlaw and Jakus (1966) who found that PA was insusceptible to attack by sodium metaperiodate, a reagent widely used to degrade carbohydrates.

In the early 1950's, three laboratory procedures were under active consideration as assay methods for pertussis PA. They were (a) the intracerebral mouse-protection test (Kendrick et al., 1947), (b) the intranasal mouse-protection test (Standfast, 1951b) and (c) the agglutinin production test (Evans and Perkins, 1953). However, the intracerebral test came to the fore as a result of the M.R.C. trials (1951; 1959) and is the only method recommended and presently accepted by the W.H.O.

Sutherland (1963) investigated the antigenic relationship of the protective antigen of Bordetella species and reported no cross reaction between the protective antigens of B. pertussis, B. parapertussis and B. bronchiseptica. Ross et al., (1969) reported that B. bronchiseptica and B. parapertussis did not protect against B. pertussis. Conversely, both B. pertussis and B. parapertussis protected against B. bronchiseptica. There is also evidence that infection with B. pertussis or B. parapertussis in man does not elicit heterologous immunity (Neimark et al., 1961)

ii. Histamine-sensitizing activity

Parfentjev and Goodline (1947; 1948) were the first to observe that the intraperitoneal injection of pertussis vaccine or cell extracts into mice made the animals up to 100-fold more sensitive to a later challenge injection of histamine or serotonin. The substance in pertussis vaccine responsible for this effect was named the histamine-sensitizing factor (HSF) by Maitland, Kohn and MacDonald (1955).

Of the three *Bordetella* species only *B. pertussis* possesses HSF (Ross et al., 1969).

HSF is very potent pharmacologically and once sensitization has been induced it may persist for months (Munoz and Bergman, 1966). Even small numbers of killed organisms when inoculated intranasally into mice were sufficient to induce histamine-sensitization (Geller and Pittman, 1973). HSF has been identified as a cell wall component (Yoshida et al., 1955; Munoz et al., 1959; Billaudelle et al., 1960; Sutherland, 1963; Preston and Garrity, 1967), which is protein in nature (Kind, 1956; Pieroni et al., 1965; Jakus et al., 1968), and carbohydrate is not required for activity (Wardlaw and Jakus, 1966). HSF isolated from intact cells contained a considerable amount of lipid (Lehrer et al., 1974) whereas HSF from culture supernatant fluids was lipid free (Morse and Morse, 1976). These latter workers suggested that this difference may be because HSF is closely associated with lipid when in the cell envelope and that this bond is broken on release of the HSF from the cells.

For some time the action of HSF was thought to be via the autonomic nervous system either by inducing a hyper-reactive state of the α -type receptors or by blockade of the β -type receptors (Fishel, Szentivanyi and Talmage, 1964; Bergman and Munoz, 1966; Pieroni and Levine, 1967; O'Bryan and Fishel, 1971). Recently there has been a counter report that *B. pertussis* does not induce β -adrenegic blockade

(Hewlett, Speigel, Wolff, Aurbach and Manclark, 1978; Parker and Morse, 1973). The latter workers have proposed that the primary effect of HSF is to alter cyclic AMP metabolism in the host either by an effect on intracellular ATP concentrations or by interfering in some way with adenylate cyclase or phosphodiesterase activity.

Thus the mechanism of action of HSF is still obscure. In addition, its role in the pathogenesis of whooping-cough is uncertain though Pittman (1970) has suggested that the apparently neurogenic nature of the paroxysmal cough of pertussis may be a manifestation of HSF.

iii. Lymphocytosis-promoting activity

Fröhlich (1897) cited by Morse (1965) first noted that leukocytosis occurs in cases of whooping-cough. In clinical infection or when experimental animals are inoculated with B. pertussis there is an increase in the overall number of circulating leukocytes, due mainly to an increase in the lymphocytes (Tuta, 1937; Morse, 1965). The component of the bacterium responsible for this increase is called the leukocytosis-promoting factor or lymphocytosis-promoting factor (LPF). Lymphocytosis has been reported to be less frequent in patients under six months of age (Lagergren, 1963) and to be absent in adults infected with B. pertussis (Mannerstedt, 1934; Lagergren, 1963; Linnemann and Nasenbury, 1977). In mice inoculated intravenously with whole cells or culture supernatant maximum leukocytosis was observed 3-5 days after injection

and then declined to base-line levels in 2-3 weeks (Morse, 1965; Morse and Bray, 1969).

Other workers have described a toxic component of B. pertussis which on inoculation into mice shows late-appearing toxicities (LAT) such as late body weight loss and late death of mice (Kurokawa, Iwasa and Ishida, 1965; Pittman and Cox, 1965; Iwasa et al., 1968; Ishida, 1968; Kurokawa, Ishida, Asakawa and Iwasa, 1978). The late toxicities occur after body weight loss due to endotoxin and deaths due to heat-labile toxin. This LAT component was also shown to have lymphocytosis-promoting activity.

Much work has been done to elucidate the mechanism of the lymphocytosis (Morse, 1965; Morse and Riester, 1967a; 1967b; Iwasa et al., 1970; and Morse and Adler, 1973). Although the dynamics of the response are still obscure, the main effect appears to be the redistribution of the mobilizable lymphocyte pool from tissue to blood rather than the rapid production of new cells.

Chemically, LPF was shown to be mainly protein (Morse and Bray, 1969) and to contain some carbohydrate (Sato and Arai, 1972; Morse and Morse, 1976). Using partially purified preparations of LPF, Sato and Arai (1972) and Lehrer et al., (1974) concluded that LPF contained up to 50% lipid. However, this was not confirmed by Morse and Morse (1976) who found no lipid in LPF. Sato and Arai (1972), Sato et al., (1973) suggested that LPF was identical with the filaments, which measured about 2x40nm, appearing on the

surface of B. pertussis, but again Morse and Morse (1976) could not confirm this observation. The molecular weight of LPF obtained by different purification methods has been estimated at 108,000 (Sato and Arai, 1972) and at 87,000 (Morse and Morse, 1976). Recent work (Sato, 1978) has shown LPF to be a spherical molecule about 6nm in diameter and with a molecular weight of 110,000.

In the laboratory, the results of LPF assays have been shown to be influenced by a number of factors which include :- the nature of the vaccine or preparation (Morse and Bray, 1969), the route of inoculation and the route of bleeding (Morse, 1965).

LPF has been reported to be absent from B. parapertussis (Morse and Morse, 1970) but there are other reports that children infected with this species may demonstrate a striking lymphocytosis (Zueler and Wheeler, 1946; Shih Man, 1950).

iv. Haemagglutinating activity

Keogh and North (1947; 1948) demonstrated that a component of B. pertussis was capable of agglutinating red blood cells of chickens and other animals and attempts have been made by several workers to isolate and characterize this component. Masry (1952) showed that in the early stages of growth of B. pertussis in liquid medium, the haemagglutinin (HA) was cell-associated, but in the later stages of growth most of the HA was extracellular. He further showed that HA was produced by most freshly isolated strains. The exact

nature of HA is still not clear. though recent studies by Morse and Morse (1976) showed it to be markedly sensitive to heat and to proteolytic enzymes and therefore must be protein. Electron microscopic examinations of Phase I B. pertussis cells revealed surface appendages commensurate with pili or fimbriae (Morse and Morse, 1970), and Sato et al., (1974) showed these pili to be similar in appearance to HA. This was confirmed by Morse and Morse (1976) who reported that the HA properties of B. pertussis resided in the filamentous particles.

Ara. ~~Arai~~ and Sató (1976) reported the separation and characterization of two distinct types of HA. One of these (LPF-HA) had very low HA activity and high LPF activity and the other (F-HA), which was associated with the fimbriae, had very high HA activity and no LPF activity. These workers further reported that the haemagglutinins had different susceptibilities to papain and that morphologically the F-HA consisted of filamentous molecules of approximately 2 x 40 nm, while the LPF-HA had a spherical structure approximately 6nm in diameter.

Irons and MacLennan (1978) stated that LPF-HA was produced under variable conditions of culture while the production of F-HA appeared to be favoured in static, poorly aerated cultures. They further reported that pure serum sialoproteins such as haptoglobin and ceruloplasmin, but not transferrin, are powerful inhibitors of LPF-HA activity. Salivary and bronchial mucins are also inhibitory to the HA.

Purified LPF-HA binds specifically to haptoglobin and this property has been used as the basis of an affinity chromatography method for purification. On the other hand, F-HA is unaffected by sialoproteins (Irons and MacLennan, 1978), but it is specifically inhibited by cholesterol (Fisher, 1949; Sato, 1978). Using sodium dodecylsulphate polyacrylamide gel electrophoresis and sucrose density gradient centrifugation methods, Arai and Satō (1976) estimated the molecular weights of F-HA at 126,000 and 133,000 respectively. The molecular weights of LPF-HA by the same methods were given as 107,000 and 103,000 respectively.

Antisera against the two haemagglutinins did not cross react in neutralization reactions, and the dominant serological specificity of LPF-HA was expressed only by Phase I B. pertussis organisms whereas that of F-HA can be detected in preparations from Phase IV organisms and in other species of Bordetella. (Irons and MacLennan, 1978).

The F-HA and LPF-HA are readily distinguished by the character of the erythrocyte agglutination they produce in test tubes. F-HA produces a diffuse pattern of settling of the red cells which can be dispersed by the gentlest resuspension; but the LPF-HA, on the other hand, is a true agglutinin which binds red cells into a coherent sheet that can be dispersed only by vigorous shaking. However, there has been no evidence of in vivo red blood cell agglutination in either natural or experimental infection (Morse, 1976).

v. Heat-labile toxicity

While B. pertussis produces a superficial infection of the respiratory tract, pathologically, there is a profound necrotising inflammation of the respiratory tract mucosa, extending through it to some depth. This suggests that toxins are produced by B. pertussis and indeed a number have been described. They have been designated variously as dermonecrotic toxin, heat-labile toxin, lethal factor and lieno-toxin; but recent evidence suggests that these various toxins may be identical (Banerjea and Munoz, 1962; Kurokawa, Ishida and Asakawa, 1969; Iida and Okonogi, 1971)

When given intraperitoneally, intravenously or intracranially, the toxin is lethal to various animals (mice, guinea-pigs and rabbits) and when given subcutaneously or intracutaneously it is dermonecrotic. This toxin has been designated heat-labile toxin (HLT) because heating at 56°C for 30 min. destroys its activity. This further distinguishes it from endotoxin which is heat-stable. The mode of action of the HLT is not known.

Its protein nature was demonstrated by Banerjea and Munoz (1962) and Munoz (1971). One of the striking characteristics of HLT in contrast to many other bacterial protein toxins is that it is not a true exotoxin. It is located in the bacterial cytoplasm and the bulk of the activity is intracellular. (Munoz et al., 1959; Billaudelle et al., 1960; Banerjea and Munoz, 1962; Munoz, 1963; 1971). The HLT is found in smooth, intermediate and rough cells of

B. pertussis (Flosdorf et al., 1941; Roberts and Ospeck, 1942; Kuwajima et al., 1958). Some strains contain more toxin than others (Lawson, 1933; Robert and Ospeck, 1942; Kasuga et al., 1954; Kuwajima et al., 1958) but usually, recently isolated cultures contain most toxin (Lawson, 1933). The amount of HLT produced by a particular strain of B. pertussis depends on the medium in which the cells are grown (Munoz, 1971). Lane (1968) found that the toxin appeared in the culture supernatants during the early logarithmic phase of growth. The HLT is found in all three species of *Bordetella* (Strizova and Trilfajova, 1964).

Although no relationship between virulence of B. pertussis and toxin production has been found (Standfast, 1951), several early workers believed that HLT was responsible for much of the observed symptomatology and pathology of whooping-cough (Gallavan and Goodpasture, 1937; Sprunt and Martin, 1943; Asada 1953a; b; c; Uchida et al., 1957; Kuwajima et al., 1958).

However, the immunology of HLT has been investigated and the opinion of some workers is that the toxin does not appear to be involved in establishing active immunity to pertussis infection since little or no antitoxin is demonstrable in convalescent sera. (Evans, 1942; 1944; Ospeck and Roberts, 1944; Proom, 1947; Verwey and Thiele, 1949; Cravitz and Williams, 1946 cited by Munoz, 1971).

vi. Heat-labile agglutinogens

Certain surface components of B. pertussis are capable of inducing agglutinating antibodies with which the organism will react in vitro. These components of the cell are the agglutinating antigens or agglutinogens. Andersen (1953) reported that the agglutinating, agglutinin-absorbing and O-agglutination-inhibitory powers of these antigens were destroyed by heating for one hour at 120°C though their immunogenic properties were not completely destroyed.

Serological studies of the agglutinogens showed the existence of serotypes of B. pertussis which possessed various combinations of agglutinogens (Andersen, 1953). Eldering, Hornbeck and Baker (1957) postulated 14 different antigenic factors (agglutinogens) which they designated agglutinogens 1-14. The various agglutinin combinations in the three species of Bordetella are shown in Table 3.

Agglutinogens 1-6 are found in cultures of B. pertussis only, and agglutinin 7 in all Bordetella species. Agglutinin 14 is specific for B. parapertussis, and agglutinin 12 for B. bronchiseptica. Agglutinogens 8, 9 and 10 account for the relationship between B. parapertussis and B. bronchiseptica. Later workers reported five serotypes of B. pertussis, each containing the principal heat-labile type 1 antigen and one or more of the heat-labile type 2, 3, 4 and 5 antigens (Preston and Te Punga, 1959; Preston and Evans, 1963; Preston, 1963; 1965). The most important antigen combinations appear to be 1, 2; 1, 3; and 1, 2, 3 (Cameron 1967). Chalvardjian (1965) and Preston (1965)

Table 3: Agglutinin content of the three species of Bordetella*

Strains	Agglutinogens (Factors)
<u>B. pertussis</u>	
strain 5373	7, 1, 3, 6, 13
strain 5374	7, 1, 2, 5, 6, 13
strain 5375	7, 1, 2, 4, 13
<u>B. parapertussis</u>	
strain 17-90 3	7, 8, 9, 10, 14
<u>B. bronchiseptica</u>	
strain 5376	7, 8, 9, 12, 13
strain 214	7, 9, 12, 13
strain 899	7, 8, 10, 11, 12

* From Eldering, Hornbeck and Baker (1957).

showed that type 1, 3 was the most commonly isolated at that time.

Agglutininogen 1 is known to be highly antigenic and easily extractable from the cells (Munoz, 1963). This agglutininogen has been purified and shown to be a simple protein of molecular weight 10,000 (Onoue, Kitazawa and Yamamura, 1961). The agglutininogen is not associated with the antigen inducing protection in mice (Schuchardt et al., 1963) and its relation to protection in children is not clear. However, Preston (1963; 1965; 1966) linked the type specific agglutinogens 2 and 3 as well as the species specific agglutininogen 1 with immunity to pertussis infection in man. He suggested that the pertussis epidemic in Britain between 1963-1964 was due to the lack of agglutininogen 3 in the vaccine then used.

vii. Heat-stable toxin (endotoxin or lipopolysaccharide)

Substances which have similar properties to the lipopolysaccharide-protein complexes isolated from the enteric group of bacteria have also been isolated from B. pertussis and other species of Bordetella (Cruickshank and Freeman, 1937; Eldering, 1942; MacLennan, 1960; Farthing, 1961). 1962).

There is very limited information on the chemical composition and structure of the lipopolysaccharide (LPS) of B. pertussis. Although hexosamine, aldoheptose, hexose, 2-keto-3-deoxyoctonate (KDO), D-glucosamine, D-glucose and lipid have been found in B. pertussis LPS (MacLennan, 1960;

Nakase et al., 1970; Aprile and Wardlaw, 1973a; Konstantinov, Kalinova and Mihailova, 1978), the precise nature and organisation of the monosaccharide units making up the polysaccharide moiety of the molecule is not known (Morse, 1976). Recent workers have reported two non-identical polysaccharide chains within the B. pertussis LPS (LeDur, Caroff, Chaby and Szabo, 1978). Each of these polysaccharides was shown to be linked to a complex Lipid A moiety of the LPS.

Thus the LPS of B. pertussis appears to be different from those elaborated by enterobacteria. The exact monosaccharide structure and arrangement of B. pertussis LPS is important because of the number of serologically specific reactions which can be obtained with the isolated LPS (Aprile and Wardlaw, 1973b). Milner et al., (1963) claimed that the electron microscopic appearance of B. pertussis LPS was that of fibres with diameters of 50-100nm.

Although the exact role of endotoxin in human pertussis is not known, the endotoxin content of the organism has been shown to contribute to certain biological effects observed after inoculation into experimental animals. Among these biological effects are the leukopenia in sheep, goats and calves (Rai et al., 1971), the induction of certain kinds of heterologous immunity (Iida and Tajima, 1971) adjuvant effects (Farthing, 1961; Farthing and Holt, 1962), sensitization to histamine (Malkiel and Hargis, 1964; Kuratsuka and Homma, 1975), an effect on certain transplanted

tumors (Malkiel and Hargis, 1961) and the induction of interferon release in cell cultures (Kojima, Yoshida and Nakase, 1973).

viii. Islets-activating protein

Recent in vivo and in vitro studies have shown that much more insulin is secreted in response to various stimuli from the pancreas of rats injected with B. pertussis cells than from the pancreas of normal rats (Sumi and Ui, 1975; Katada and Ui, 1976; 1977; 1978). This unique action of the bacterium has recently been attributed to the so-called Islets-activating protein (IAP) purified from the culture medium of B. pertussis strain Tohama (Phase I) (Yajima, Hosoda, Kanbayashi, Nakamura, Nogimori, Mizushima, Nakase and Ui, 1978a).

Purified IAP, when injected intravenously at a dose as low as 0.02 μ g-0.1 μ g, doubled the insulin secretory response of rats to glucose loading three days later. The maximal, about 12-fold, response was obtained with 1 μ g of IAP. The action persisted for longer than one month, with the peak value from 3 to 10 days after injection (Yajima, Takahashi, Kurokawa, Nakase and Ui 1978b; Yajima et al., 1978a; Toyota, Kakizaki, Kimura, Yajima, Okamoto and Ui, 1978; Ui, Katada and Yajima, 1978).

Purified IAP injected into mice at higher doses of more than 0.5 μ g exhibited HSF and LPF activities. Lower doses were effective in potentiating the insulin-secretory response of mice and dogs without inducing leukocytosis. Thus, IAP

appears to potentiate the insulin secretory response of animals rather selectively (Yajima et al., 1978b). Injection of IAP at a dose of 10 μ g/Kg into spontaneously diabetic animals successfully improved the diabetic state for a month. (Goto, Kakizaki and Masaki, 1975; Toyota et al., 1978).

Further studies showed purified IAP to be mainly protein with a carbohydrate content of 1.0-1.5% and no lipid. The molecular weight by polyacrylamide gel electrophoresis was estimated to be 77,000 (Yajima et al., 1978a; Ui et al., 1978). Upon incubation with 8M urea, the IAP molecule was dissociated into three subunits, F-1, F-2, F-3, none of which was biologically active by itself. However, the same biological activity as the native IAP was recovered when F-3 was incubated with either one of the other subunits. No biological activity was detected when F-1 was combined with F-2. Thus, the subunit F-3 was essential for the biological activities of IAP while F-1 and F-2 were only permissive (Ui, et al., 1978).

Since the enhancement induced by IAP is comparable to the potentiation of insulin secretion observed upon pertussis vaccination of rats, it is very likely that IAP is one of the factors responsible for the diverse pathophysiological actions of B. pertussis.

ix. Adjuvanticity

Immunological adjuvants potentiate the immune response to a given antigen. Numerous micro-organisms and some of

their structural components, such as the endotoxins from Gram-negative bacteria, possess significant adjuvant activity (Johnson et al., 1956; Neter, 1969). B. pertussis appears to be of special interest because pertussis organisms function as an adjuvant to other bacterial components which may be present in vaccines.

There is increasing evidence that B. pertussis is an effective adjuvant, for example, in the induction of experimental encephalomyelitis (Levine et al., 1966), a disease which is largely due to direct activity of sensitized lymphocytes rather than serum antibody and is therefore a product of the cell-mediated immune response.

The organism has also been found to augment markedly the sensitization of mice to inhaled antigens (Chang and Gottshall, 1972). This finding correlated with the observation that the administration of pertussis organisms enhanced the production of reaginic antibody (Binaghi and Benacerraf, 1964; Reed et al., 1972; Tada et al., 1972) an IgE-like homocytotropic antibody whose production is regulated by thymus-dependent lymphocytes. The most recent evidence suggests that it is the effect on T-lymphocyte helper functions which result in an augmented humoral immune response in general (Taub et al., 1972). The organism seems to contain no components affecting the secretory immune response (Tada et al., 1972).

B. pertussis has been reported to decrease the growth of tumours implanted into experimental animals (Likhite, 1974) and this effect may also be due to enhanced cell-mediated

immunity, although critical evidence on this point has not been obtained. Conversely there are instances where pertussis apparently enhances the growth rate of tumours (Floersheim, 1967; Likhite, 1974).

The factor or factors of B. pertussis involved in these immunological reactions has not been defined. One candidate for the role of adjuvant in B. pertussis is endotoxin, since endotoxins from other Gram-negative organisms are known adjuvants. However, a heat-labile component is also believed to be important in pertussis-induced adjuvanticity, and it has been suggested that this component plays a role in the cell-mediated reactions (Levine et al., 1966; Levine and Pieroni, 1966).

The mode of action of B. pertussis and other adjuvants is still unknown. In principle B. pertussis may affect the multiplication of antibody-producing cells, increase the antibody-sensitizing activity of cells or act by a combination of these two mechanisms (Finger, 1975).

x. Mitogenicity for lymphocytes

Recent work (Kong and Morse, 1975; 1976) has revealed that pertussis culture supernatant fluids contain a potent mitogen for murine lymphocytes. The active material appears to be identical to LPF. Spleen and lymph node cells respond fully but pertussis mitogen does not stimulate normal thymocytes.

A variety of experimental procedures have been employed to delineate the proliferating cell and there is evidence that it is the T-cell that responds, although a second cell type, probably the macrophages, is required to initiate the stimulatory events, perhaps by processing the mitogen (Morse, 1976).

Recent studies by Kong and Morse (1975; 1976; 1977a; 1977b) showed that human lymphocytes also proliferate in response to the pertussis mitogen. This response was not due to sensitized lymphocytes since cord blood cells were also stimulated. The type of human lymphocytes involved, B or T, has not as yet been established.

xi. Adenylate cyclase

Wolff and Cook (1973) first detected the presence of adenylate cyclase activity in the supernatant of a merthiolate preserved whole cell pertussis vaccine. This observation stimulated further studies on the adenylate cyclase of B. pertussis. Hewlett and Wolff (1976) demonstrated the presence of adenylate cyclase in the supernatant fluid of exponentially growing organisms. This indicated that the adenylate cyclase might be an exoenzyme and would therefore differ from previously studied bacterial adenylate cyclases, which are intracellular and either membrane associated or soluble in the cytoplasm (Ide, 1969). Hewlett and Wolff (1976) purified the enzyme by ion-exchange chromatography and gel filtration and showed that from its presence in the culture supernatant,

its low molecular weight (70,000), its insensitivity to α -keto-acids and nucleotides, and its inhibition by fluoride, the enzyme from B. pertussis differed from other bacterial adenylate cyclases previously described.

Hewlett, Urban, Manclark and Wolff (1976), proposed the following four compartments of adenylate cyclase in B. pertussis: (1) Soluble enzyme in the culture supernatant (up to 20% of the total activity); (2) enzyme associated with intact cells and measurable without cell disruption (20% - 45%); (3) extracytoplasmic enzyme sensitive to trypsin, but not measurable in intact cells at standard substrate concentration (40% - 60%); (4) intracellular enzyme (7% - 9%). These workers further added that in comparison with previously studied bacterial adenylate cyclases, the extracytoplasmic location appears to be unique to the B. pertussis enzyme.

Some workers measured the adenylate cyclase activity in a number of commercial pertussis vaccines and observed that although these vaccines met the required limits for potency, toxicity, adjuvant content and organism concentration, they differed significantly in their adenylate cyclase activity (Hewlett, Manclark and Wolff, 1977). Although no explanation was offered for these differences, they speculated that several factors might be influencing the persistence of the enzyme activity during the handling of the organisms in the preparation of vaccines.

The activity of adenylate cyclase was shown to vary with growth medium. On Bordet-Gengou agar with rabbit

blood, the organism had very high adenylate cyclase activity (approximately 10-1000nmol cAMP/min/mg protein), but when grown on a synthetic Stainer and Scholte medium, the adenylate cyclase activity decreased as much as by 1000-fold (Hewlett, Underhill, Vargo, Wolff and Manclark, 1978). These workers isolated from rabbit erythrocytes a protease-sensitive activator which increased the adenylate cyclase activity of B. pertussis organisms grown in Stainer and Scholte medium by 100-1000 fold.

Parton and Durham (1978) recently reported the loss of adenylate cyclase activity in degraded strains of B. pertussis. This observation is currently directing the interest of workers towards attempting to determine whether adenylate cyclase is associated with any of the well known toxic effects of B. pertussis and what its role may be in regulating the metabolism of the organism. Cyclic AMP in other organisms appears to play an important regulatory role. In E. coli, for example, cyclic AMP appears to control many cellular functions of the type that are not required under all conditions of growth (Rickenberg, 1974).

SECTION 6 : INTERRELATIONSHIP BETWEEN
THE VARIOUS IMMUNOLOGICAL
AND PATHOPHYSIOLOGICAL
ACTIVITIES

Numerous attempts have been made to isolate, purify and characterize the cell components responsible for the pathophysiological activities of B. pertussis. The protective antigen (PA) has been particularly intensively investigated.

An aspect of recent fractionation studies which has generated much interest is the relationship between the PA and the HSF. Early reports indicated that both the PA and HSF activities could be attributed to the same fraction of the cell envelope (Maitland and Guerault, 1958; Munoz, Ribi and Larson, 1959; Niwa, Hiramatsu, Kawasaki and Kuwajima, 1965; Nakase, Fujita, Takatsu, Yoshioka and Kasuga, 1965; Munoz and Hestekin, 1966).

Levine and Pieroni (1966) postulated a "Unitarian Hypothesis" in which they stated that PA, HSF activities and other pathophysiological activities of B. pertussis were attributable to a heat-labile protein substance which was presumably the whooping-cough immunogen. This "Unitarian Hypothesis" was supported by results of certain other workers who showed that purified preparations of HSF still had PA activity (Munoz and Bergman, 1968). Further work by Manclark, Hansen, Treadwell and Pittman (1975) suggested some relationship between the PA and HSF when they reported that mice of a particular line were more sensitive to HSF and more immunisable with pertussis vaccine, and vice versa. The most recent support for the Unitarian Hypothesis was demonstrated by the "Pertussigen Theory" of Munoz (1976). He isolated a substance "pertussigen" from B. pertussis which contained PA, HSF, LPF, IgE and EAE adjuvant activities. However, the purity of this substance is questionable.

Contrary to the Unitarian Hypothesis, other workers indicated that the PA and HSF were attributable to different components of the cell (Dolby, 1958; Pusztai, Joó and

Juhasz, 1961; Griffith and Mason, 1964; Wilson and Jakus, 1970). Using different colonial forms, Cameron (1976) reported some discrepancies between the levels of HSF and PA in the resulting cultures. Zakharova (1978) reported that the PA was located in the cytoplasmic membrane while the LPF, HSF and endotoxin were located on the outer structures of the cell wall. From fractionation studies on B. pertussis, Nagel and Graaf (1978) obtained a sediment which contained endotoxin and PA but was devoid of HSF and LPF activities. Nakase et al., (1972) found that purified preparations of HSF had no significant PA activity in the mouse. Partially purified PA had no detectable HSF activity (Nakase, 1978).

Apart from the HSF, the relationship between the PA and the other pathophysiological properties has been studied. Keogh and North (1948) suggested that the HA was a PA. Sato's fimbriae-haemagglutinin fraction had PA activity (Sato et al., 1974; Arai and Sato, 1976; Sato, 1978). The relation between HA and PA was also suggested by Morse and Morse (1976). However, Nakase et al., (1975) showed that their purified preparation of HA had no significant PA activity in mice but it was not clear which of the two haemagglutinins they isolated.

The relationship between the PA and agglutinogens has also been examined. Agglutinogens had no PA activity (Andersen and Bentzon, 1958; Schuchardt, Munoz, Verwey and Sagin, 1963; Eldering, Holwerda and Baker, 1966; Nakase et al., 1969; Nakase et al., 1970; 1971; Ross and Munoz,

1971, Zakharova, 1978). But other workers reported the converse : that agglutinogens had PA activity (Preston, 1963; Preston and Evans, 1963; Preston, 1966; Sato, 1978).

Nagel and Graaf (1978) in their fractionation studies obtained a sediment which contained endotoxin and some PA activity. A relationship between the LPF and PA activities has also been reported (Nakase, 1978).

Lehrer, Vaughan and Tan (1975; 1976) partially purified the HSF component and found it also had LPF activity and enhanced the production of homocytotropic but not of conventional antibody. It has also been shown that highly purified LPF served as an adjuvant for reaginic antibody production (Tada et al., 1972). Morse and Morse (1974; 1976) purified LPF, which also induced histamine-sensitization, hypoglycemia, unresponsiveness to the hyperglycemic effect of epinephrine and in vitro proliferation of lymphocytes.

Two distinct types of HA were separated and characterized by Arai and Satō (1976) and one of these was shown to have LPF activity. Sato (1978) purified LPF which possessed high HSF activity and also showed strong T-cell mitogenicity, T-cell adjuvant and insulin secretive activities.

The IAP was shown to stimulate insulin secretion, suppress epinephrine hyperglycaemia and induced lymphocytosis and histamine-sensitization. The IAP appears to be very similar to the LPF preparation of Morse and Morse (1976).

Other reports on the interrelationship of the pathophysiological properties have shown that the heat-stable

toxin (endotoxin) has (a) some HSF activity (Malkiel and Hargis, 1964; Kuratsuka and Homma, 1975), (b) an adjuvant effect (Farthing and Holt, 1962) and (c) LPF activity (Kurokawa, Ishida, Asakawa and Iwasa, 1978).

Thus, there is still no conclusive evidence either for or against the relationship between PA and HSF or other pathophysiological activities because of the heterogeneity of the preparations so far obtained. From evidence available it appears that there are at least five components which may contribute to the immunological and pathophysiological properties of B. pertussis ; 1) component with PA and HA 2) component with LPF, HA, HSF, IAP, heat-labile adjuvant and mitogen, 3) endotoxin, 4) HLT and 5) adenylate cyclase.

SECTION 7: ANTIGENIC VARIATION IN B. PERTUSSIS

One of the most striking features of B. pertussis is its great propensity for variation both culturally and serologically. Two main types of variations have been described; Phase variation, which is a genotypic change during which the organism undergoes a series of mutational changes; and antigenic modulation, which is a freely-reversible phenotypic process induced by changes in the environment.

i. Phase variations

Bordet and Sleswyk (1910) cited by Aprile (1971), first observed that anti-sera raised against old cultures of B. pertussis maintained on nutrient agar did not agglutinate fresh isolates grown on Bordet-Gengou medium and vice-versa. Krumwiede, Mishulow and Oldenbusch (1923) also observed that strains grown on chocolate agar fell into two different serologic groups. Leslie and Gardner (1931) studied these phenomena in greater detail. They analysed 32 strains of B. pertussis by agglutination and agglutinin absorption and distinguished four serologically and culturally different groups which they called phases I, II, III and IV. They suggested that Phases I and II corresponded to the smooth or pathogenic forms of other bacteria and Phases III and IV to the rough, relatively avirulent forms. They also showed that a culture derived from a single colony could be converted by subculture on various media, through Phases II and III to the stable Phase IV. This work was essentially confirmed by Lawson (1932) who was however unable to obtain Phase II. Flosdorf, Dubois and Kimball (1941) also confirmed the existence of Phases I, III and IV but failed to find Phase II. These workers suggested that variation in B. pertussis was considerably more complex in minor relationships than stated by Leslie and Gardner (1931). Shibley and Hoelscher (1934) suggested that the terms smooth and rough be used for the stable Phases I and IV respectively without differentiation of the intermediate forms.

Phase IV strains have lost many of the biologically active components associated with Phase I or fresh isolates. These include PA (Kasuaga et al., 1954; Aprile, 1972), HSF (Kind, 1953; Aprile, 1972; Parton and Wardlaw, 1975), agglutinin 1 (Eldering, Eveland and Kendrick, 1962), HA (Keogh and North, 1948; Standfast, 1951), 28k and 30k envelope polypeptides found in Phase I strains (Parton and Wardlaw, 1975) and adenylate cyclase activity (Parton and Durham, 1978).

Parker (1976) proposed a "genetic hypothesis" for degraded Phase IV strains whereby Phase I to Phase IV transition was affected by a series of spontaneous mutations and selected for by the presence of inhibitors in the medium. This hypothesis suggests that intermediate strains will show a variety of types, depending on the sequence of mutations, but leading ultimately to the fully degraded Phase IV strains.

Earlier workers thought that Phase I strains were of a single homogeneous serotype (Leslie and Gardner, 1931; Standfast, 1951). However, Andersen (1952) clearly showed the existence of several Phase I serotypes. The works of Andersen (1953); Eldering, Hornbeck and Baker (1957), Preston and Te Punga (1959); Preston (1963; 1965) and Preston and Evans (1963) showed five serotypes in B. pertussis, each containing the principle heat-labile type 2, 3 antigens. The most important antigen combinations appear to be 1, 2; 1, 3; and 1, 2, 3.

Cameron (1967) suggested that B. pertussis undergoes a stepwise loss of heat-labile antigens converting a parent type 1, 2, 3 to a degraded strain of type 1. This suggestion was supported by similar findings in the child (Preston and Stanbridge, 1972) and in the marmoset (Stanbridge and Preston, 1974a) in which these workers showed that the parent (1, 2, 3) and the intermediates (1, 2; 1, 3) could each establish infection and could change to a different serotype during the course of the infection. Type 1 organisms were found only at a late stage of the infection and even then, did not constitute a predominant serotype.

Further study by Stanbridge and Preston (1974b) showed that the four main serotypes of B. pertussis (1, 2, 3; 1, 2; 1, 3; 1) undergo spontaneous variation involving loss or gain of antigen 2 or antigen 3. By serial subculture from single colonies on charcoal-blood-agar medium they detected loss mutations from type 1, 2, 3 to 1, 2 or 1, 3 and from type 1, 2 to type 1. Similarly, they found gain mutations from type 1 to 1, 2 or 1, 3 and from 1, 2 to 1, 2, 3. They reported that these mutations occurred with a high frequency in some strains, while other strains had a low mutation rate and were more stable antigenically.

Dolby and Bronne-Shanburry (1975) induced L-forms with glycine and by a combination of antibody and complement. The L-forms were called "1" variants because they contained agglutinin 1 but lacked agglutinogens 2-6. There was little difference in HSF, HA and mouse weight-gain toxicity between the parent types and their "1" variants and it

seemed that properties other than the agglutinogens were unchanged. Vaccines made from "1" variants protected mice challenged intracerebrally with virulent strains of B. pertussis.

These various findings appeared to explain the changes in serotype that occurred during the course of a pertussis infection in the child and in the marmoset. They also indicated the possible antigenic instability of laboratory strains, especially relevant in the production, absorption and testing of diagnostic antisera and in the preparation of pertussis vaccine.

ii. Antigenic modulation

A number of environmentally induced phenotypic changes have been reported for B. pertussis. The two, most fully described are "Antigenic Modulation" (Lacey, 1960) and changes due to growth in high levels of nicotinic acid (Pusztai and Joo, 1967).

Lacey (1951; 1960), while developing a selective medium for B. pertussis, observed that a crude rabbit serum against B. parapertussis which agglutinated B. pertussis to a titre of 800 failed even at a titre of 2, to agglutinate the same strain of B. pertussis grown on BG medium containing $MgSO_4$ instead of NaCl. Further experiments showed that the change was not the result of mutation and selection, for it occurred simultaneously in the whole population, was regularly reproducible, completely reversible within one subculture and shown by all freshly isolated strains.

Lacey showed that this change was quite different from the relatively stable, unpredictable and mutational phase variation described by Leslie and Gardner (1931). He called this variation "antigenic modulation" and described three antigenic modes : X, I and C which were equilibrium states in a continuous change from X- to C- mode. X stands for "xanthic" which describes the yellowish-ochre hue of confluent growth of freshly isolated strains on Bordet-Gengou medium containing NaCl; C for "cyanic", describes the greyish-blue appearance of growth on BG medium containing a high level of $MgSO_4$. The I-mode is the intermediate state between these two extremes.

Lacey (1960) reported that the following factors can cause modulation - (a) the ionic composition of the growth medium, e.g. $MgSO_4$ in place of NaCl (b) growth at low temperatures, e.g. $28^{\circ}C$ and (c) miscellaneous factors, e.g. fatty acids, tellurite or old blood. More detailed study on the effect of salts showed that merely suspending the X- and C- mode cells in salt solutions did not cause modulation and this indicated that cell division was necessary for modulation to occur. Conclusions about modulation were based on differences in agglutinability of the modes with X and C antisera and with B. parapertussis antiserum from a variety of animals. By replacing the NaCl in the medium with a chemically-equivalent amount of another salt, he investigated the influence of over 100 salts on modulation. He concluded that cations and anions had independent potencies for modulation, and that it was

the ratio of the ionic species which determined the final mode of the cells. Furthermore, he studied the combined effect of salts and temperature and showed that they were independent but that the sum of their individual potencies for modulation determined the final mode of the cells. He showed that at 25°C, cells grew in the C-mode irrespective of the salt added to the medium. During growth at any temperature above 27°C, the mode changed from X through I to C as the proportion of the MgSO₄ was increased. Similarly at any NaCl:MgSO₄ ratio greater than 2:1 a similar change of mode occurred as the temperature was lowered.

From calculations involving the equivalent concentrations and conductance ratio of salts, Lacey (1960) concluded that butyrate and glutarate had about the same pro-C-mode power as sulphate but in both the mono- and dicarboxylic acid series, pro-C mode power rose steeply with increase in chain length. Sodium pelargonate was seventy times more powerful (equivalent for equivalent) than magnesium sulphate and was the most active pro-C substance so far discovered.

Lacey (1960) postulated an agglutinogen, "X-antigen", which induced agglutinins to B. parapertussis and X-mode B. pertussis. This agglutinogen was present on the surface of the X-mode cells but "serologically submerged" in the C-mode. He further identified a C-mode agglutinogen but found it to be a very poor immunogen compared with the X-antigen. Whereas, the X-antigen induced a high slide

agglutination titre of 2560 or more by every route and in almost every individual animal injected, the C-mode antigen was unproductive in raising antibody to intraperitoneal, intracardiac and intravenous inoculations of various animals except turkey and fowls. In rabbits, intravenous inoculation with C-antigen yielded titres of up to 640 but only when the animal was first given a subdermal injection of C-cells two weeks before the intravenous course. Thus apart from rabbit and fowl, no other animal was found to produce useful amounts of antibody to C-mode B. pertussis. In both rabbits and fowls the C-mode antibodies appeared to be maximal about seven days after a course of five to six inoculations. Thereafter, they diminished even if further inoculations were given. This contrasted with the X-mode titre which increased with extra inoculations. Lacey also reported a number of other characters which were altered during modulation of X to C mode (Table 4).

His results showed that in the change from X- to C-mode, equilibrium was reached between the 21st and 36th hour which was after 7 to 12 subdivisions. On the time sequence of loss of characters, Lacey (1960) observed that under pro-C mode conditions, the HA activity of the X-mode disappeared long before the X-antigen was lost. The HA activity returned rapidly under pro-X mode conditions and this suggested that HA, though highly associated with the X-antigen, may not be entirely dependent on the presence of the X-antigen.

Table 4: Distinguishing characteristics of the X- and C- mode cultures *

Character	X-mode	C-mode
Zone of haemolysis	0.5mm	nil
Colour of confluent growth	ochre	pale greenish blue
Haemagglutinin titre	64	< 2
Agglutination titres in sera :		
i. to <u>B. parapertussis</u> grown on BG at 35°C	1280	< 2.5
ii. to <u>B. bronchiseptica</u> grown on BG at 35°C	2560	< 2.5
iii. of normal rabbits	6 of 120 > 5	All of 120 < 2.5
LD ₅₀ Mice inoculated :		
i. intraperitoneally	c. 2×10^8	c. 2×10^9
ii. intranasally	c. 10^7	c. 10^{10}

* from Lacey (1960)

Summing up his results, Lacey (1960) speculated that modulation in B. pertussis was not a saltative change but a process of continuous change leading at equilibrium to one of a large number of antigenic states. He postulated two possible hypotheses to explain modulation. The first was that modulation could be attributed to changes in the position of constant amounts of antigens and the second that modulation could be attributed to changes in position accompanying or resulting from changes in amount of antigens. In the first case, environment would primarily determine which antigen, solely because of its position, would determine the antigenicity. In the second case, the environment would primarily determine the rates of synthesis of the various antigens; the most rapidly synthesized would be present in greatest amount and for this reason would both dominate the antigenicity and monopolize the surface. He further stated that the first hypothesis could not explain how low temperature or the ionic balance had an effect on modulation. In contrast, since the influence of the physico-chemical environment on synthesis of antigens in other organisms and on enzyme activity is well known, the second hypothesis seemed much more probable.

Lacey (1960) investigated primarily the haemagglutinating and agglutinating properties of the X- and C- mode cells. With the exception of virulence, he did not investigate the other pathophysiological properties associated with freshly isolated strains (X-mode). However, later workers have shown that many of the properties associated with the X-mode cells are lost during modulation. These properties include

PA activity, HSF activity, 28k and 30k cell envelope polypeptides (Parton and Wardlaw, 1975; Wardlaw, Parton and Hooker, 1976), HLT (Livey, Parton and Wardlaw, 1978), adenylate cyclase (Parton and Durham, 1978) and heat-labile adjuvant for reaginic antibody production and hyperacute EAE (Wardlaw, Parton, Bergman and Munoz, 1979). These workers noted the loss of these activities in modulated C-mode cells but gave no attention to the time of loss or the sequence of loss of the various pathophysiological activities during the actual process of modulation.

Apart from antigenic modulation, other phenotypic variations have been reported.

Pusztai and Joó (1967) described a phenotypic change which occurred when B. pertussis Phase I strains were grown in high levels of nicotinic acid. Such cells had lost the PA, HSF and agglutinogens but the HLT was unaffected. The process was completely reversible by subculture onto normal medium containing low levels of nicotinic acid and this was thought to be the result of impaired cell wall synthesis as only cell wall antigens (PA, HSF and agglutinogens) were affected and not the cytoplasmic antigens e.g. HLT. Wardlaw et al., (1976) showed that growth in high nicotinic acid, but not in high nicotinamide, resulted in a loss of PA and HSF. They also showed, on electrophoretic analysis, that the cell envelopes of the high nicotinic acid cells had a protein profile almost identical to those of Phase IV and C-mode cells i.e. two envelope polypeptides (28k and 30k) associated with the normal Phase I cells were lost.

Andersen (1952) observed differences in the colony sizes of B. pertussis after a series of intracerebral passages in mice. She showed that the small colonies were more virulent, more toxic and more easily agglutinable than the large colonies.

Rowatt (1957) reported that a freshly isolated strain of B. pertussis grown on horse blood-agar became avirulent and filamentous but the same strain grown on sheep or human blood-agar remained virulent and was coccobacillary.

There are also reports of changes in the pathophysiological activities resulting from alterations in the carbon source of the growth medium. Goldner et al., (1966) reported that an increase in the glutamic acid content of the growth medium increased the antigenic potency of the cells. Replacing casein hydrolysate in the growth medium with glutamate also led to loss of toxicity, HSF potency and intracerebral protective potency. Stainer and Scholte (1971) reported a reduction of the mouse-protective activity and agglutinability of Phase I strains after 29 serial transfers in one of their chemically defined media. They showed that only 5%-10% of the original mouse-protective activity was present after 21 transfers.

The reports discussed in this section tend to confirm the unique ability of B. pertussis to undergo a wide range of genotypic and phenotypic variations. Recently Bemis, Greisen and Appel (1977) examined the biochemical, cultural and morphological characteristics of B. bronchiseptica

isolates from dogs and other species and reported a type of phase variation which involved changes in colonial morphology, haemagglutination, haemolysis, flagellation and fimbriation. In addition, Kumazawa and Yoshikawa (1978) reported the conversion of B. pertussis to B. parapertussis by treatment with N'-methyl-N'-nitro-N nitrosoguanidine. They postulated that B. parapertussis was a mutant of B. pertussis which appeared in these experiments probably by the selective pressure of antibiotics. It is interesting to note that the apparent mutation of B. pertussis to B. parapertussis resulted in a gain of the ability to split urea and utilize citrate. It also resulted in a loss of the histamine-sensitizing ability.

SECTION 8 : PRESENT DAY CONTROVERSY
OVER PERTUSSIS VACCINES AND
ATTEMPTS TO PRODUCE A SAFER AND
MORE EFFECTIVE VACCINE

Despite the reported control of pertussis infection by immunization, a number of problems are still associated with the present day vaccines.

Almost all pertussis vaccines in current use are whole cell preparations. Because the present vaccines are manufactured by various companies, they vary in quality, safety and efficacy according to the production strains of B. pertussis, the method of manufacture and quality control procedures (Griffith, 1978). Even vaccines produced by the same company have been found to vary from batch to

batch. These problems are related to the fact that the antigenic profile of B. pertussis strains varies continuously with environmental conditions of growth (Lacey, 1960; Goldner et al., 1966; Lane, 1970; Stainer and Scholte, 1971).

The other problem of present vaccines has been the possible side effects of the vaccine on children. Such side effects include shock, abnormal screaming fits, vomiting, convulsions and brain damage. The most controversial at the moment is the reported occasional brain damage associated with vaccination. Byres and Moll (1948) first reported that brain damage appeared to follow the use of the vaccine. This complication was so uncommon that its incidence was difficult to estimate. Strom (1967) reported the incidence of brain damage in Sweden as 1 in every 170,000 children vaccinated, whereas, Dick (1974) put the incidence in the United Kingdom at 1-2 in 10,000. This statement has been challenged though not substantiated by Griffith (1974). Kulenkampff et al., (1974) reported 50 cases of brain damage, in children, recorded from January 1961 to December 1972 in a London hospital. Dick (1974) and Kulenkampff et al., (1974) raised serious questions regarding the safety of the vaccine and this stimulated considerable interest in the neurological complications following pertussis vaccination. This interest was fostered by the formation in Britain of an Association of Parents of Vaccine-Damaged Children which sought some compensation from the government.

Stephenson (1977) and Ounstead (1977) pointed out the chance association of side effects and immunization from the fact that pertussis vaccines are routinely administered to the infants at an age when serious clinical disorders of unknown causes are particularly likely to occur or become evident. Stewart (1977) suggested that the incidence of brain damage following pertussis vaccination might be 1 per 168,000 injections, but Wilson (1977) reported that there was no case of reported brain damage in the 180,000 children that received immunization in the Greater Glasgow area from 1961-1975. The report of Wilson was supported by Grist (1977) whose survey found no cases of brain damage in the whole of Scotland from 1968-1969. Bassili and Stewart (1976) and Stewart (1977) claimed that the changing socio-economic conditions have been more important than vaccination in the reduction of the incidence of pertussis and that the risk of serious neurological complications following pertussis vaccination was too great to justify national use of the vaccine. They therefore advocated the withdrawal of the vaccine. This led the Joint Committee on Vaccination and Immunization in Britain to review the vaccination problems of whooping-cough in 1977. The Committee concluded that the risks of vaccination were far outweighed by the risks of infection and recommended that vaccination should continue (Joint Committee on Vaccination and Immunization Report, 1977).

As a result of this controversy, there has been a decline in the acceptance rate of the pertussis vaccine.

The acceptance rate in 1977 was 40% or less as compared to 70%-80% before 1974 (Stuart-Harris, 1978). This present low acceptance rate may in part be responsible for the sharp outbreak of pertussis which began in October 1977 and in fact the intensity of the infection as judged by notifications in the first quarter of 1978 was higher than at anytime since the 1950's.

These present problems have prompted the need for a safer and more effective vaccine. However, a major difficulty at present in preparing an entirely satisfactory pertussis vaccine is the inability to identify precisely, by reliable laboratory tests, the antigen or antigens necessary for the protection of children. The mouse-protection test used in control of pertussis vaccines gives some guide to the efficacy in children but the test cannot be regarded as precise and the immunological mechanisms involved are not understood. Furthermore, there are no in vitro tests by which the protective antigens of pertussis vaccine can be identified or assayed. Because of these gaps in knowledge, pertussis vaccines are at present prepared as whole bacterial suspensions. As stated earlier, B. pertussis cells have an array of antigens, some of which are protective and others quite toxic and appear to be responsible for some of the reported side effects. To produce a safer and more effective vaccine, it is necessary to retain the protective antigen and remove or inactivate the toxic components of the cells.

One of the possible approaches to this is the isolation and purification of the biologically active components of

B. pertussis. This will help identify and purify the actual component responsible for protection and eventually lead to replacing the present whole-cell vaccine with preparations containing the purified protective immunogen extracted from the cells. This approach is at the moment attracting the interest of many workers (e.g. Nagel and Graaf, 1978; Nakase, 1978; Helting and Blackkolb, 1978).

Another approach to a safer and more effective vaccine might be to utilize the ability of B. pertussis to undergo antigenic variations. Since it has been reported that the manipulation of cultural conditions affects the amount of various antigens present on the bacterial surface, it might be possible to determine cultural conditions which would yield cells with high levels of protective antigens and very low levels of toxic components.

OBJECT OF RESEARCH

OBJECT OF RESEARCH

Lacey (1960) originally showed that during antigenic modulation of B. pertussis, surface agglutinogens, haemagglutinin and mouse virulence were lost in C-mode cells. Later workers reported that the protective antigen, the histamine-sensitizing factor, the heat-labile toxin and adenylate cyclase activity were also lost in C-mode cells. (Wardlaw et al., 1976; Livey et al., 1978; Parton and Durham, 1978). However, in all these studies, there was no indication of the time-sequence of loss of these various properties during modulation.

Furthermore, because of a number of undesirable toxic side effects associated with the present vaccine, efforts are being made by many workers to develop a vaccine free from toxicity. Attempts to isolate and purify a single potent, protective antigen from the numerous other biologically-active components of B. pertussis, or to produce cells with altered levels of activities by manipulation of cultural conditions, have so far not been successful.

The aims of this investigation were :

- (a) To determine if the immunological and pathophysiological properties associated with X-mode cells were lost at the same rate or at different rates during modulation.
- (b) To determine whether one component or several distinct components of the cell were responsible for the various pathophysiological activities.

- (c) To explore the possibility of utilizing the process of modulation for the production of a safer vaccine by attempting to produce cells with high protective activity and low toxicity.
- (d) To attempt to determine the mechanism of antigenic modulation.

MATERIALS AND METHODS

SECTION 1 : BACTERIOLOGICAL MATERIALS
AND METHODS

i. Strains

Four Phase I strains of B. pertussis were studied :-

Strain 18334, a vaccine strain from the Connaught Laboratories Limited, Toronto;

Strain 134, a strain originally used by Pillemer (1950) in the preparation of his protective antigen;

Strain 18323, the original mouse-virulent Kendrick strain used in the mouse-protection test (Kendrick, Eldering, Dixon and Misner, 1947);

Strain D30042, originally supplied by Dr. Jean Dolby of the Lister Institute of Preventive Medicine, Elstree, Hertfordshire, England.

All strains were maintained in the freeze-dried state

ii. Media

The following were used for the cultivation of

B. pertussis :-

Charcoal agar (Difco Laboratories, Detroit, Michigan, U.S.A.).

Bordet-Gengou medium (Gibco) containing 20% (v/v) citrated horse blood (Gibco Bio-Cult Diagnostics, Glasgow, Scotland).

A modified Hornibrook medium (Parton and Wardlaw, 1975)

based on the original recipe of Hornibrook (Hornibrook,

1939). For C-mode growth, the 0.5% (w/v) NaCl in modified

Hornibrook medium was replaced by 0.5% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,

other ingredients being the same. The medium with added

NaCl is designated X-medium and the medium with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, C-medium (Parton and Wardlaw, 1975).

Stainer and Scholte defined liquid medium (Stainer and Scholte, 1971). C-mode growth in Stainer and Scholte medium was obtained by replacing the 0.25% (w/v) NaCl in the medium with 0.5% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

The composition and preparation methods for all media are given in Appendix 1.

iii. Mice

Mice used were from a randomly-bred closed colony originally derived from the Ham/ICR strain (Charles River, U.K. Ltd., Manston Road, Margate, Kent).

Mice from a similar colony of strain NIH originally obtained from Anglia Laboratories (Alconbury, Huntingdon, Cambridgeshire, England) by the Wellcome Institute of Parasitology, Veterinary College, University of Glasgow, Scotland, were also used in some experiments.

iv. Growth of cultures

Freeze-dried cultures were reconstituted with 1% (w/v) casamino acids solution and grown on charcoal agar slopes or on Bordet-Gengou agar plates.

Slopes and plates were incubated at 35°C for 2-3 days. Plates were placed in a closed plastic box containing a beaker of water to saturate the atmosphere.

Seed cultures were grown by inoculating 2-litre dimpled conical flasks, containing 500ml of Hornibrook X- or C-medium

with a loopful of cells from growth on Bordet-Gengou plates incubated for 72h. Flasks were incubated in an orbital incubator (Gallenkamp, London) at 80 r.p.m. and 35°C for 48h.

For larger scale growth, 2-litre dimpled flasks containing 1-litre amounts of X- or C-medium, were inoculated with 50ml or 100ml of seed culture with sterile 50ml pipettes. Flasks were then incubated in an orbital incubator at 80 r.p.m. and 35°C. Cultures were harvested at various intervals during growth. Purity of each culture was checked by Gram staining and by inoculation onto nutrient agar (Oxoid) plates.

Challenge cultures for the mouse protection test had been passaged several times through mouse brain to enhance virulence. These cultures were suspended in casamino acids solution containing 20% (w/v) glycerol, sealed in sterile plastic ampoules (Sterilin Ltd., Teddington, Middlesex, England) and stored frozen in liquid nitrogen. For use, ampoules were allowed to thaw at room temperature and the suspension was plated onto two or three Bordet-Gengou plates which were then incubated at 35°C for 48h. Fresh Bordet-Gengou plates were heavily inoculated with cells from the 48h plates and incubated for 24h at 35°C. Challenge suspensions were prepared from these 24h cultures.

v. Harvesting of cultures

Cultures were harvested by centrifugation at 8000 r.p.m. (10,000 x g) in 250ml bottles at 4°C for 30 min. on an MSE 18 centrifuge. Supernatant fluid was carefully decanted and the cell pellets were evenly resuspended in 0.85% (w/v) sterile saline. The concentration of the cell suspension was adjusted to 100 opacity units (see section 1(vi)) and heated in a water bath at 56°C for 30 min to kill the cells. Heated cell suspensions were then preserved by the addition of thiomersal (BDH Chemicals Ltd., Poole, England) to a final concentration of 0.1mg/ml.

vi. Standardization of bacterial concentration by opacity

The concentration of bacterial suspensions was estimated by comparison with the International Opacity Reference Preparation designated as having 10 international opacity units.

The dilution factor used to obtain matching of cell suspension with the reference preparation was calculated and multiplied by 10 to give the concentration in opacity units of the undiluted bacterial suspension. A concentration of 1 o.u. was considered to be equivalent to approximately 10^9 organism per ml. The opacity reference was supplied by the World Health Organisation International Laboratory for Biological Standards (National Institute for Biological Standards and Controls, Holly Hill, Hampstead, London).

vii. Estimation of optical density

The optical density of bacterial suspensions and other optical density measurements were made at a wavelength of 540nm in a Pye-Unicam, model SP500 spectrophotometer using polystyrene cuvettes with a 1cm light path.

viii. Estimation of viable cell numbers

Serial ten-fold dilutions of cultures were made in sterile saline. Duplicate 0.2ml amounts of each dilution were spread onto BG plates. After incubation at 35°C for 72h, plates with countable number of colonies were selected. The average number of colonies per dilution was multiplied by the dilution factor to obtain an estimate of the number of viable cells per millilitre of the original culture.

SECTION 2 : BIO-ASSAY METHODS

i. Expression of bacterial dose

Having standardized a bacterial suspension in terms of opacity units (which is a measure of concentration), the doses injected into mice for PA, HSF and LPF tests were expressed in "opacity unit millilitres" (ou. ml) as suggested by Wardlaw (personal communication). Ou. ml. were calculated as opacity units X volume in millilitres.

ii. Estimation of protective activity

Protective activity of samples was determined by a slight modification of the original method of Kendrick et al., (1947).

Three-week old Ham/ICR mice of either sex were injected intraperitoneally, in randomized groups of ten, with 0.5ml of graded doses of heated (56°C for 30 min) B. pertussis cell suspensions. Fourteen days later the mice were anaesthetized with ether and challenged intracerebrally with 0.03ml of caseamino acids solution containing approximately 10^5 organisms of B. pertussis strain 18323 of enhanced virulence (Section 1 (iv)).

The virulence of the challenge culture was checked by injecting groups of unimmunized mice with 10^5 , 10^4 , 2×10^3 and 2×10^2 organisms per mouse, respectively. Surviving mice were counted daily for 14 days. Mice dying within three days of challenge were excluded from the results. Mice moribund on day 14 were counted as dead.

ii. Histamine-sensitizing activity

Seven to nine-week old Ham/ICR mice of either sex were injected intraperitoneally, in groups of five, with 0.5ml of graded doses of heated (56°C for 30 min) B. pertussis cell suspensions or 0.5ml of heated whole culture or heated culture supernatant.

Five days later the mice were injected intraperitoneally with 3mg of histamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.) contained in 0.5ml of saline. Survivors were counted 4h after challenge with histamine.

iii. Leukocytosis-promoting activity

Unless otherwise stated, five-week old female Ham/ICR mice were used. Groups of five mice were inoculated intravenously with 0.2ml or intraperitoneally with 0.5ml amounts of B. pertussis samples. Control mice were inoculated with saline only. Four days after inoculation mice were anaesthetized and bled from the orbital plexus with heparinized capillary tubes (Harshaw Chemicals Ltd., Daventry, Northants, England). The blood sample (40µl) was immediately transferred with a Coulter pipette to plastic vials containing 20ml Isoton II diluent (Coulter Electronics Ltd., Harpenden, Herts, England) to give a final blood concentration of 1:501.

Immediately before counting, six drops of Zap-Oglobin (Coulter Electronics Ltd., Harpenden, Herts, England) were added to each vial to lyse the red blood cells. Enumeration of leukocytes was done using a Coulter Counter model FN (Coulter Electronics Ltd., Harpenden, Herts, England). Duplicate or triplicate counts of each diluted blood sample were made and the mean of these counts was calculated for each sample. To correct for coincidence losses during counting, mean counts above 10,000 were adjusted with a coincidence correction chart. Mean background counts, from diluent alone, were subtracted from each corrected count to obtain the final corrected mean count for each animal or group of mice.

iv. Heat-labile toxicity

Three to four-week old Ham/ICR male mice were injected intraperitoneally, in groups of five, with 0.5ml of graded doses of unheated samples of B. pertussis. Deaths were recorded over the next three days.

When samples were heated (56°C for 30 min) lethality was completely abolished.

v. Toxicity as measured by the mouse weight-gain test

Three to four-week old Ham/ICR male mice were injected intraperitoneally, in groups of five (of approximately same weight) with 0.5ml of graded doses of heated (56°C for 30 min) cell suspensions. Mice were weighed just before inoculation and then daily for seven days.

vi. Estimation of the distribution of biological activities between cells and culture supernatant

Aliquots of 50ml of culture were harvested at various stages of growth by centrifugation at 8000 r.p.m. ($10,000 \times g$) in 50ml bottles at 4°C for 30 min in an MSE 25 centrifuge. Supernatant fluid was carefully removed and cell pellets were thoroughly resuspended to the original volume with saline. The cell suspension and supernatant samples were heated in a water bath at 56°C for 30 min, then cooled and stored at -20°C until they could be used. Samples were assayed for HSF and LPF activities. Samples for assay of HLT activity were not heated.

vii. Disruption of cells

Packed cells were adjusted to 100 o.u. and after freezing in dry ice, were broken by three passages through an X-press (LKB, Instruments Ltd., South Croydon, Surrey).

SECTION 3 : BIOCHEMICAL MATERIALS AND
METHODS

i. Protein estimation

Compositions of reagents used are shown in Appendix 2. Estimates were done by the method of Herbert, Phipps and Strange (1971) with bovine serum albumin (Sigma Chemical Co., St. Louis, U.S.A.) as a standard).

a) 0.5ml of distilled water (or buffer) were dispensed into duplicate tubes as blanks.

b) 0.5ml of samples were dispensed into duplicate test tubes.

c) Dilutions of the standard protein (BSA) (500, 400, 300, 200, 100 and 50 μ g/ml) were prepared. 0.5ml of each dilution was dispensed into duplicate test tubes.

d) 0.5ml of 1N NaOH was added to each of the tubes (a, b and c above). All the tubes were placed in a boiling water bath for 5min, then removed and allowed to cool.

e) 2.5ml of reagent C was added to each of the tubes, after which the tubes were allowed to stand for 10 min.

f) 0.5ml of reagent D was added to all the tubes. Tubes were allowed to stand for 30 min for full colour development.

g) The optical density of the solution in each tube was measured at 750nm against the blanks (a).

h) A curve of the optical density values against concentrations of the standard protein was drawn and from this curve the protein concentrations in samples were estimated.

ii. Slab-gel electrophoresis

The method used was based on that of Laemmli (1970) as modified by Ames (1974). Electrophoresis was done in the presence of sodium dodecyl sulphate (SDS) in a discontinuous gel and buffer system. Separating and stacking gels contained respectively 10% (w/v) and 5% (w/v) acrylamide (British Drug Houses Ltd., Poole, England). Both the gels and buffers contained 0.1% (w/v) SDS.

The dimensions of the slabs were approximately 8.0 x 7.0 x 0.3cm, the separating gels being 6.0 x 7.0 x 0.3cm. Details of reagents, preparation of gels, staining and destaining are given in Appendix 3. Samples were adjusted to contain 2mg protein/ml and 0.5ml of sample was added to 0.5ml of 0.125M Tris-HCL buffer pH 6.8 containing 4% (w/v) SDS, 10% (v/v) β -mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue. The mixture was heated at 100°C for 5 min prior to application of between 20 and 100 μ l to the gel.

Electrophoresis was done in a Uniscil slab gel electrophoresis unit (Universal Scientific Ltd., London) at a constant current of 15 mA/gel for approximately 3h. Staining and destaining was by the method of Weber and Osborn (1969). Destaining was accomplished with several

changes of the destaining solution.

For molecular weight estimations, the following mixture of protein standards was run under identical conditions : bovine serum albumin (Mol. wt. 67,000. Sigma Chemical Co., St. Louis, U.S.A.), ovalbumin (43,000. Sigma Chemical Co., St. Louis, U.S.A.), bovine chymotrypsinogen (25,700. Miles Seravac, Maidenhead, Berkshire), trypsin (23,000. Armour Pharmaceutical Co., Eastbourne, Sussex) and horse-heart cytochrome c (11,700. Koch Light, Colnbrook, Buckinghamshire).

iii. Tube-gel electrophoresis

The same method and reagents were used as for slab-gel electrophoresis except that gels were prepared in clean glass tubes and run in a Shandon analytical polyacrylamide gel electrophoresis outfit (Shandon Southern Instruments Ltd., Surrey, England) at a constant current of 1mA per tube for approximately 2½h.

iv. Uptake of labelled glutamate

Twenty ml volumes of 48h cultures of B. pertussis strain 134 in Stainer and Scholte X-medium were spun down in sterile universals at 4000 r.p.m. (3000 x g) for 30 min in a bench centrifuge (MSE super minor). The cell pellets were evenly resuspended in 40ml of Stainer and Scholte X- or C-medium in 50ml conical flasks. These media contained one tenth of the normal amount of glutamate (1.072g) and ten times the normal amount of proline (2.4g).

200 μ l of ^{14}C glutamate ($>250\text{mCi/m mol}$) (Radiochemical Centre, Amersham) was added to each flask. Flasks were incubated in a shaking water bath at 35°C for 8h. Cultures were harvested by centrifugation and the cell pellets obtained were washed twice and resuspended in distilled water. To determine the distribution of ^{14}C glutamate in the cellular proteins, cell suspensions were adjusted to a concentration of 80 o.u. and prepared for tube-gel electrophoresis as described in Section 3 (iii).

v. Densitometry and gel slicing

Densitometer traces of stained gels were obtained by means of a Joyce-Loebl Ultraviolet Scanner with an effective scanning aperture of 0.05mm and fitted with a 280nm interference filter (Joyce-Loebl and Co. Ltd., Gateshead, England).

For gel slicing, destained gels were carefully wrapped in aluminium foil and frozen in crushed dry ice. Frozen gels were sliced into 1.00mm sections starting from the bottom end, with a Mickle gel slicer (Mickle Laboratory Engineering Co. Surrey, England).

vi. Determination of ^{14}C in gel slices

Gel slices were transferred to numbered test tubes and allowed to dry under an infra red lamp for 2h. To each tube was added 0.25ml of a mixture of one volume of concentrated NH_4OH added to 99 volumes of 30% H_2O_2 which had been cooled to 0°C in ice. This mixture was used

immediately after preparation. Tubes were incubated at 37°C for 3-4h. The gel slices were completely dissolved by this treatment.

The content of each tube was transferred to a scintillation vial, the tubes rinsed with distilled water and the washing also transferred to the vial. The volume in each vial was made up to 1.5ml with distilled water and 10ml of Triton-toluene scintillant was added. The vial was capped, mixed and counted for one minute in an Automatic Spectrometer, Model NE8312 (Nuclear Enterprises Ltd., Edinburgh). Composition of the Triton-toluene scintillant is given in Appendix 2.

vii. Estimation of proteolytic activity of samples

Two colourimetric methods were used for the estimation of the proteolytic activity of samples. The methods involved the use of Azocoll and Azocasein respectively as substrates.

a. Use of Azocoll

Azocoll (Calbiochem., San Diego, California) is an insoluble powdered cowhide to which is bound a red dye. It contains an assortment of peptide linkages characteristic of all proteins and is used for a non-specific assay for proteolytic activity.

In the presence of a proteolytic enzyme, some of these linkages are split, leading to the release of the dye into solution. The colour intensity of the solution after

centrifugation indicates the degree of splitting of the peptide bonds and thus the level of proteolytic activity.

For the preliminary estimation of proteolytic activities of X and C cultures, the enzyme pronase (Sigma Chemical Co., St. Louis, U.S.A.) was used as a standard. Serial dilutions of a stock solution of the enzyme (5mg/ml) was made in 0.1M phosphate buffer at pH 7.0. To 5ml of each enzyme dilution, and 5ml of culture samples in 6" x 5/8" test tubes, 25mg of Azocoll was added. Tubes were incubated in a water bath at 37°C for 30 min with constant mixing. After incubation the insoluble azocoll was deposited by centrifugation at 4000 r.p.m. (3000 x g) for 30 min and the optical density of the supernatant solutions measured at 520nm.

b. Use of Azocasein

Azocasein (Sigma Chemicals Co., St. Louis, U.S.A.) is essentially fat-free casein to which a red-orange dye has been coupled. The principle of the assay method is the same as for azocoll.

The substrate solution used in the assay contained 25mg of azocasein and 5mg NaHCO_3 per ml. This was prepared by dissolving 2.5g of azocasein in 50ml of 1% (w/v) NaHCO_3 at 60°C with stirring. The pH was adjusted to 8.3 and the solution diluted to 100ml with distilled water.

The enzyme subtilisin (Sigma Chemicals Co., St. Louis, U.S.A.), a protease prepared from Bacillus amyloliquefaciens, was used as the standard enzyme. Serial dilutions of the enzyme were made in bicarbonate buffer, pH 8.3.

For the estimation of proteolytic activity, 0.3ml of each of the X- and C- mode samples in duplicates and the various enzyme dilutions were pipetted into 6" x 5/8" test tubes. To each tube 0.3ml of the substrate solution was added. Control tubes with medium only were set up. All the tubes were incubated in a water bath at 37°C. After 30 min the digestion was stopped and undigested azocasein precipitated from the solution by the addition of 2.4ml of 5% (w/v) trichloroacetic acid to each tube. The contents of each tube was centrifuged. To 2ml aliquots of supernatant from each tube was added 2ml of 0.5N NaOH and the optical density was measured at 440nm.

SECTION 4 : IMMUNOLOGICAL MATERIALS AND METHODS

i. Preparation of rabbit anti-serum to B. pertussis

Rabbit anti-B. pertussis serum was raised by immunizing female adult rabbits with X- and C-mode cell suspensions adjusted to a concentration of 10 o.u. Each rabbit was given four subcutaneous injections of 1.5ml amounts and one intravenous injection of 4ml of the cell suspensions. The subcutaneous injections were repeated at weekly intervals for six weeks. After a rest period of two weeks, the rabbits were given similar subcutaneous injections for two further weeks. The animals were bled through the ear vein seven days after the last injection. The blood was allowed to clot, then refrigerated for four hours and the serum collected by centrifugation and stored at -20°C.

ii. Preparation of specific X and specific C anti-sera

Aliquots of 25ml of 48h X- and C- cultures in modified Hornibrook medium were spun down in universal containers at 3000 r.p.m. for 30 min. X-mode cell pellets obtained were resuspended in 5ml of undiluted anti-C serum. Similarly, C-mode cell pellets were resuspended in 5ml of undiluted anti-X serum. Both cell suspensions were incubated at 37°C for 1h, then shaken for 20 min at room temperature with a wrist-action shaker (Griffin and Tatlock Ltd., London). Bacterial cells were removed by centrifugation and the absorption repeated three times with fresh batches of cells.

iii. Agglutination tests

Fresh, unheated cell suspensions were used for agglutination tests. X- and C- mode cultures were harvested at various intervals during growth. Cell pellets obtained were evenly resuspended in 0.85% (w/v) saline and adjusted to a concentration of 10 o.u.

Doubling dilutions of anti-sera in normal saline were made in 2½" x 3/8" test tubes. Each dilution was dispensed in 0.05ml amounts into wells in plastic disposable Cooke micro-titre trays (Sterilin Ltd., Sussex, England).

0.05ml amounts of cell suspensions were added to each well. Controls with no antiserum (cell suspension and saline) were also included. Trays were shaken periodically for 30 min at room temperature and incubated

at 37°C for 1h. After incubation, trays were refrigerated for another 1h and results were read with a plate microscope. The end point was taken as the reciprocal of the highest dilution still showing definite agglutination.

iv. Haemagglutination tests

Tests were performed in Cooke plastic micro-titre trays (Sterilin Ltd., Sussex, England) with microdroppers and microdiluters (Cooke Engineering Co., Alexandria, Virginia).

Serial two-fold dilutions of samples were made in Dulbecco A phosphate buffered saline pH 7.3 (Oxoid Ltd). Two drops (0.05ml) of 2% (v/v) horse erythrocytes in PBS were added to each well. Controls with no B. pertussis samples (only PBS and red cells) were included. Each test was done in duplicate. Mixtures were shaken and incubated at 37°C. After 24h, results were read, then trays were left at 4°C overnight and read again. The haemagglutination titre was taken as the reciprocal of the highest dilution showing complete agglutination of the erythrocytes.

SECTION 5 : STATISTICAL METHODS

i. Probit analysis

Results from the HSF, mouse-protection and heat-labile toxicity assays were analysed by the probit method (Finney, 1952) using a computer programme developed in the Department of Epidemiology and Biometrics, School of Hygiene, University of Toronto, Canada. One sample was used as a standard and

the potencies, with the 95% confidence limits, of other samples relative to the standard were estimated. In some experiments, a local B. pertussis 18334, vaccine produced in this laboratory was used as standard. The ED₅₀ values of the samples were also calculated.

ii. Rankit analysis of leukocyte counts

Rankit plots of leukocyte counts were done to see if the data could reasonably be regarded as random samples from a normal distribution. This was to facilitate subsequent statistical processing.

The leukocyte count for each group of mice was arranged in rank order, from the highest to the lowest counts. The log₁₀ of the arithmetic counts were also arranged in rank order. From a rankit table (Bliss, 1967), rankit values were assigned to the counts. Results were analysed by plotting the graphs of the rankit values against arithmetic counts. Similar rankit plots were done with the log₁₀ values of the leukocyte counts.

iii. The 4-point and 6-point assays

LPF activities of unknown samples and standards were compared by the 4-point or 6-point assay methods (A.C. Wardlaw, personal communication).

The 4-point assay design involves testing low and high doses of standard and unknown preparations. The 6-point assay tests three evenly spaced doses of the unknown against the same three similarly spaced doses of the standard.

The C-mode preparations were compared with the X-mode preparations as standards. X-mode samples from various stages of growth were also compared with a standard X-mode preparation.

Results of the 4-point or 6-point assays were analysed by an Olivetti computer programme (A.C. Wardlaw, personal communications) using a Programma 101 Electronic desk-top computer (British Olivetti Ltd., Berkeley Square, London). The relative potency values and the 95% confidence limits of the unknown preparations to the standards were obtained.

iv. Bartlett's test and the analysis of variance

Variances were tested for homogeneity by the Bartlett test and analysis of variance was done according to schemes prepared by Wardlaw (personal communication).

RESULTS

SECTION I : DEVELOPMENT OF AN ASSAY
METHOD FOR THE LEUKOCYTOSIS-
PROMOTING FACTOR (LPF)

Preliminary experiments in this study showed that LPF was lost in C-mode cells. One of the aims of this project was to determine the time sequence of loss of the various immunological and pathophysiological activities during modulation. The experimental approach was to quantitate the potency of each of the active factors in the C-mode cells at various intervals. At the beginning of this study, methods were available for quantitating the potencies of HSF, PA and HLT in B. pertussis preparations but there was no suitable method for determining LPF potency. Thus initial experiments were set up to devise a suitable assay system.

1. Factors influencing the LPF response
in mice

In order to obtain the optimal conditions for an LPF assay, it was first necessary to examine the effect of a number of variables on the LPF response of mice. This was important because factors like sex, age and strain have been reported to influence histamine-sensitization in mice (Bergman and Munoz, 1964; Munoz and Bergman, 1968; Morse, 1977).

i. Time interval between inoculation and bleeding

Seven-week old female Ham/ICR mice were inoculated intraperitoneally, in groups of five, with 0.5ml of heated (56°C/30 mins) cell suspensions obtained from a 48h X-mode culture of B. pertussis strain 18334. Another group of mice received saline only. Mice were bled for leukocyte counts on days 1, 3, 4 and 8 after inoculation.

As depicted in Figure 2A, striking leukocytosis occurred in all animals inoculated with the cell suspensions. The response reached maximum levels by about day 4 and began to show a slight decline by day 8. Figure 2B shows that the leukocyte count was dose-dependent. In subsequent LPF experiments mice were routinely bled for leukocyte counts on day 4 after inoculation in order to obtain the maximum LPF response.

ii. Sex

To determine the effect of the sex of mice on the LPF response, seven-week old male and female Ham/ICR mice were injected intraperitoneally with heated cell suspensions obtained from a 48h X-mode culture of strain 18334. Similar groups of mice were inoculated with saline only and all animals were bled 4 days later for leukocyte counts.

Figure 3 shows that the dose-response curve for the male mice was more elevated than for the female mice. Analysis of variance of the data confirmed that the LPF responses in the male mice were significantly higher than in the female mice at all doses tested (Appendix 4a). There was no significant difference in the leukocyte counts

Figure 2 :

A. Determination of peak of LPF response with time.

Groups of five 7-week old female Ham/ICR mice were injected intraperitoneally with graded doses of B. pertussis strain 18334 X-mode cell suspensions and bled at intervals for leukocyte counts.

Dose per mouse

○—○ 8 ou.ml
□—□ 2 ou.ml
●—● 0.5 ou.ml
▲—▲ 0.125 ou.ml
△—△ 0.5ml saline

B. LPF dose-response curves obtained in the above experiment.

○—○ day 1
●—● day 3
□—□ day 4
▲—▲ day 8

Bars in both graphs represent standard deviations from the mean leukocyte counts.

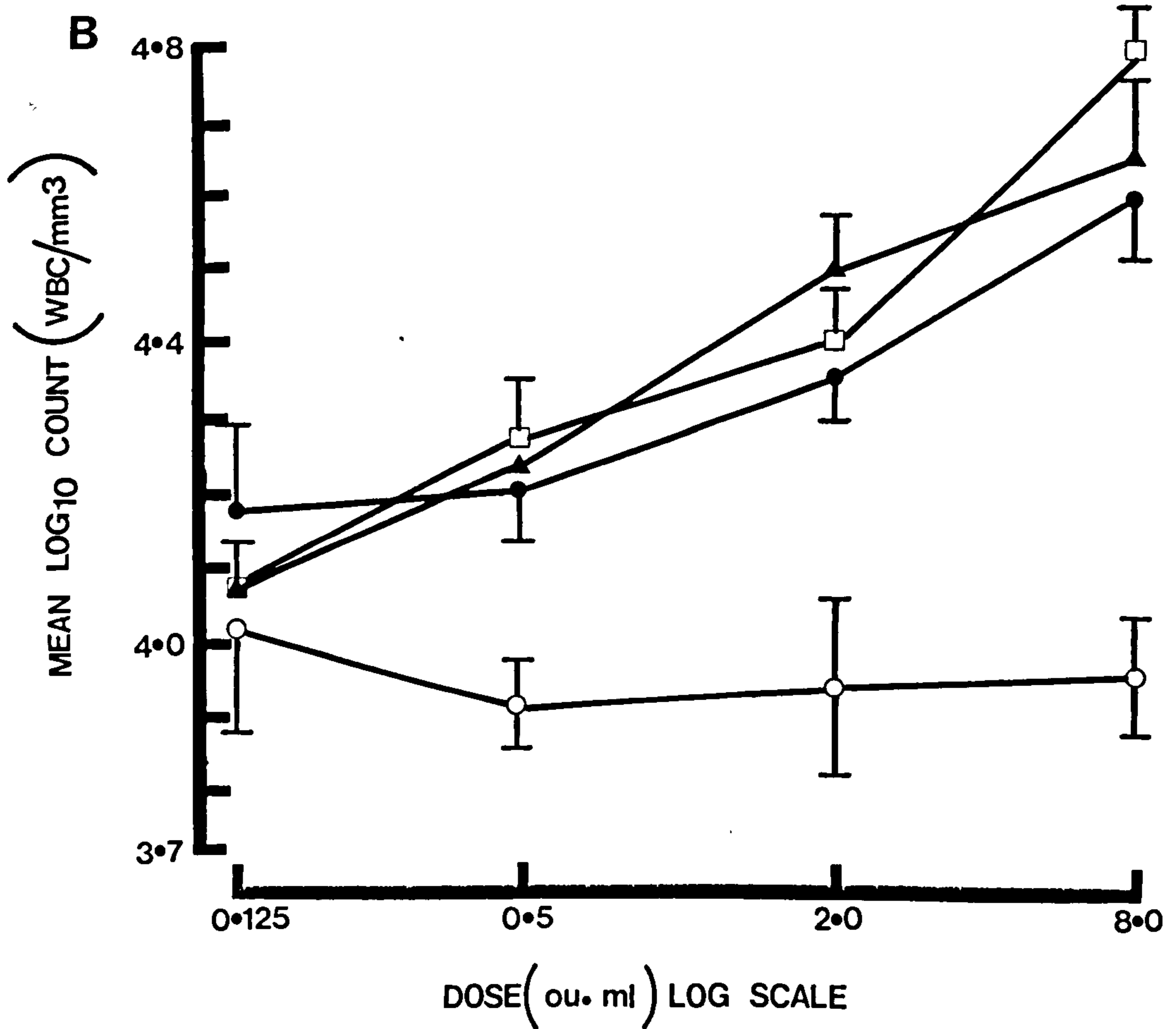
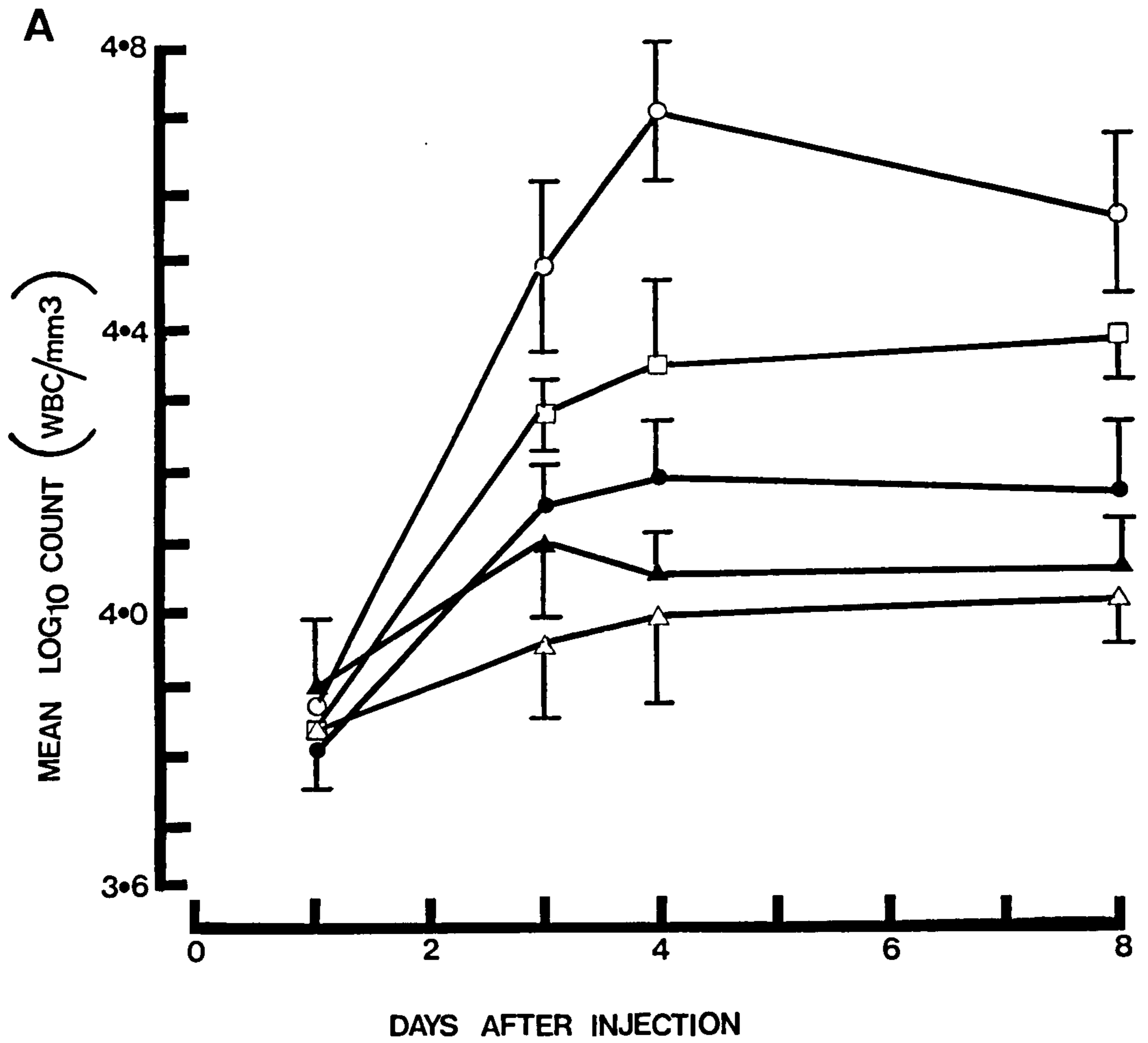


Figure 3 : Effect of sex of mouse on LPF response.

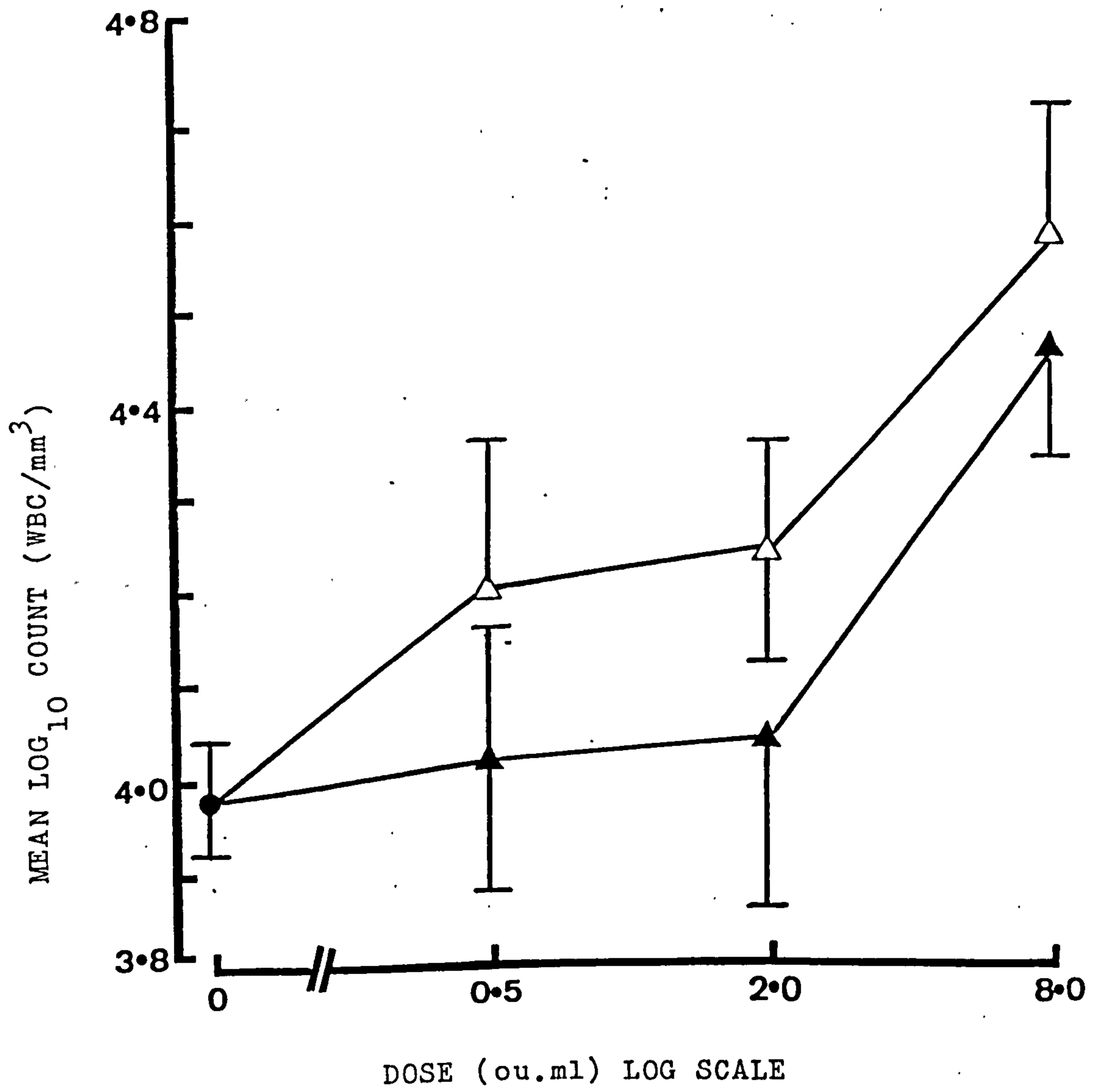
Groups of five 7-week old male and female Ham/ICR mice were injected intraperitoneally with graded doses of heated X-mode cell suspensions from a 48h culture of B. pertussis strain 18334. Mice were bled four days later for leukocyte counts.

△——△ males

▲——▲ females

● saline control (males and females)

Bars represent the standard deviations from the mean leukocyte counts.



of male and female mice inoculated with saline alone.

Although the males were somewhat more responsive and therefore would be more suitable for LPF assay, female mice were used in subsequent LPF experiments because they were more readily available in the numbers required.

iii. Route of inoculation

The intraperitoneal and the intravenous routes of inoculation were compared. Groups of five-week old female Ham/ICR mice were inoculated intravenously or intraperitoneally with identical graded doses of a 48h heated X-mode culture of strain 18334 contained in 0.2ml or 0.5ml volumes respectively.

Figure 4 shows that the dose response curve for the intravenous route was much steeper than that for the intraperitoneal route. Further analysis of data by the Student t-test showed that mice were four times more responsive by the intravenous route than by the intraperitoneal route (Appendix 4b). Based on this observation the intravenous route of inoculation was therefore used in subsequent LPF experiments.

iv. Age

Groups of female Ham/ICR mice of various ages were inoculated intravenously with 0.2ml of heated cell suspensions obtained from a 48h X-mode strain 18334 culture. Similar groups of mice of approximately equal weights were inoculated with saline alone. In another experiment female

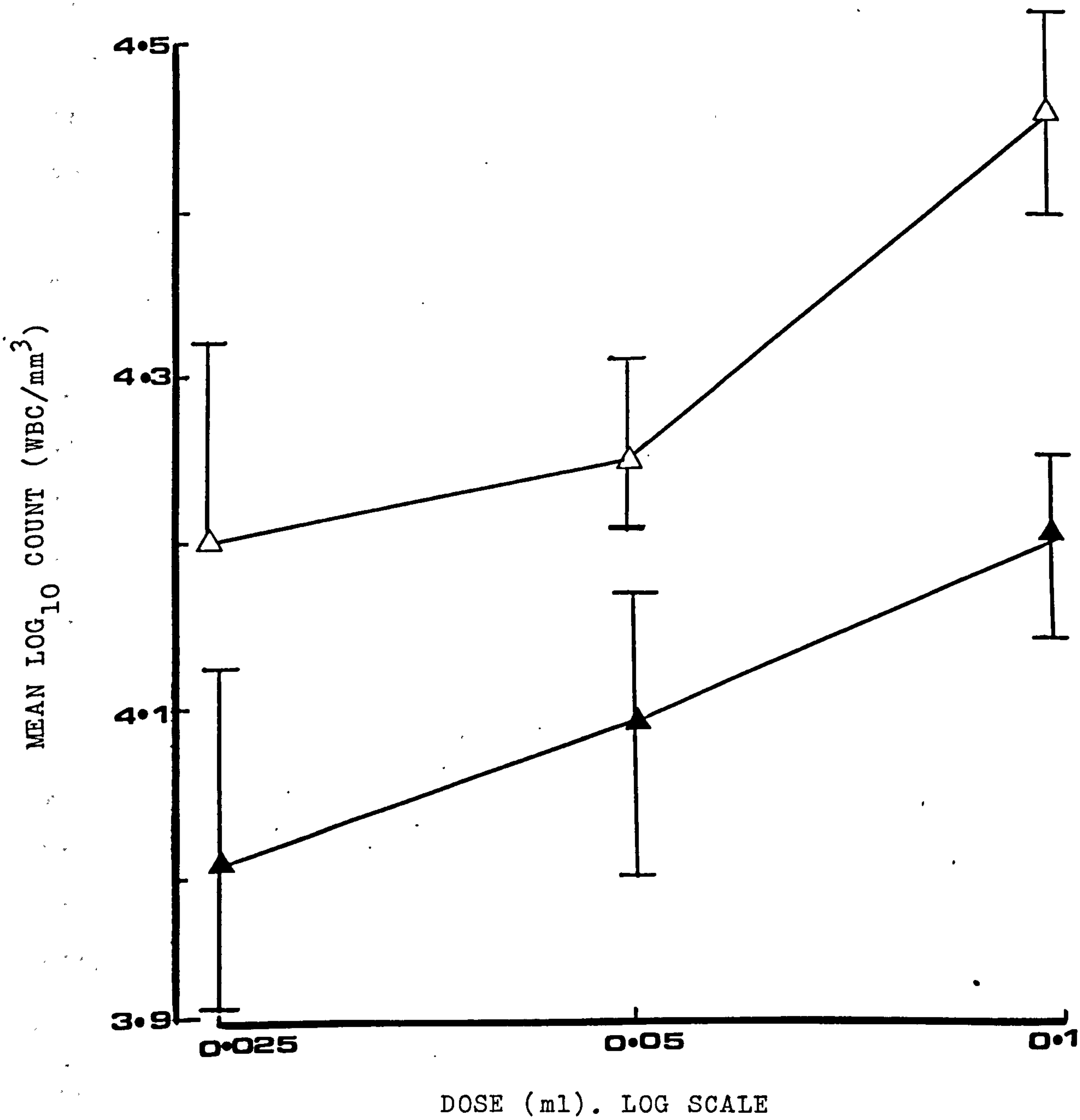
Figure 4 : Effect of route of inoculation on LPF response.

Five-week old Ham/ICR mice were inoculated intravenously or intraperitoneally with graded doses of B. pertussis strain 18334 X-mode whole culture.

△——△ intravenous route

▲——▲ intraperitoneal route

Bars represent the standard deviations from the mean leukocyte counts.



NIH mice of various ages were used.

With both strains of mice the various ages tested were equally responsive to LPF and there was no apparent trend for leukocytosis to increase or decrease with age (Figures 5A and 5B). It was concluded that the age of the mice, from 3 to 20 weeks, had no significant effect on the LPF response. However, for the sake of uniformity and because they were more convenient to handle, five-week old mice were used in all subsequent LPF experiments.

v. Strain

The possible effect of the strain of mouse on LPF response was examined further. Groups of five-week old and eight-week old female NIH and Ham/ICR mice each containing five animals, were inoculated intravenously with 0.2ml of heated X-mode cell suspension. Similar groups of the two strains of mice were inoculated with saline only.

Table 5 shows both the mean arithmetic and the mean \log_{10} leukocyte counts obtained. \log_{10} leukocyte counts were further examined by analysis of variance (Appendix 4c). It was apparent that there was no significant difference in the LPF responses of the different groups with regards to either strain or age.

In subsequent experiments, Ham/ICR mice were routinely used because they were more readily available.

Figure 5 : Comparison of LPF responses in mice of different ages.

Strains Ham/ICR and NIH mice of different ages were inoculated intravenously in groups of five with B. pertussis strain 18334 X-mode whole cultures. Similar groups of different ages of mice were inoculated with saline alone. Mice were bled four days later for leukocyte counts.

A. Strain Ham/ICR

- experiment 1. (pertussis treated)
- experiment 2. (pertussis treated)
- experiment 1. (saline control)
- experiment 2. (saline control)

B. Strain NIH

- △—△ experiment 3. (pertussis treated)
- ▲—▲ experiment 3. (saline control)

Bars represent the standard deviations from the mean leukocyte counts.

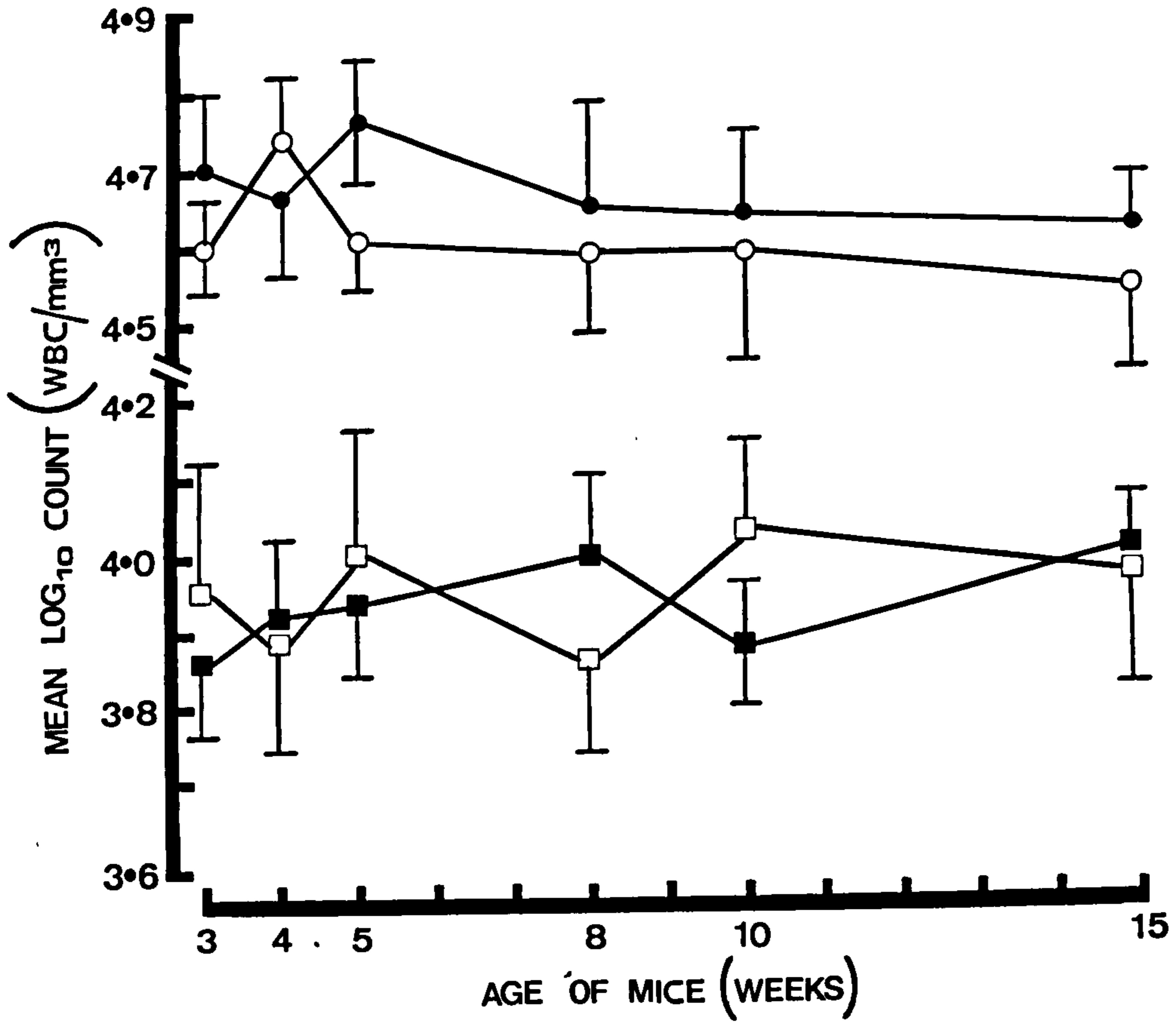
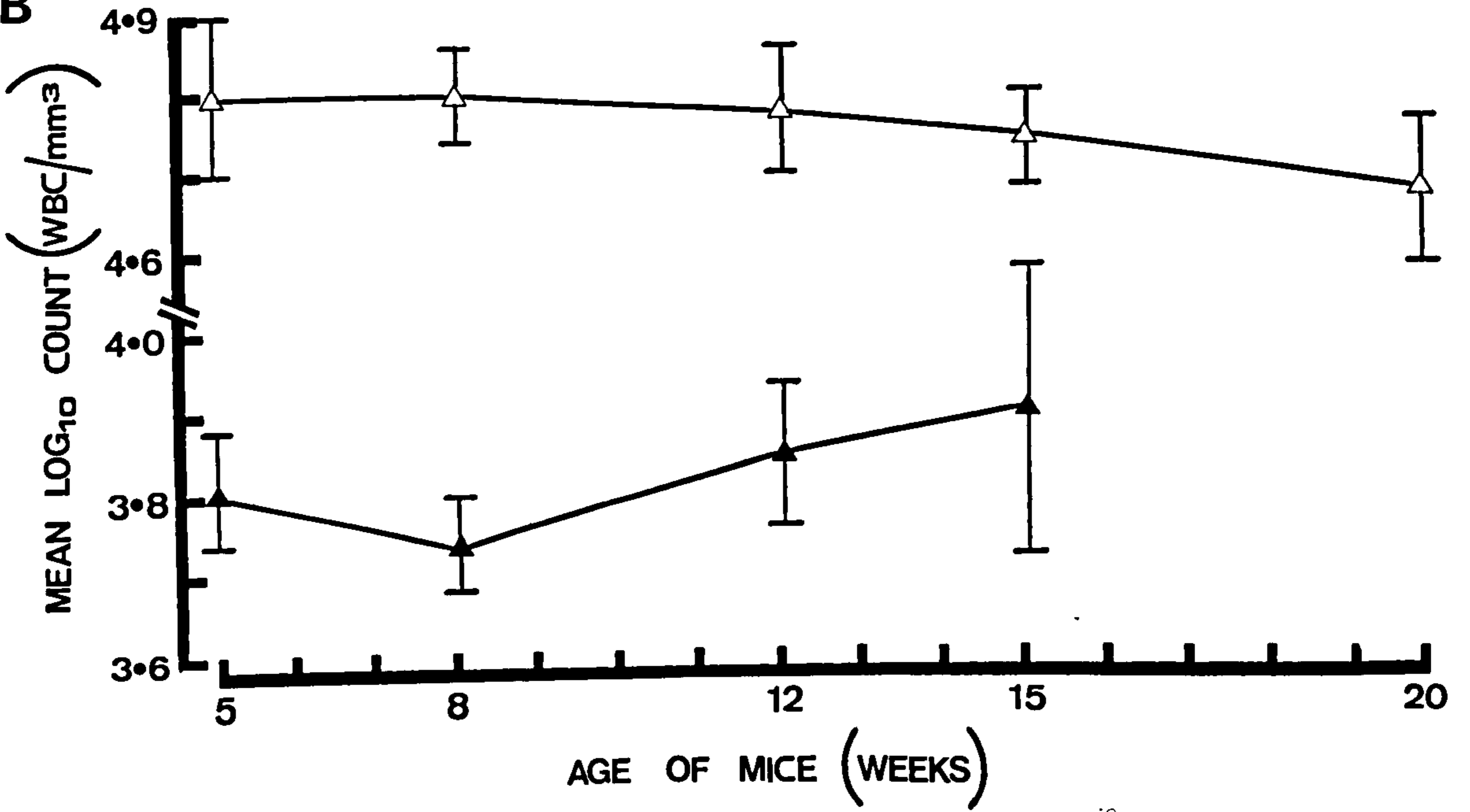
A**B**

Table 5: Comparison of LPF responses in
two mouse strains

	Ham/ICR		NIH	
Age	arithmetic mean	\log_{10} mean	arithmetic mean	\log_{10} mean
8 weeks	47,483 ± 1052	4.6669 ± 0.11	46,574 ± 7,797	4.66 ± 0.09
5 weeks	59,031 ± 1449	4.7588 ± 0.12	57,327 ± 1033	4.75 ± 0.08
5 weeks (saline control)	5,657 ± 1028	3.75 ± 0.09	6,391 ± 784	3.802 ± 0.06

2. Quantitation of LPF response

i. Rankit analysis of leukocyte counts

For t-test and analysis of variance to be valid, the following conditions should be satisfied :

- a. data within each group should approximate to a normal distribution.
- b. the standard deviations within the various groups should not be significantly different.

Since the application of statistical analysis of LPF results in later experiments was intended, it was important to show that the data obtained from the particular assay method used satisfied the above conditions. Groups of equal numbers of mice were used and leukocyte counts obtained were checked for their normal distribution.

Figures 6A and 6B show that both the arithmetic and the \log_{10} leukocyte counts were evenly distributed about straight lines by the rankit method. On application of the Bartlett test for homogeneity of variances, it was found that the variances of the arithmetic counts were highly heterogenous.

However, with the \log_{10} counts, variances within groups had a satisfactory degree of homogeneity (Appendix 4d). Thus although the rankit plots did not discriminate between normal and \log_{10} normal distribution, the transformation to \log_{10} values was desirable because of the homogeneity of variance which then followed.

Figure 6 :

Rankit analysis of arithmetic and
 \log_{10} leukocyte counts

A. Arithmetic counts

Dose per mouse

- 0.5ml of culture
- 0.25ml of culture
- ▲————▲ 0.125ml of culture
- △————△ 0.5ml of saline (control)

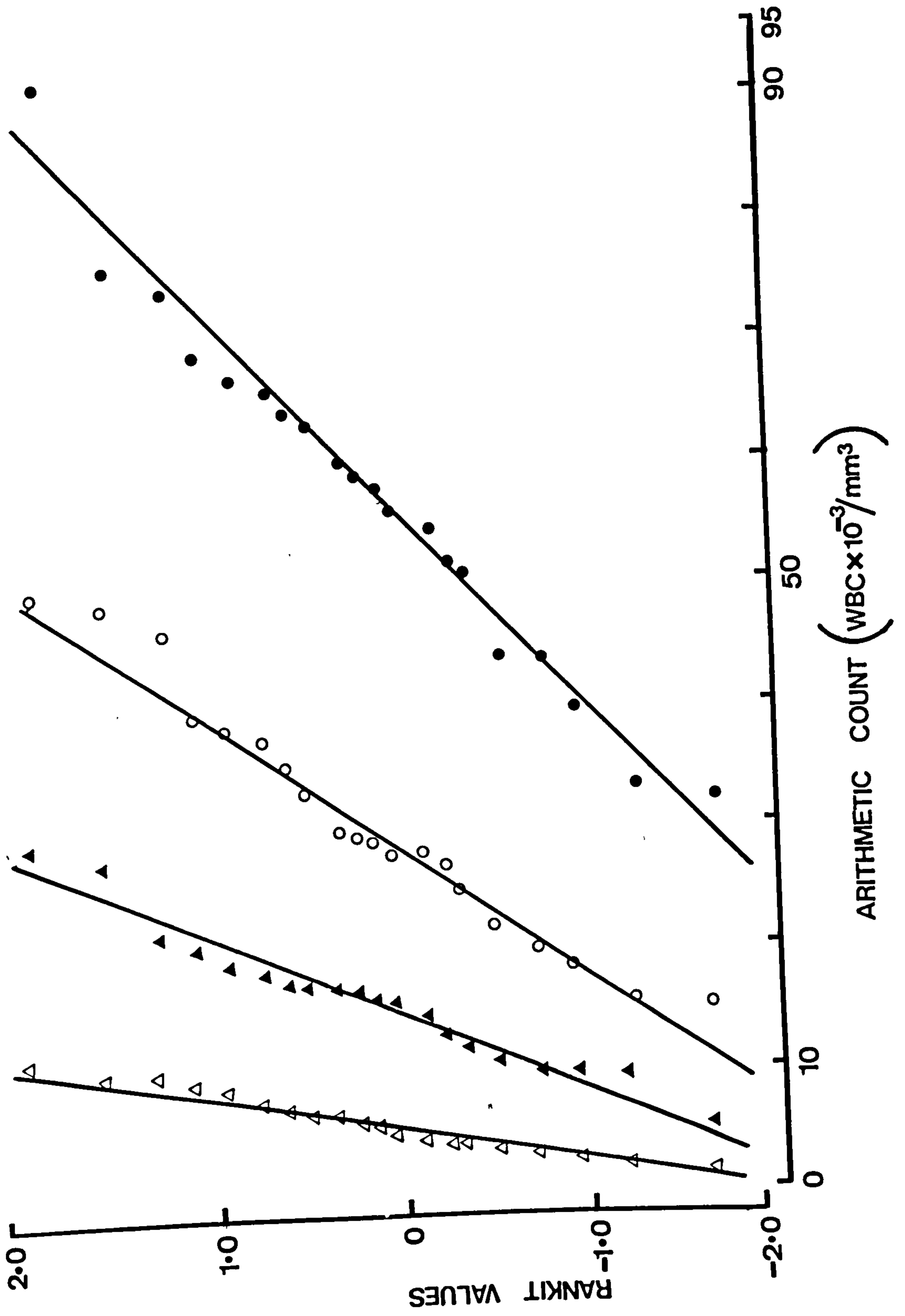


Figure 6B : Log_{10} counts

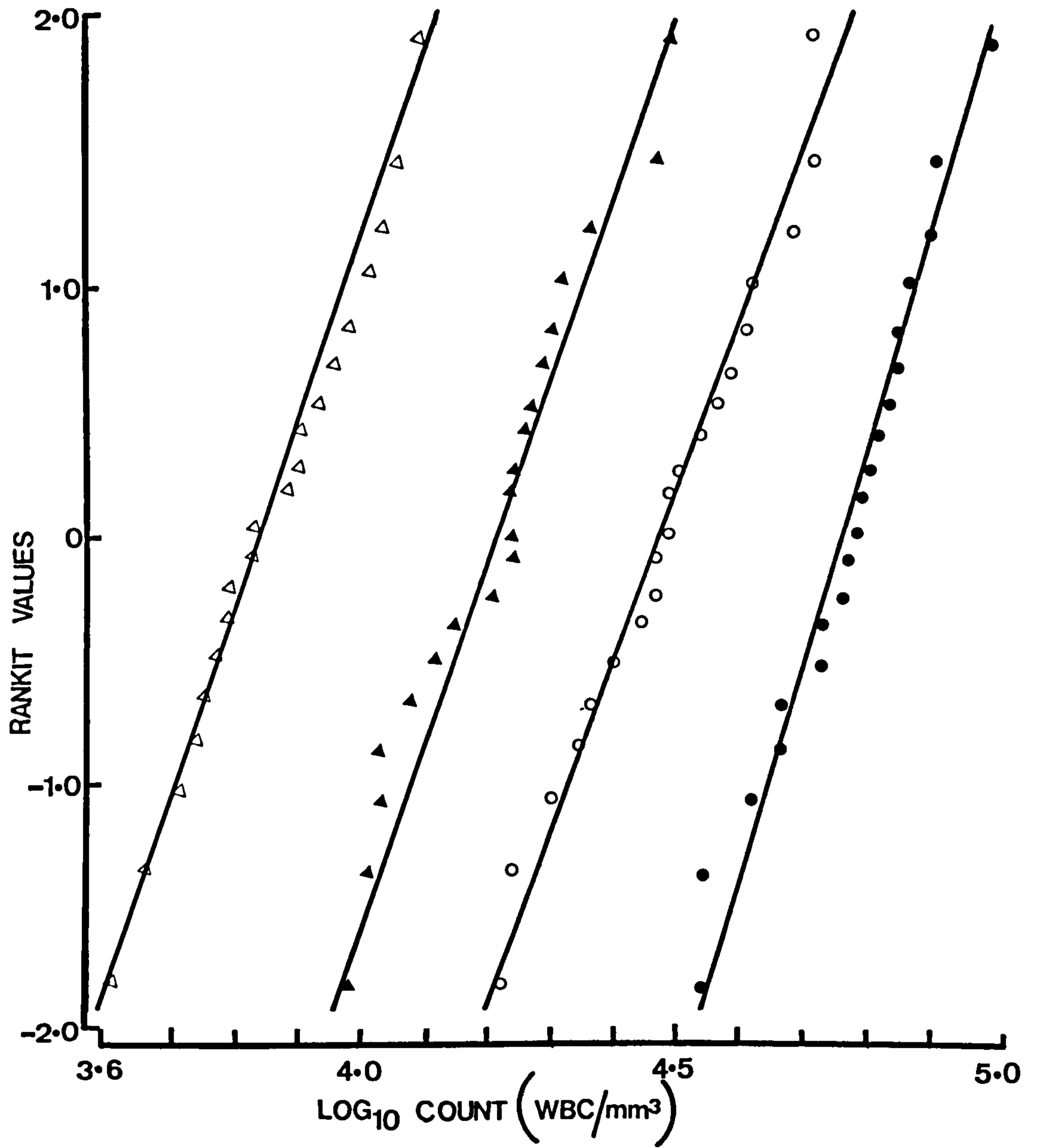
Dose per mouse

●—● 0.5ml of culture

○—○ 0.25ml of culture

▲—▲ 0.125ml of culture

△—△ 0.5ml of saline (control)



ii. Estimation of LPF potencies

Having worked out suitable conditions for an LPF assay, attempts were made to estimate the LPF potencies of samples using the 4-point and 6-point assay methods. (A.C. Wardlaw, personal communication). These are parallel line assays in which unknown preparations are compared with standard preparations. In the 4-point assay, two doses of the unknown preparation are compared with the same two doses of the standard; in the 6-point assay three doses of the unknown preparation are compared with the identical doses of the standard preparation. For these assays to be valid, the dose-response curve of the unknown preparation must parallel that of the standard preparation over the particular dose range tested.

Groups of five-week old female Ham/ICR mice were inoculated intravenously with graded doses of heated cell suspensions harvested at various intervals after inoculation of X-mode cells into X- and C-media. Cells harvested from C-medium after 10h growth, although they still had some X-mode characteristics, were referred to as C-mode cells in the present study. With X-mode samples as standards, an attempt was made to estimate the relative LPF potencies of the C-mode samples by the 4-point and 6-point assay programmes using an Olivetti computer (Programma 101).

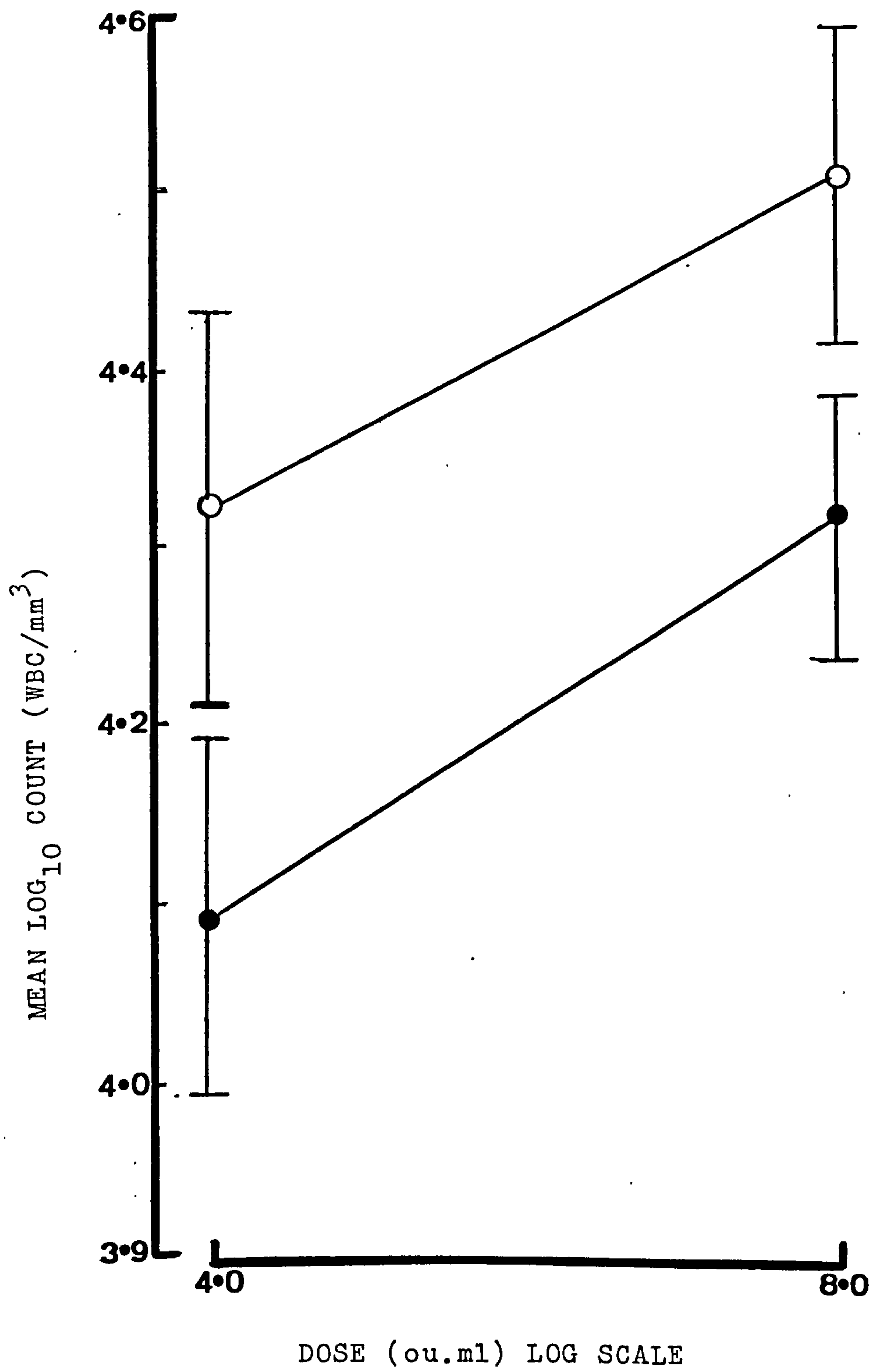
The dose-responses of X and C-mode 10h samples showed some degree of parallelism in the 4-point assay apparently because these C-mode cells still retained some LPF activity (Figure 7). However, the dose-responses of older C-mode

Figure 7 : LPF dose-response curves for 10h X-
and C-mode cell suspensions.
4-point assay.

○ — ○ X-mode

● — ● C-mode

Bars represent the standard deviations
from the mean leukocyte counts.



samples which contained very little LPF activity showed no parallelism with dose-responses of their X-mode counterparts at the doses tested. The assay method was valid only when the 10h samples were compared but the method was invalid when the older C-mode samples were compared against their X-mode counterparts (Appendix 4e). At 10h, the LPF activity of the C-mode sample was only 20% of the activity of the X-mode sample (Table 6).

With the 6-point assay method, there was lack of parallelism between the dose-responses for the 10h X- and C-mode samples (Figure 8), suggesting that the assay method would not be valid for comparing the potency of any C-mode sample against those of the X-mode. Confirmation of this observation was obtained statistically from the tabulated F-ratio results (Appendix 4f).

Both the 4-point and 6-point assay methods were valid when comparing any of the X-mode samples against another X-mode sample as standard (Figure 9). The LPF potencies of the various X-mode samples obtained by these assay methods are shown in Tables 6 and 7 and are reported in detail in Section 2, part 2iii.

In conclusion, the LPF potencies of preparations with low levels of activity can be estimated only by the 4-point assay method while the potencies of preparations with relatively high levels of LPF activity can be estimated by either the 4-point or 6-point assay methods.

Table 6: LPF potencies of X- and C-mode
cell suspensions by 4-point assay

Age of culture Relative potency (and 95% confidence limits)

	X-mode	C-mode
10h	1.00	0.20* (0.17-0.44)
24h	1.5 (0.65-5.6)	**
48h	1.5 (0.57-8.1)	**
72h	0.96 (0.55-5.7)	**

** assay not valid at doses tested

* expected potency based on observed number of cell divisions is 0.8

Figure 8 : LPF dose-response curves for 10h
X- and C-mode cell suspensions.
6-point assay.

○—○ X-mode

●—● C-mode

Bars represent the standard
deviations from the mean leukocyte
counts.

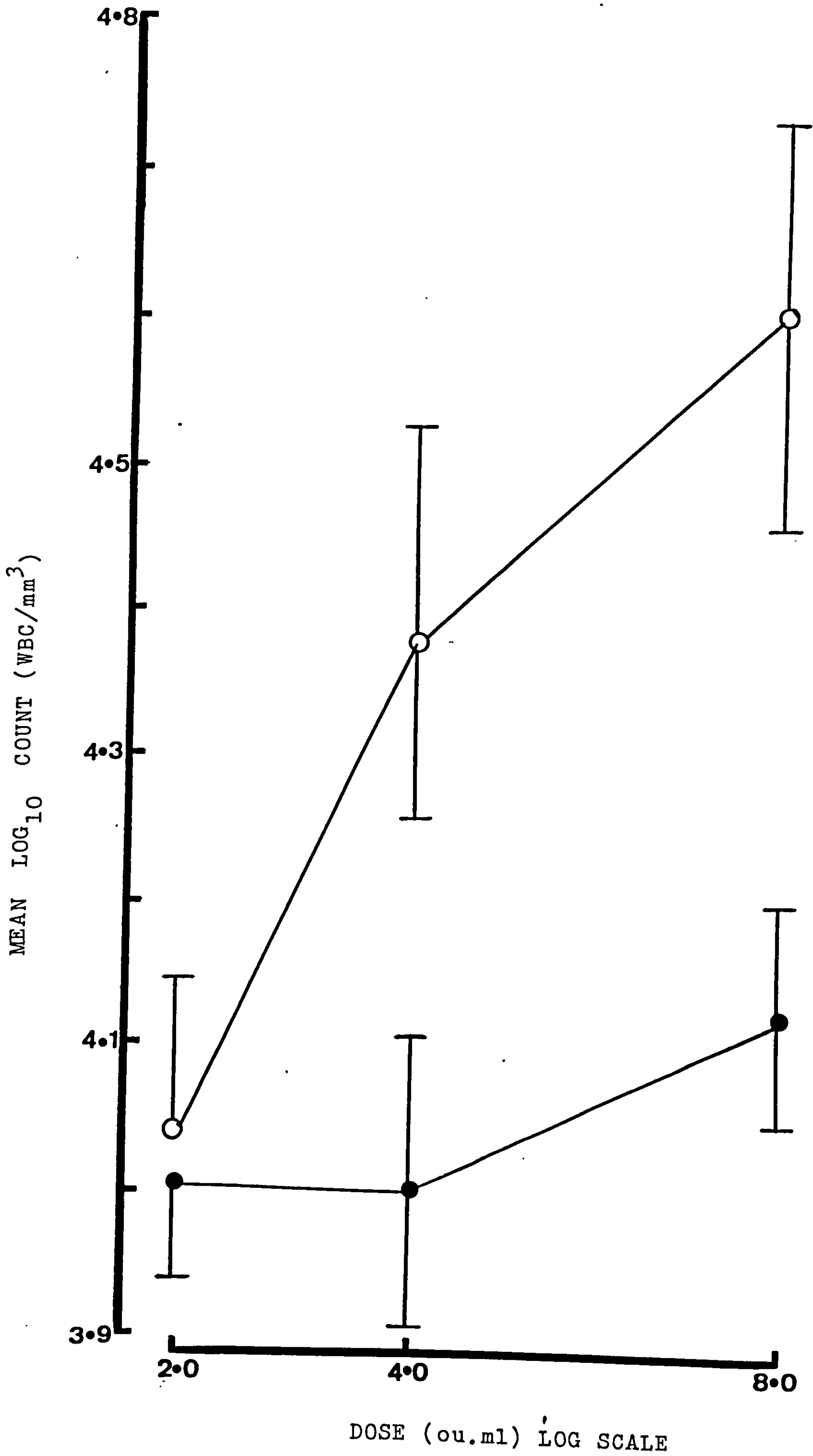


Figure 9 : LPF dose-response curves for 10h and
48h X-mode cell suspensions.
6-point assay.

▲————▲ 10h X-mode

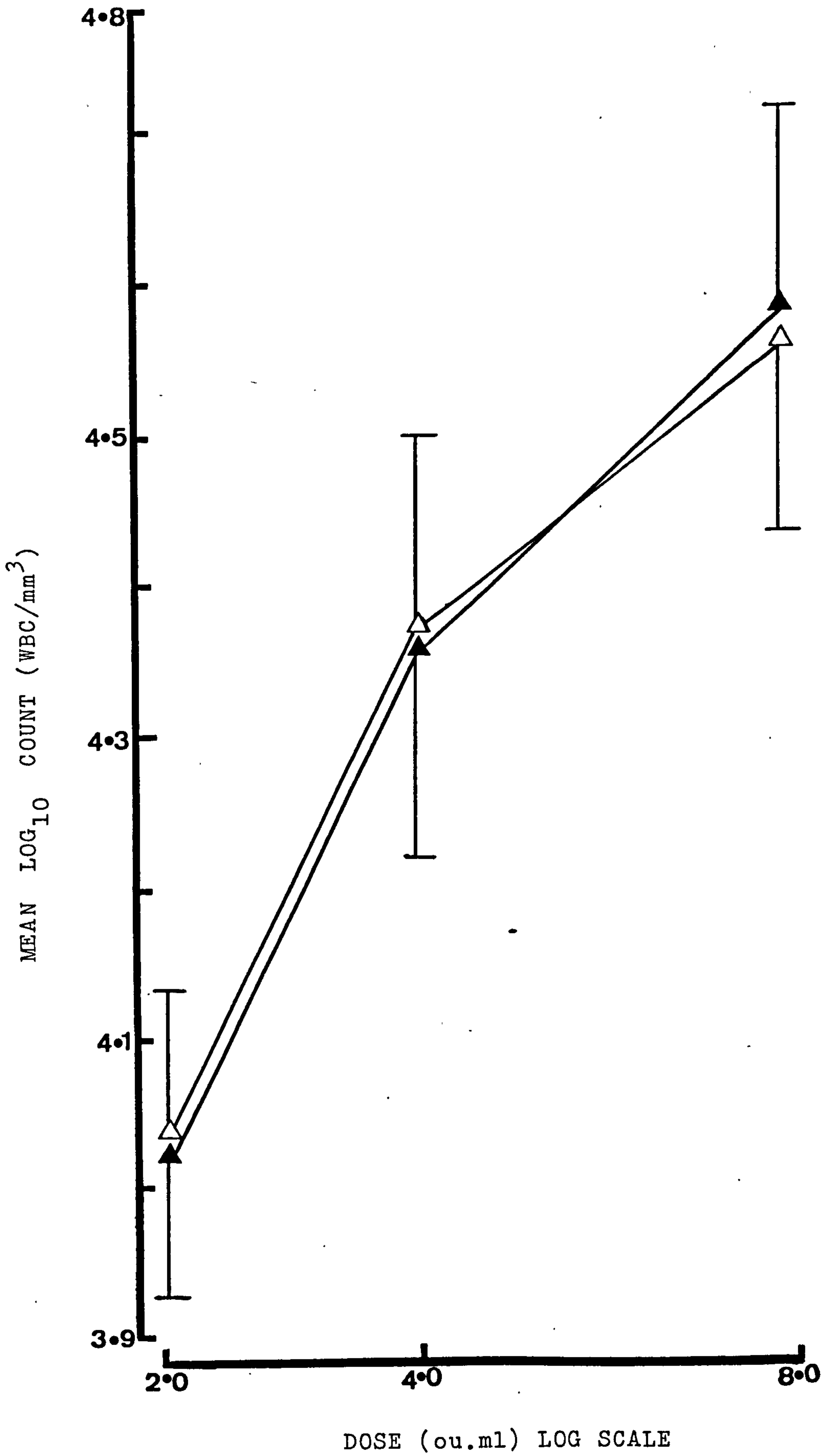
△————△ 48h X-mode

Bars represent the standard deviations
from the mean leukocyte counts.

Table 7: LPF potencies of X- and C-mode
cell suspensions by 6-point assay

Age of culture	Relative potency (and 95% confidence limits)	
	X-mode	C-mode
Inoculum (48h X-mode)	1.00	**
10h	0.93 (0.70-1.2)	**
24h	0.95 (0.76-1.2)	**
48h	0.99 (0.78-1.3)	**
72h	0.97 (0.55-1.5)	**

** assay not valid at doses tested.



SECTION 2 : CULTURAL CHANGES DURING
MODULATION

Subsection A : X to C modulation

Modulation was induced by inoculating X-mode B. pertussis into modified Hornibrook medium containing high levels (5g/l) of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (C-medium). A number of shake flasks containing either X- or C-medium were inoculated with equal amounts of a 48h X-mode liquid culture. Flasks were incubated and harvested at various intervals during growth. Samples obtained were later assayed for various pathophysiological and immunological activities.

1. Growth characteristics

i. Comparability of shake-flask cultures

In early experiments, one X-mode and one C-mode shake-flask culture were used and sampled repeatedly at various intervals during growth. However, this led occasionally to problems with contamination and did not provide enough cells for all tests undertaken. To overcome these initial problems, a number of flasks of X- and C-mode cultures were set up in parallel so that the contents of one flask could be harvested at each sampling time. In order to check the validity of this procedure the following experiment was done. At various intervals, estimation of viable cell numbers was done on three X-mode and three C-mode flasks. The aim was to determine if parallel shake flasks receiving the same size of inoculum and grown under the same conditions

showed any difference in viable cell numbers at any sampling time during growth. The experiment was repeated several times and results obtained showed that viable counts of the parallel cultures did not differ significantly at any time during growth. Thus in later experiments this parallel shake-flask culture method was adopted.

ii. Viable count and optical density

At each sampling time during growth, the viable cell numbers and optical densities of X- and C-mode cultures were measured.

The viable counts in a typical experiment showed that the X- and C-mode cells grew at approximately the same rate, though there seemed to be an initial lag in the C-mode cultures (Figure 10A). The mean generation time for both X- and C-mode cells was found to be approximately 6-7h.

Despite the close similarity of the viable counts of X- and C-mode cultures at any given time, the optical densities of the C-mode cultures were consistently higher than those of the X-mode cultures throughout growth (Figure 10B). There was an indication that for the same viable cell number the C-mode cells had a higher optical density than the X-mode cells.

The viable cell numbers and optical density values of X- and C-mode cultures obtained in a number of independent experiments, in which 5% (v/v) or 10% (v/v) inocula were used, are shown in Figures 11A to 12D. A 10% (v/v) inoculum was used in some experiments to provide sufficient cells at

Figure 10 : Changes in viable cell numbers and optical density during growth. (Typical growth experiment).

Data were obtained from growth of B. pertussis strain 18334 in X- and C-media. A 5% (v/v) inoculum of 48h X-mode culture was used.

A. Viable cell numbers

○—○ X-mode culture

●—● C-mode culture

B. Optical density

○—○ X-mode culture

●—● C-mode culture

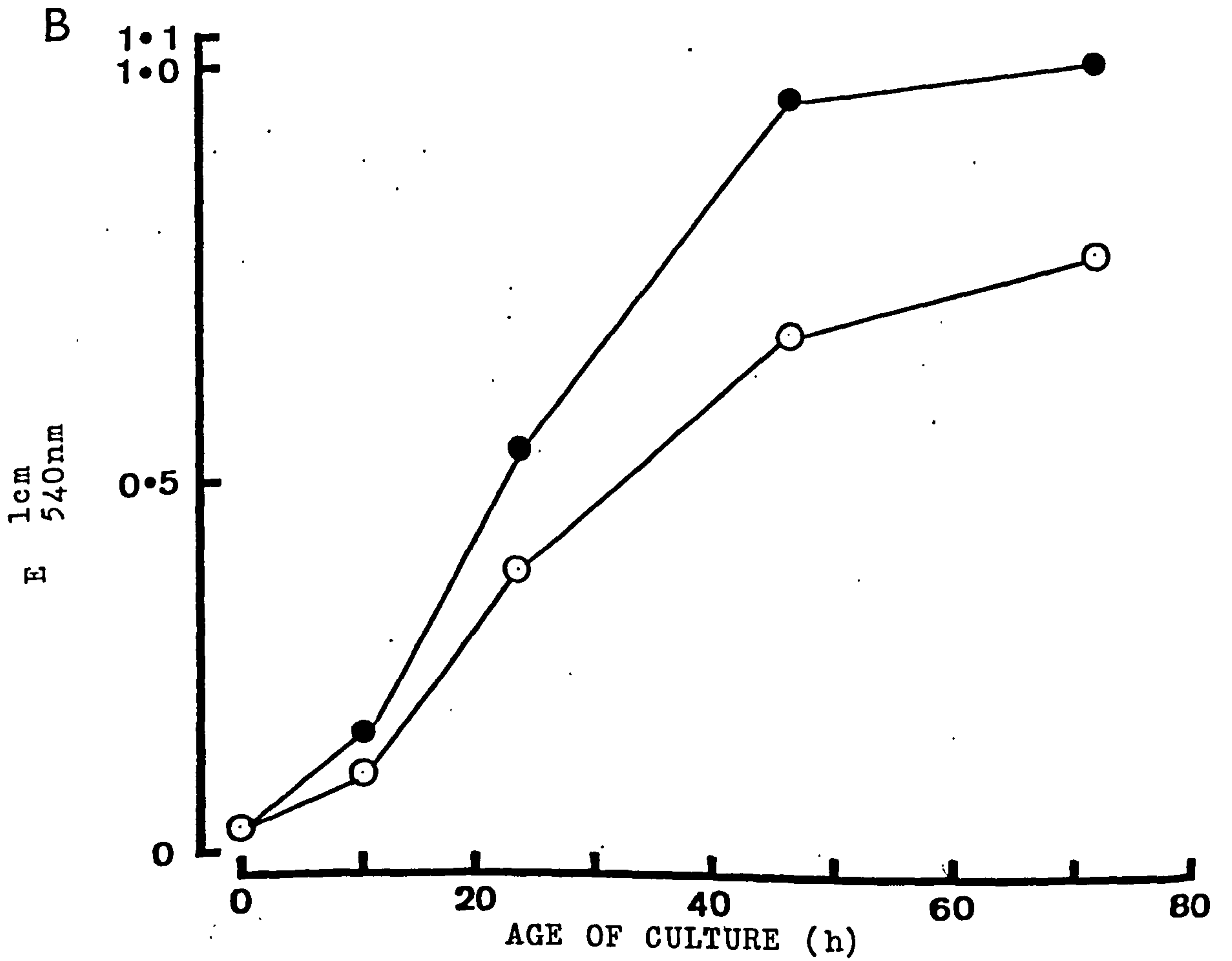
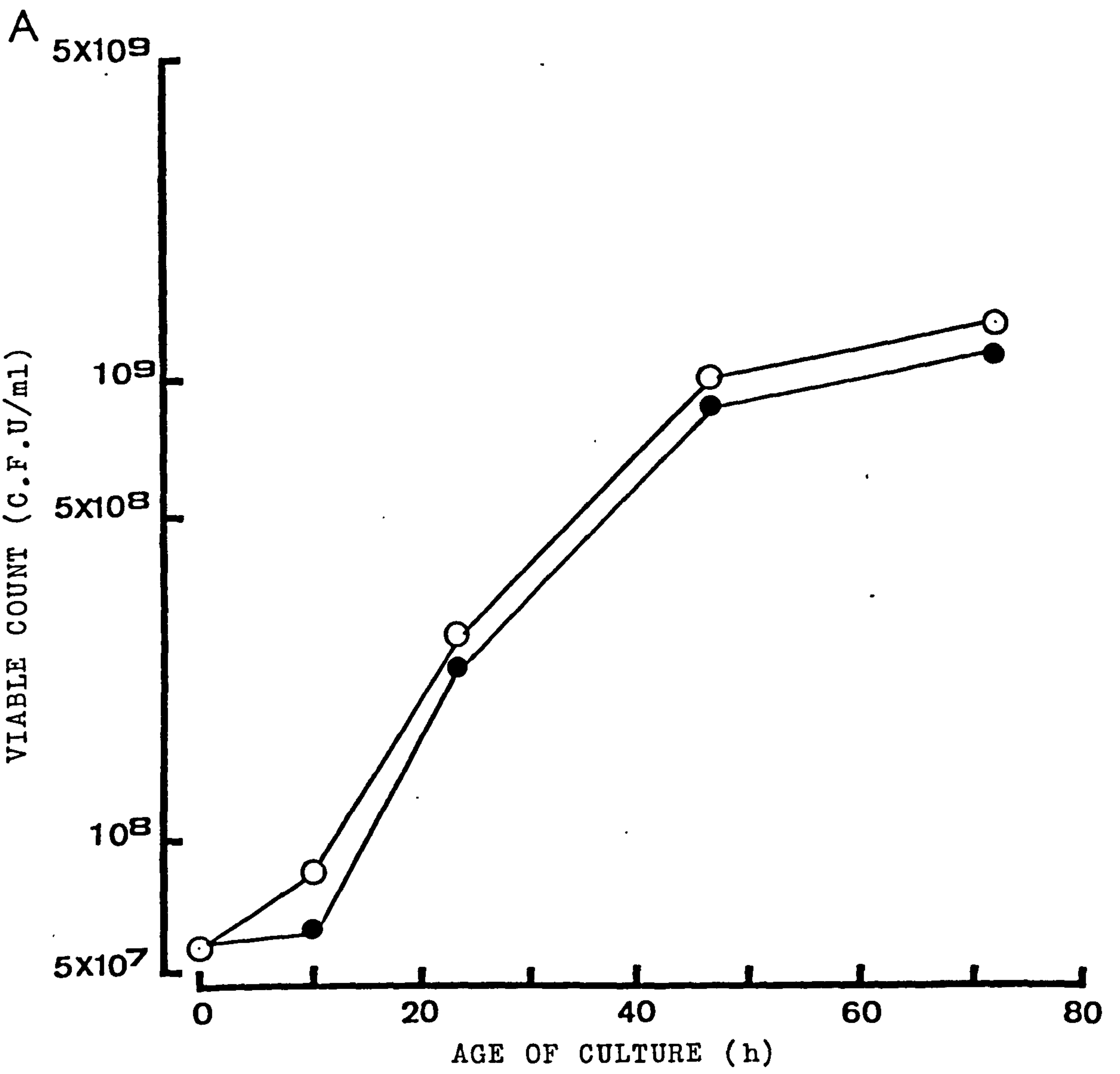


Figure 11 : Changes in viable cell numbers and optical density during growth. (5% (v/v) inoculum)

Data were obtained from four similar experiments in which B. pertussis strain 18334 was grown in X- and C-media. In each experiment, inoculum was obtained from a 48h X-mode culture.

A. Viable cell numbers in X-medium

●—● experiment 1
○—○ experiment 2
▲—▲ experiment 3
△—△ experiment 4

B. Optical density in X-medium

●—● experiment 1
○—○ experiment 2
▲—▲ experiment 3
△—△ experiment 4

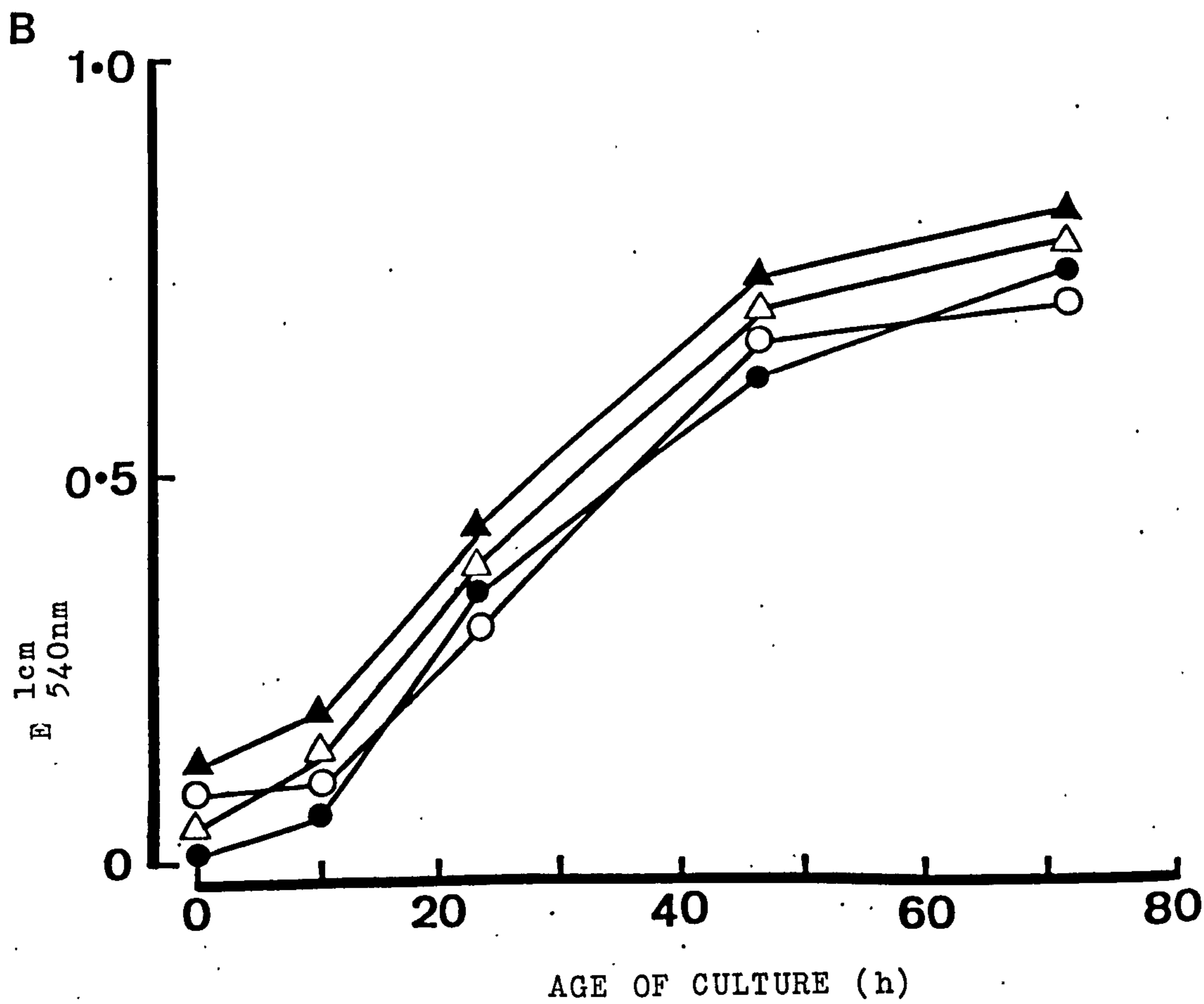
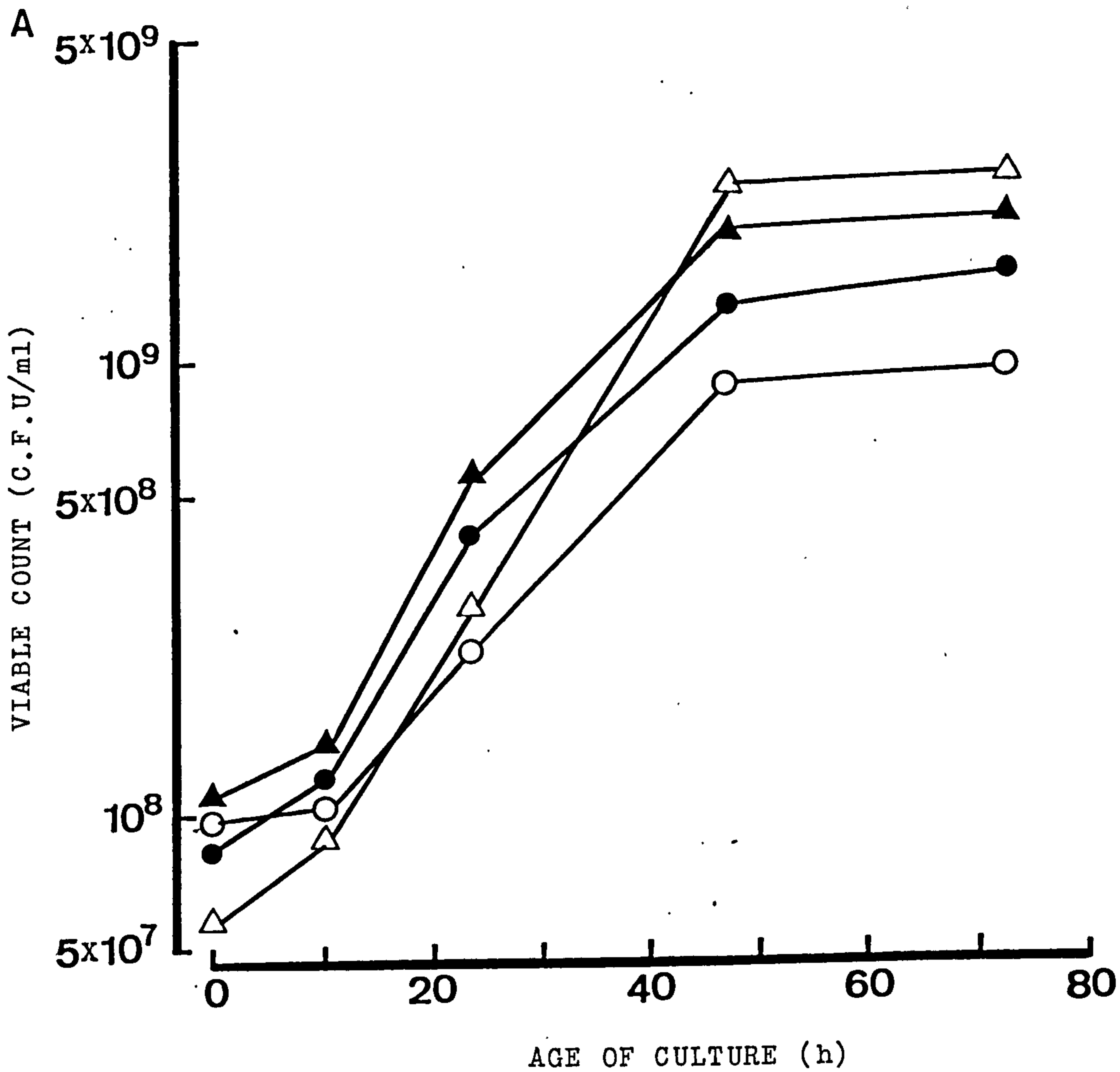


Figure 11C :

Viable cell numbers in C-medium

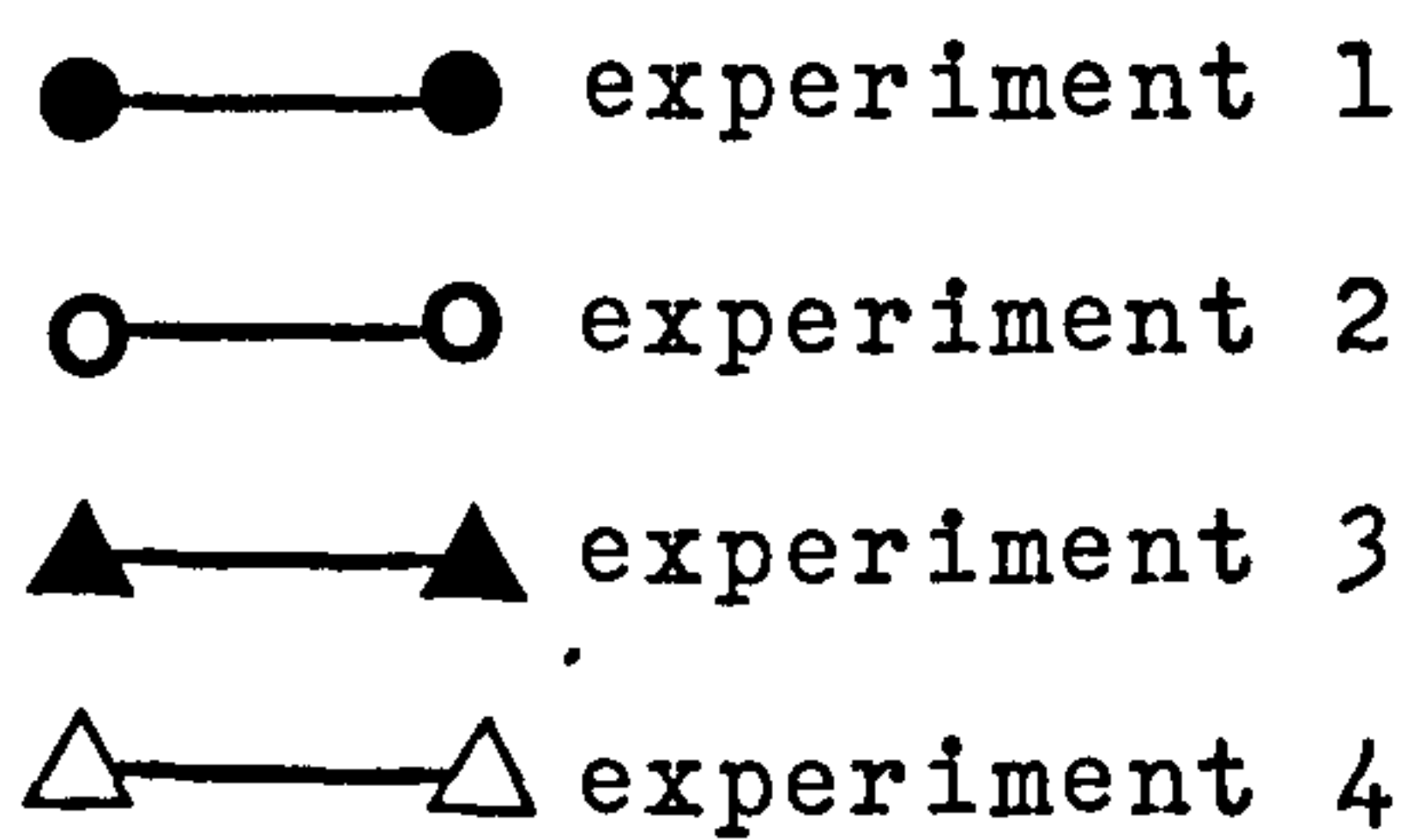
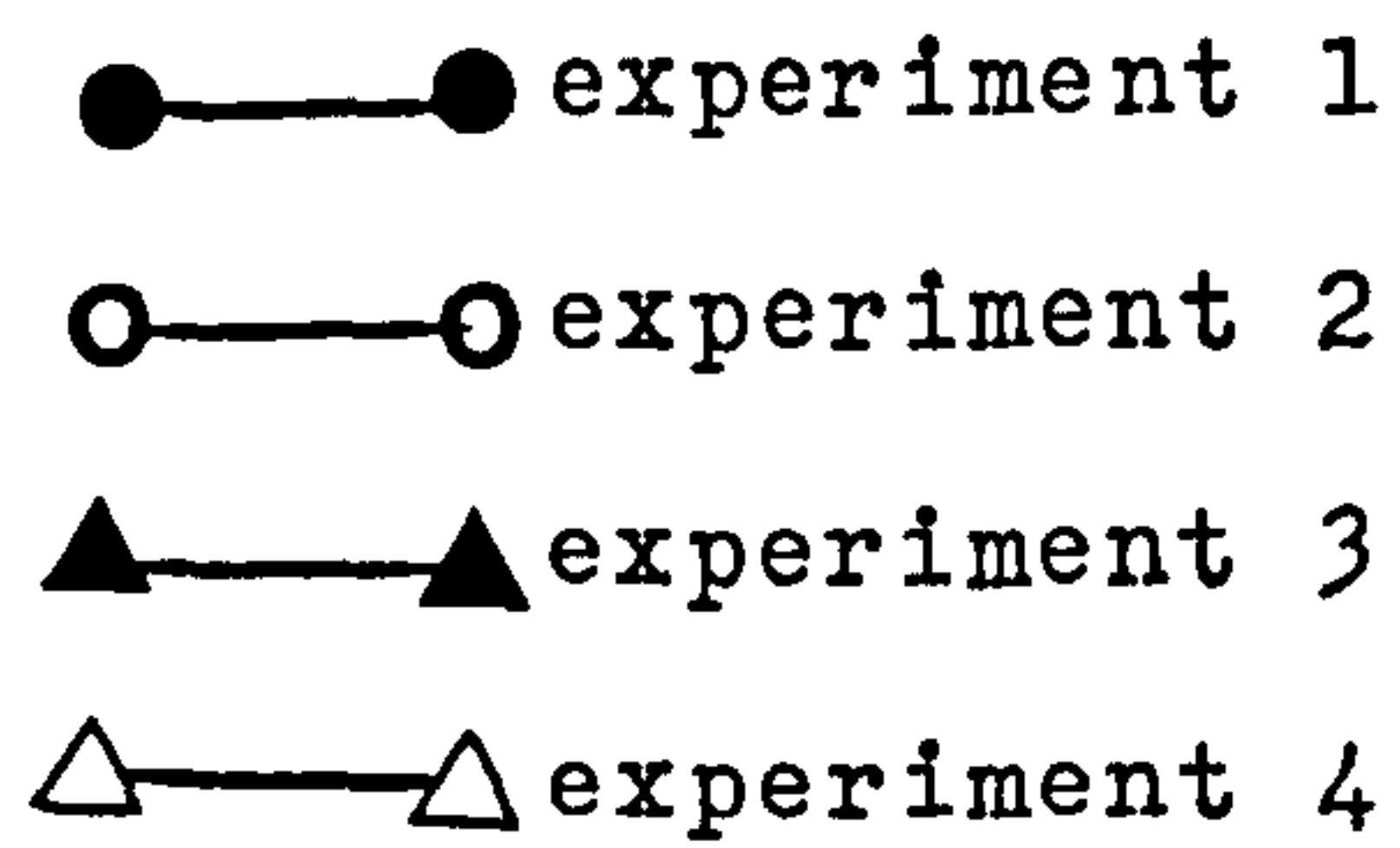


Figure 11D :

Optical density in C-medium



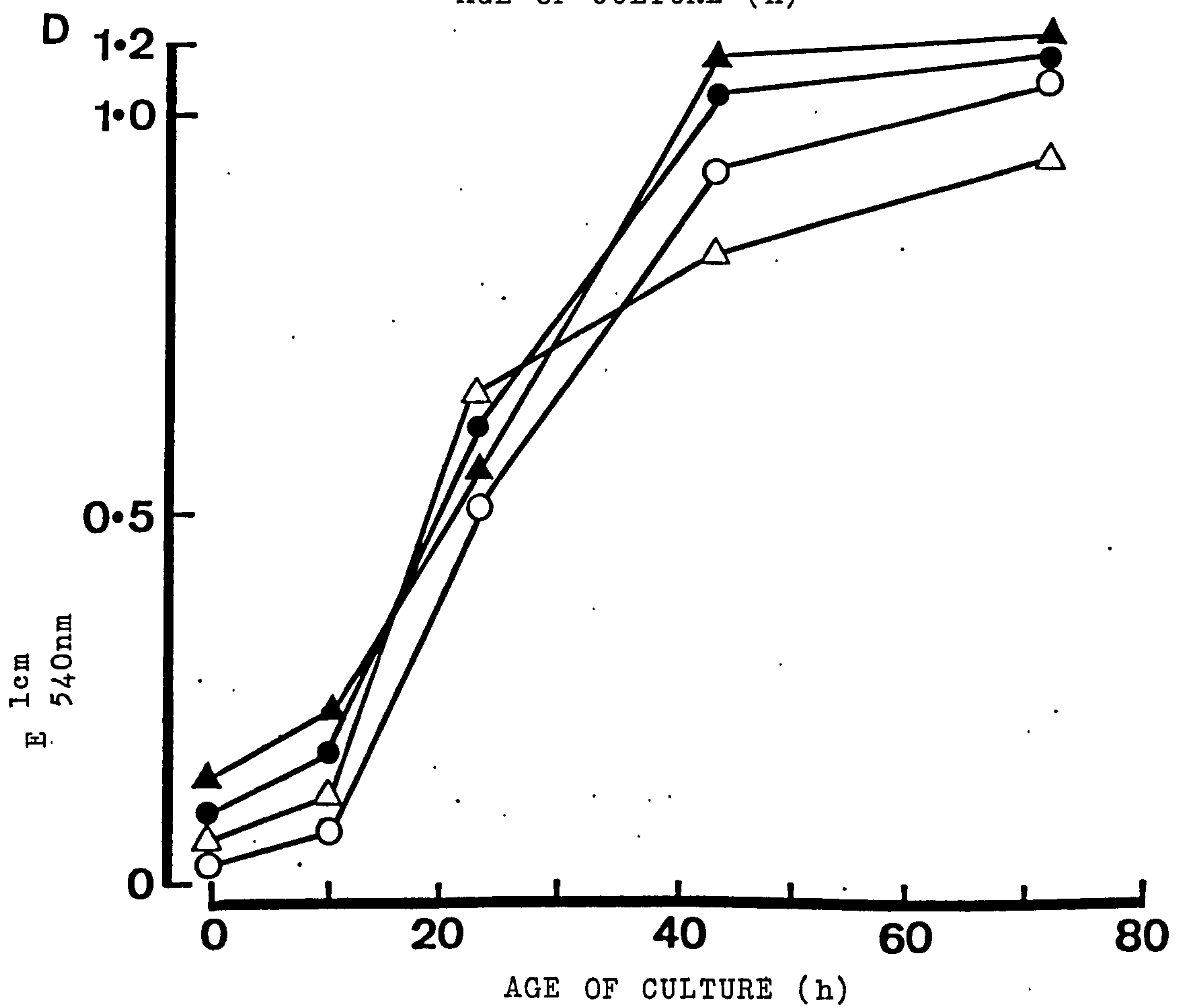
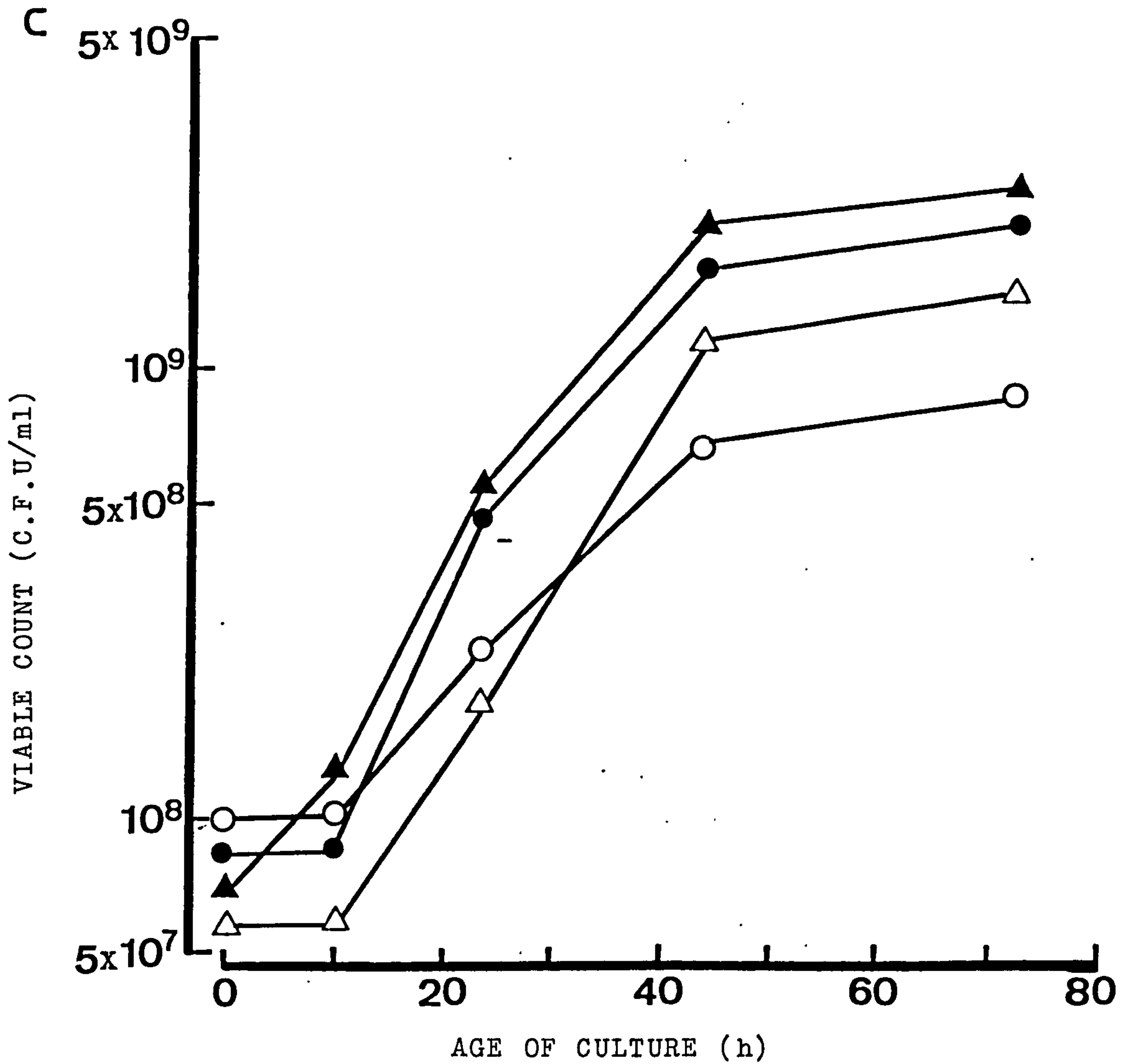


Figure 12 :

Changes in viable cell numbers and optical density during growth. (10% (v/v) inoculum).

Data were obtained from three similar experiments in which B. pertussis strain 18334 was grown in X- and C-media. In each experiment, inoculum was obtained from a 48h X-mode culture.

A. Viable cell numbers in X-medium

○—○ experiment 1
●—● experiment 2
△—△ experiment 3

B. Optical density in X-medium

○—○ experiment 1
●—● experiment 2
△—△ experiment 3

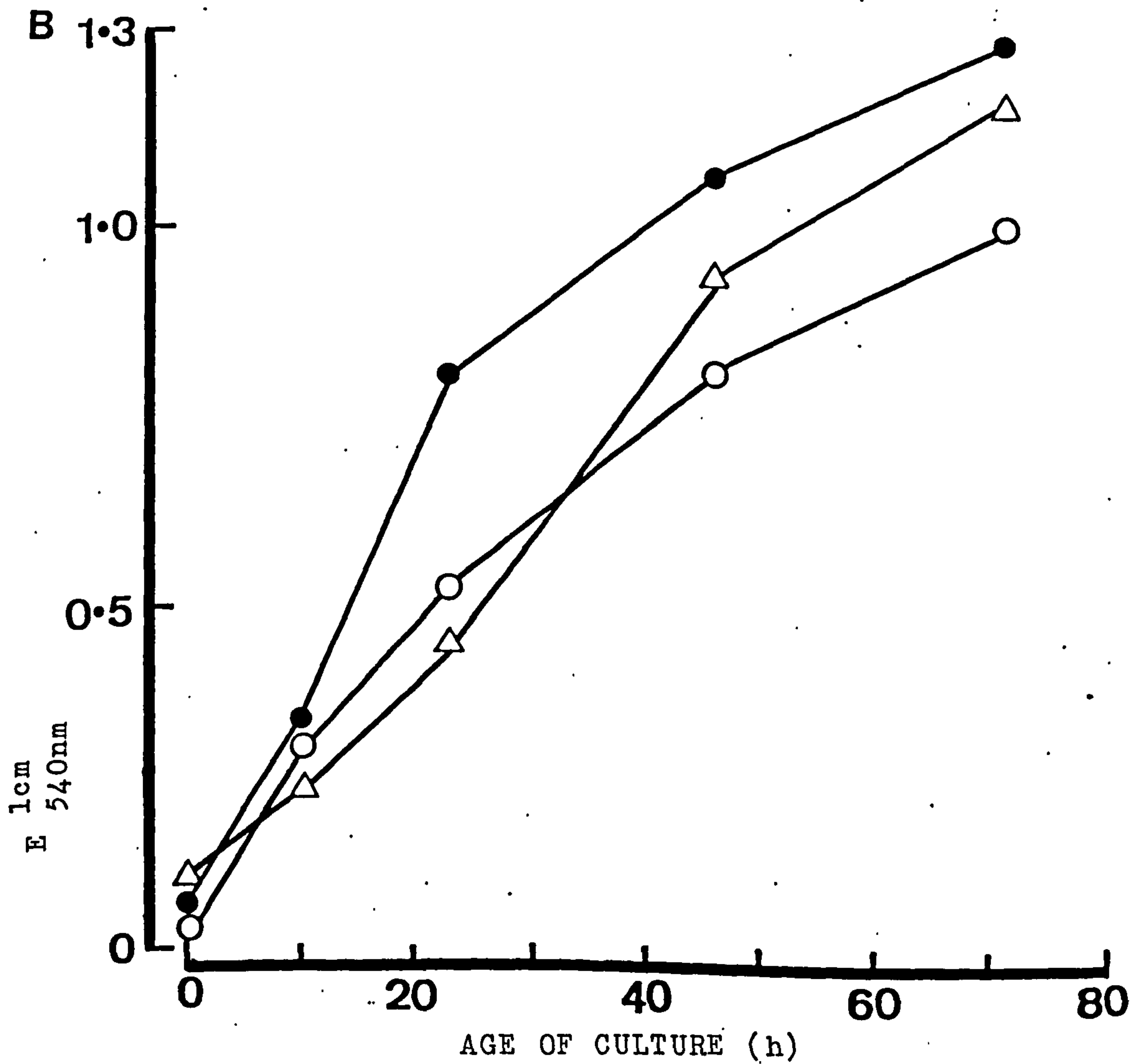
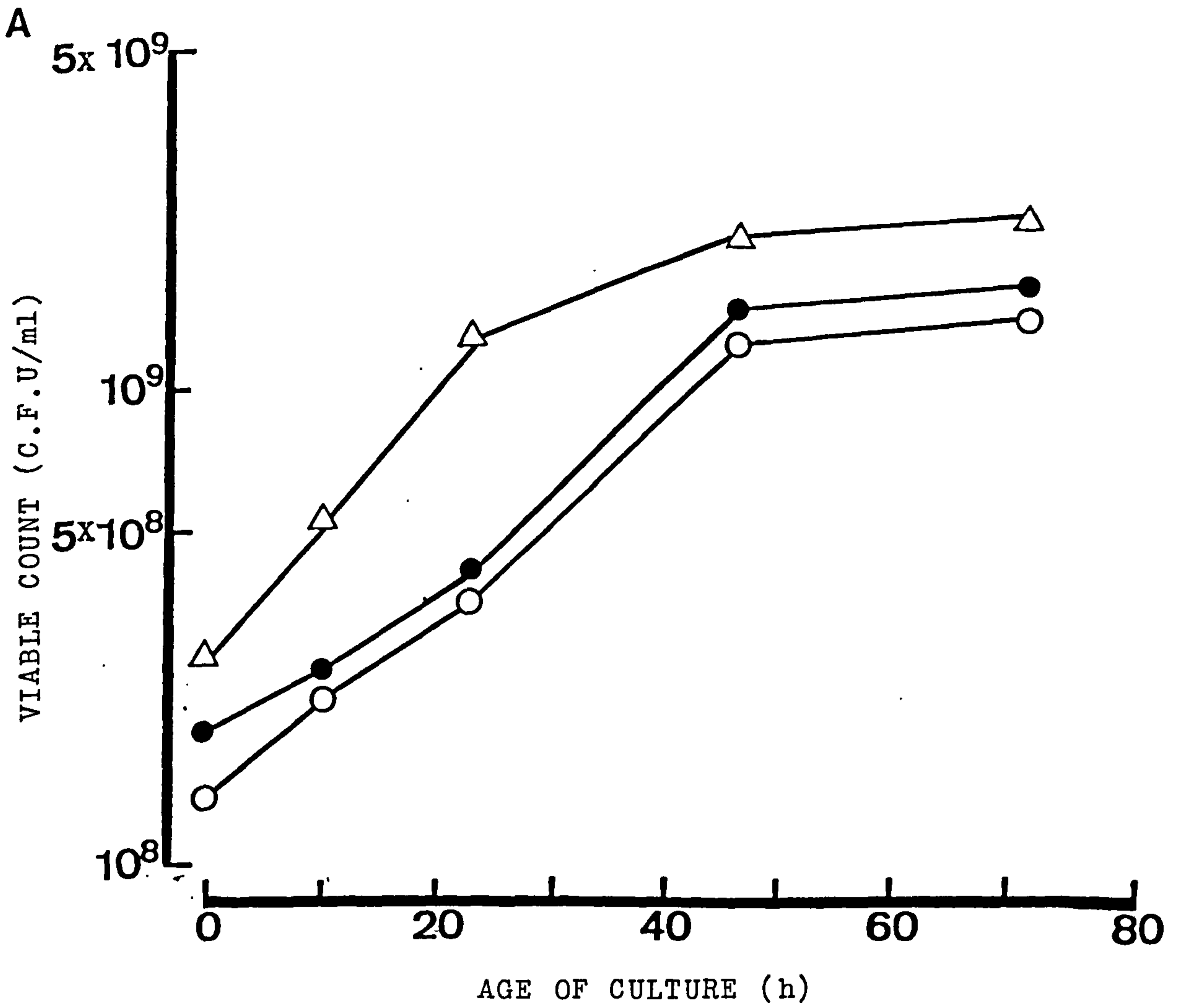


Figure 12C :

Viable cell numbers in C-medium

○—○ experiment 1

●—● experiment 2

△—△ experiment 3

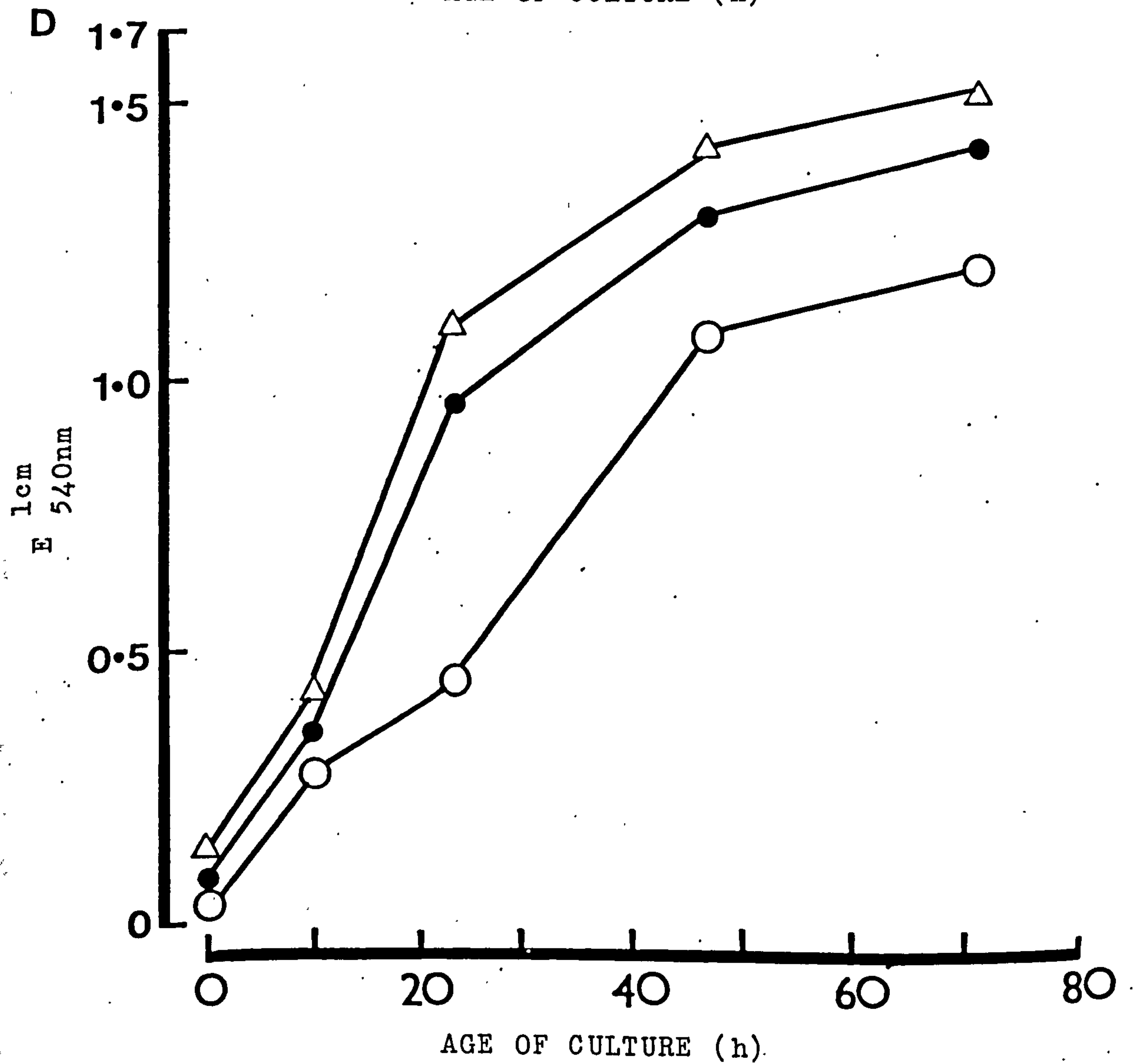
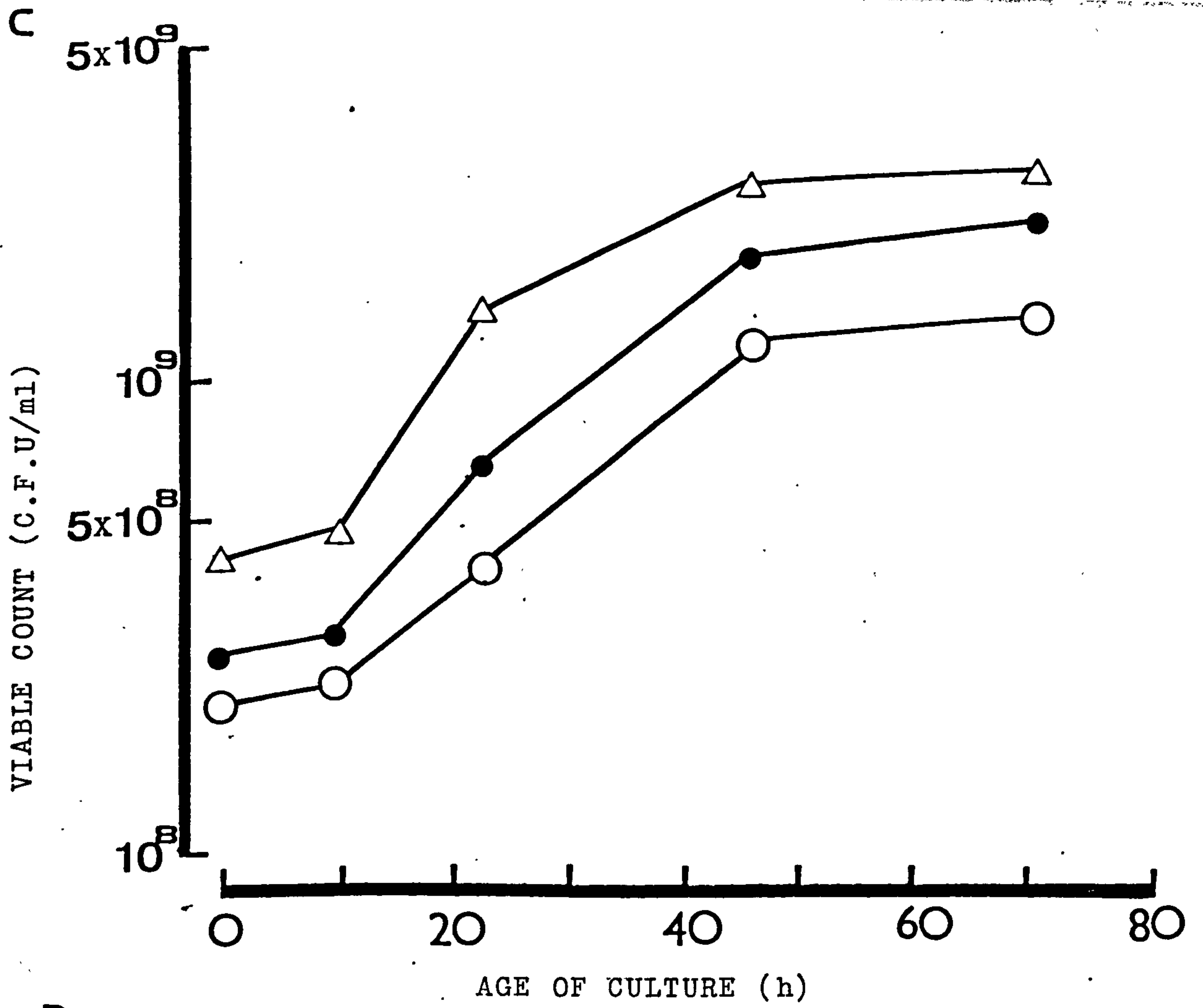
Figure 12D :

Optical density in C-medium

○—○ experiment 1

●—● experiment 2

△—△ experiment 3



10h to allow bio-assays to be repeated where necessary.

2. Immunological and pathophysiological
activities

i. Protective activity

Heated (56°C/30 min) X- and C-mode cell suspensions harvested at various intervals during growth were assayed for their mouse-protective activities. In each mouse-protection test the 48h X-mode sample was used as a standard against which the protective potencies of the other samples were compared.

Results (Appendix 5) were analysed by the probit method using a computer programme. In four independent experiments the relative potency values obtained for each sample were very similar, ranging from 0.52-0.81 for X 10h samples; 0.006-0.015 for C 10h samples and 0.001-0.008 for C 48h samples (Table 8). The best-estimated relative potency values (obtained as shown in Appendix 4g), from the four mouse-protection experiments showed that at 10h, cells grown in C-medium had only 2% of the PA activity of the same age of cells grown in X-medium. At 48h, the level of activity in the C-cells had decreased to 0.5% of the level of activity in their X-mode counterparts, although based on the 95% confidence limit values, the C 10h and C 48h samples were not significantly different in their protective potencies. There was also no significant difference between the levels of activities in the 10h and 48h X-mode samples because the protective potency value of

Table 8:

Summary of mouse-protection test data

Sample	Relative potencies (and 95% confidence limits) from four independent experiments	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Best-estimate relative potency values (and 95% confidence limits)	Expected relative potency values based on observed number of cell divisions
X-mode cells (10h)		0.52 (0.02- 8.6)	0.52 (0.09- 2.4)	0.81 (0.19- 3.4)	0.81 (0.16- 3.9)	0.70 (0.29- 1.7)	1.00
X-mode cells (48h)		1.00	1.00	1.00	1.00	1.00	1.00
C-mode cells (10h)		0.006 (0.000- 0.06)	0.009 (0.001- 0.04)	0.015 (0.003- 0.06)	0.013 (0.002- 0.06)	0.012 (0.005- 0.03)	0.77
C-mode cells (48h)		0.001 (0.000- 0.008)	0.005 (0.001- 0.024)	0.008 (0.002- 0.04)	0.004 (0.000 0.02)	0.005 (0.002- 0.01)	0.15

the 48h X-mode sample was within the 95% confidence limits of the potency value for the 10h X-mode sample (Table 8).

In view of the rapid loss of PA activity in C-medium, it was of interest to determine the potencies of C-mode cells which would be expected if there was no further synthesis of the PA when X-mode cells were inoculated into C-medium. If the original PA activity in the inoculum was diluted among the progeny cells, the expected potencies of the C-mode samples based on the number of cell divisions which had occurred in C-medium, would be 0.77 and 0.15 for C 10h and C 48h samples respectively (Table 8). The actual results obtained, 0.012 and 0.005 for C 10h and C 48h samples respectively, deviated greatly from these expected results. This suggested that the loss of PA activity in the C-mode cells was greater than would be expected from a simple dilution effect. It thus appeared that the original PA contained in the X-mode inoculum was degraded or denatured during modulation.

ii. Histamine-sensitizing activity

The HSF activities of heated X- and C-mode cell suspensions harvested at various intervals during growth were also estimated. In some experiments, the HSF activities of X- and C-mode whole cultures were estimated.

The dose of each sample required to sensitize 50% of the test animals to subsequent challenge with histamine (HSD_{50}) was obtained by probit analysis using a computer programme.

Results obtained from eight independent experiments, in which 5% (v/v) inocula were used, were very consistent. Similarly the individual results from six independent experiments in which 10% (v/v) inocula were used, did not differ significantly. In three of these experiments with a 10% inoculum, whole culture samples were also assayed and again results were consistent. From the various individual experiments, the best-estimated HSD_{50} values were calculated (Tables 9A, 9B and 9C) and the reciprocal of these values were plotted against sampling time. Figures 13A and 13B show that with a 5% (v/v) inoculum, the level of HSF activity in the X-mode cell suspensions remained relatively constant during growth. However, with the C-mode cell suspensions, the level of activity decreased rapidly with time. After 10h, the C-cells had only 12% of the activity of their X-mode counterparts and by the 72h the activity in the C-mode cells had dropped to 1% of that of the 72h X-mode cells (Table 9A).

Results from experiments with a 10% (v/v) inoculum further showed that the activity in X-mode cells per unit of opacity again stayed relatively constant during growth but decreased rapidly with time in the C-mode cells (Figures 14A and 14B). By 10h and 72h, the percentage activities of the C-mode cells relative to their X-mode counterparts were 11% and 2% respectively (Table 9B). Thus these values were very similar to those obtained with a 5% inoculum.

Table 9A:

Histamine-sensitizing activity
in X- and C-mode cell suspensions
harvested at various intervals during
growth (5%(v/v) inoculum)

Sample HSD₅₀ in opacity unit.ml (Best-estimate values from eight independent experiments).

	10h	24h	48h	72h
X-mode	0.36	0.47	0.29	0.23
C-mode	3.1	4.9	16.4	20.8
Percentage activity of C-mode relative to X-mode	12%	10%	2%	1%

Table 9B:

Histamine-sensitizing activity
X- and C-mode cell suspensions
harvested at various intervals during
growth (10%(v/v) inoculum).

Sample HSD₅₀ in opacity unit.ml (Best-estimate values from six independent experiments).

	10h	24h	48h	72h
X-mode	0.62	0.55	0.61	0.57
C-mode	5.6	12.9	33.3	33.3
Percentage activity of C-mode relative to X-mode	11%	4%	2%	2%

Table 9C: Histamine-sensitizing activity in X- and C-mode whole cultures obtained at various intervals during growth (10%(v/v) inoculum).

Sample HSD₅₀ (ml of whole-culture sample). (Best-estimate values from three independent experiments)

	10h	24h	48h	72h
X-mode	0.13	0.07	0.06	0.08
C-mode	0.56	0.70	1.3	2.9
Percentage activity of C-mode relative to X-mode	23%	10%	5%	3%

Figure 13 : Changes in the levels of histamine-sensitizing activity and viable cell numbers. (5% (v/v) inoculum).

Histamine-sensitizing activity was measured in cells harvested at various intervals during growth of X-mode B. pertussis strain 18334 in X- and C-media. Data represent the reciprocal of the best-estimated HSD_{50} values and mean viable cell numbers obtained from eight similar experiments.

A. Histamine-sensitizing activity.

○—○ X-mode cells

●—● C-mode cells

B. Viable cell numbers.

○—○ X-mode culture

●—● C-mode culture

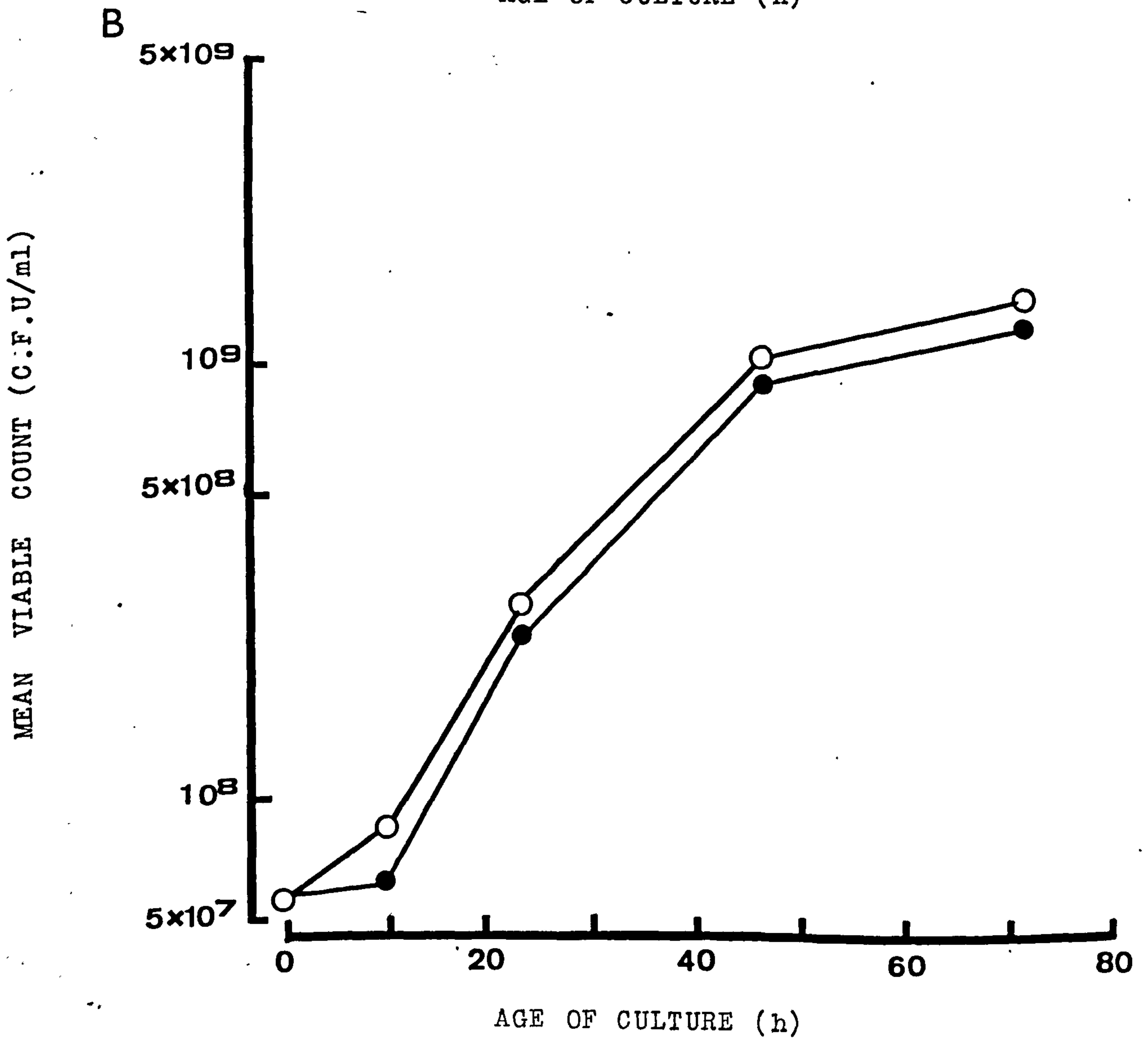
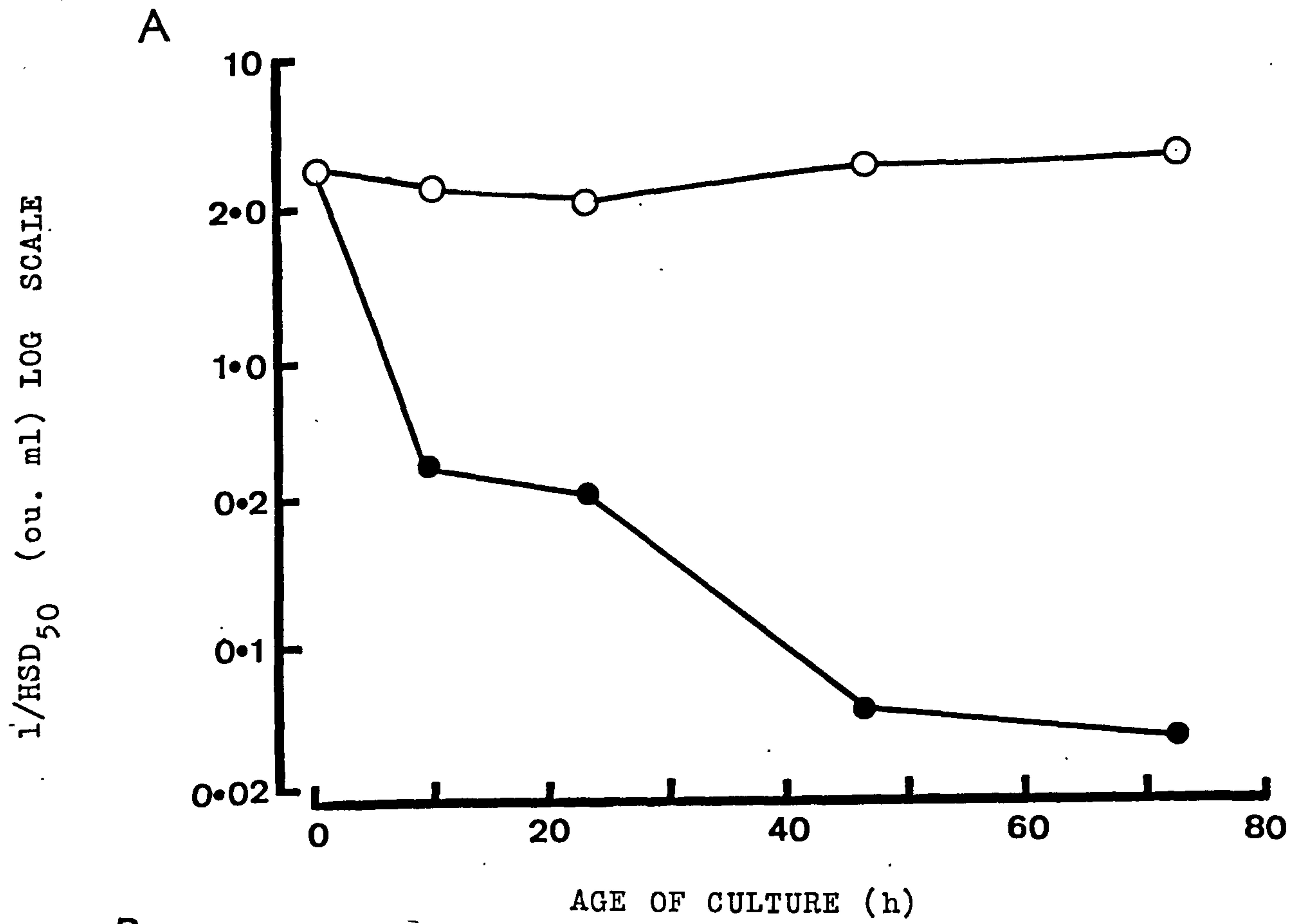


Figure 14 :

Changes in the levels of histamine-sensitizing activity and viable cell numbers (10% (v/v) inoculum)

Histamine-sensitizing activity was measured in cells harvested at various intervals during growth of X-mode B. pertussis strain 18334 in X- and C-media. Data represent the reciprocal of the best estimated HSD_{50} values and mean viable cell numbers obtained from six similar experiments

A. Histamine-sensitizing activity

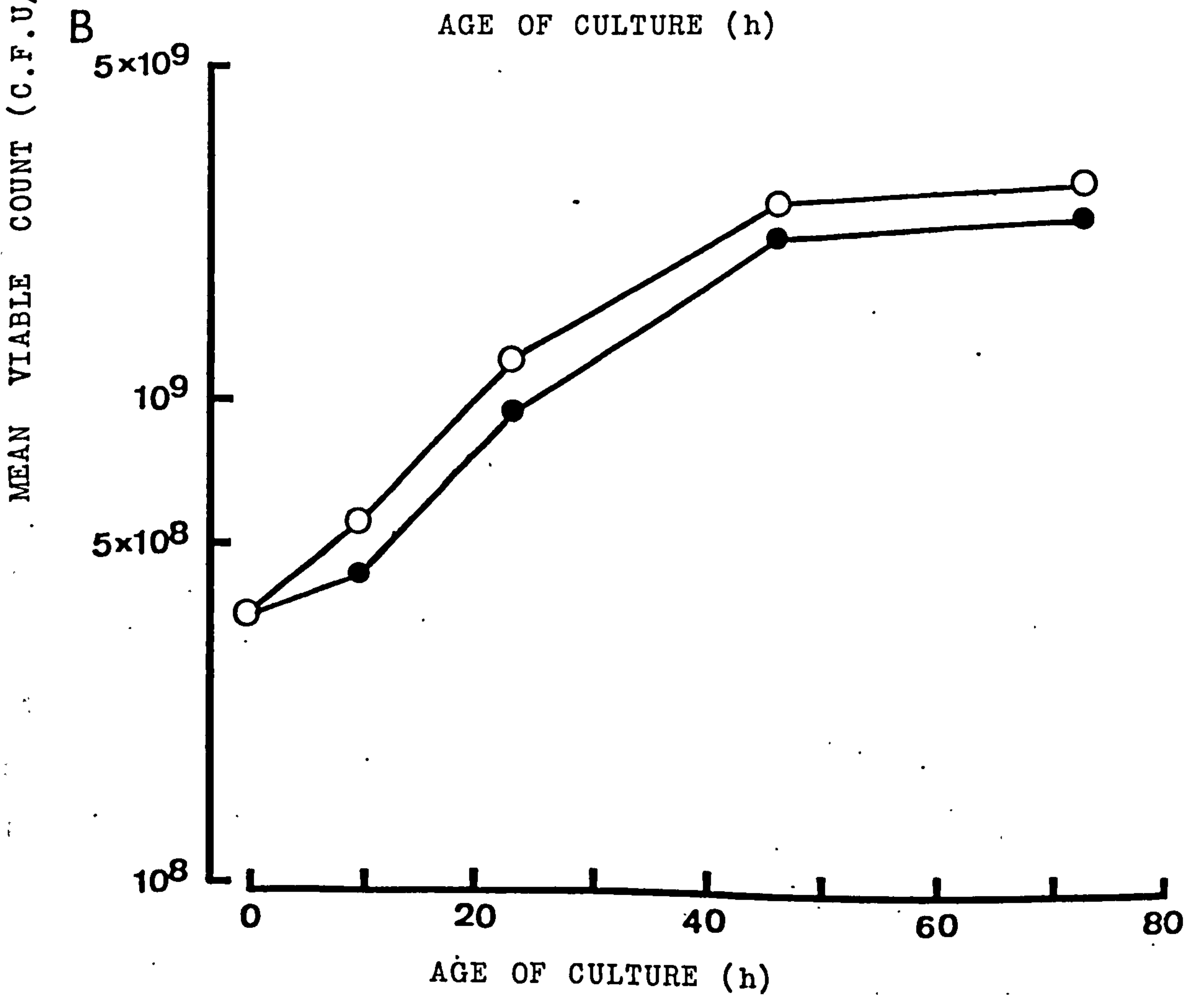
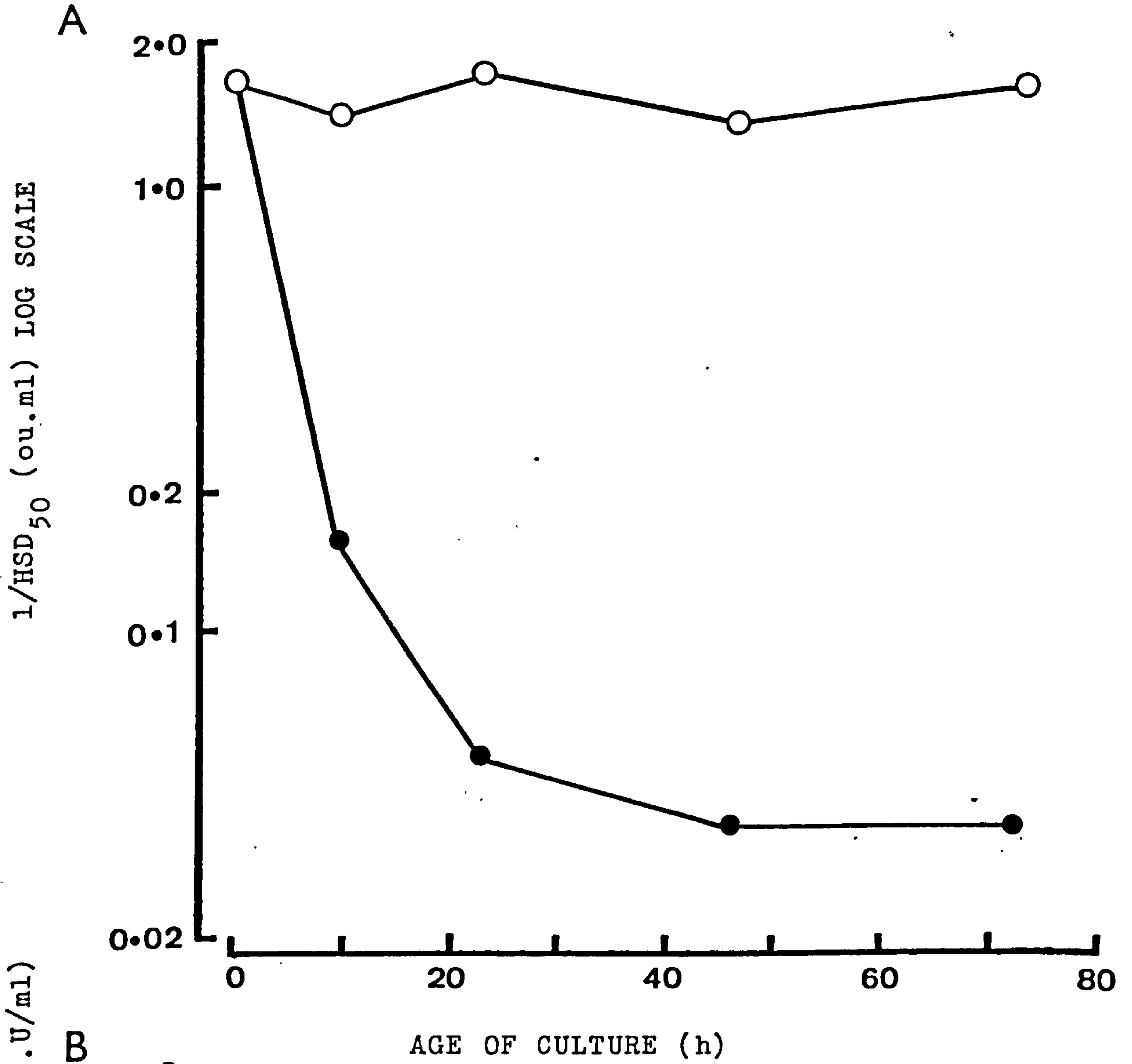
○—○ X-mode cells

●—● C-mode cells

B. Viable cell numbers

○—○ X-mode culture

●—● C-mode culture



When the total HSF activity present in the culture was estimated by testing graded doses of whole culture samples, it was found that the activity of the X-mode cultures increased simultaneously with cell numbers whereas the activity in the C-mode cultures showed a slight increase initially then decreased gradually with time (Figures 15A and 15B). As depicted in Table 9C, the total levels of activity in 10h and 72h C-mode cultures were only 23% and 3% respectively of the levels of activity in the 10h and 72h X-mode cultures. The results suggested that HSF may be transiently synthesized immediately after transfer of X-mode cells to C-medium but as the culture aged, the level of activity declined.

In order to determine if loss of HSF activity in C-mode cells could be accounted for solely by dilution among progeny cells, the levels of HSF activities of X- and C-mode cell suspensions were correlated with the viable cell numbers up to the end of log phase. Results from experiments with 5% (v/v) and 10% (v/v) inocula are shown in Figures 16A and 16B. Line 1 in both graphs represents the expected level of HSF activity in the X-mode cells assuming that cells from any part of the growth curve would contain similar amounts of HSF. The actual results obtained were in accordance with the expected results.

On the other hand, if in C-medium there was no further synthesis of HSF and the original activity in the inoculum was diluted among progeny cells, the expected levels of activity in the C-mode cells would be represented by line 2

Figure 15 :

Changes in the levels of histamine-sensitizing activity and viable cell numbers (10% (v/v) inoculum)

Histamine-sensitizing activity and viable cell numbers were estimated in whole culture samples obtained at various intervals during growth of X-mode B. pertussis in X- and C-media. Data represent the reciprocal of the best-estimated HSD_{50} values and mean viable cell numbers obtained from three similar experiments.

A. Histamine-sensitizing activity

○—○ X-mode

●—● C-mode

B. Viable cell numbers

○—○ X-mode

●—● C-mode

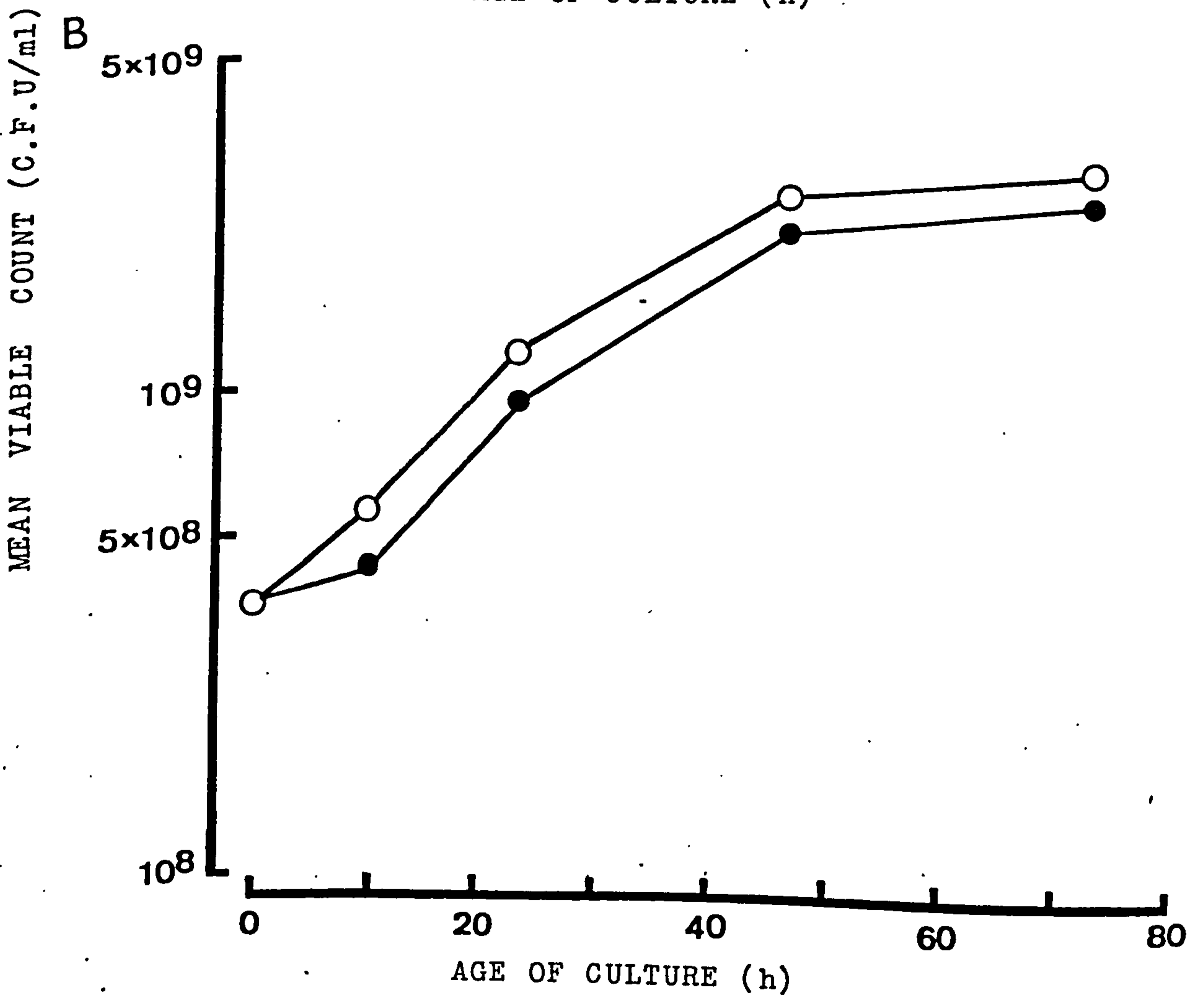
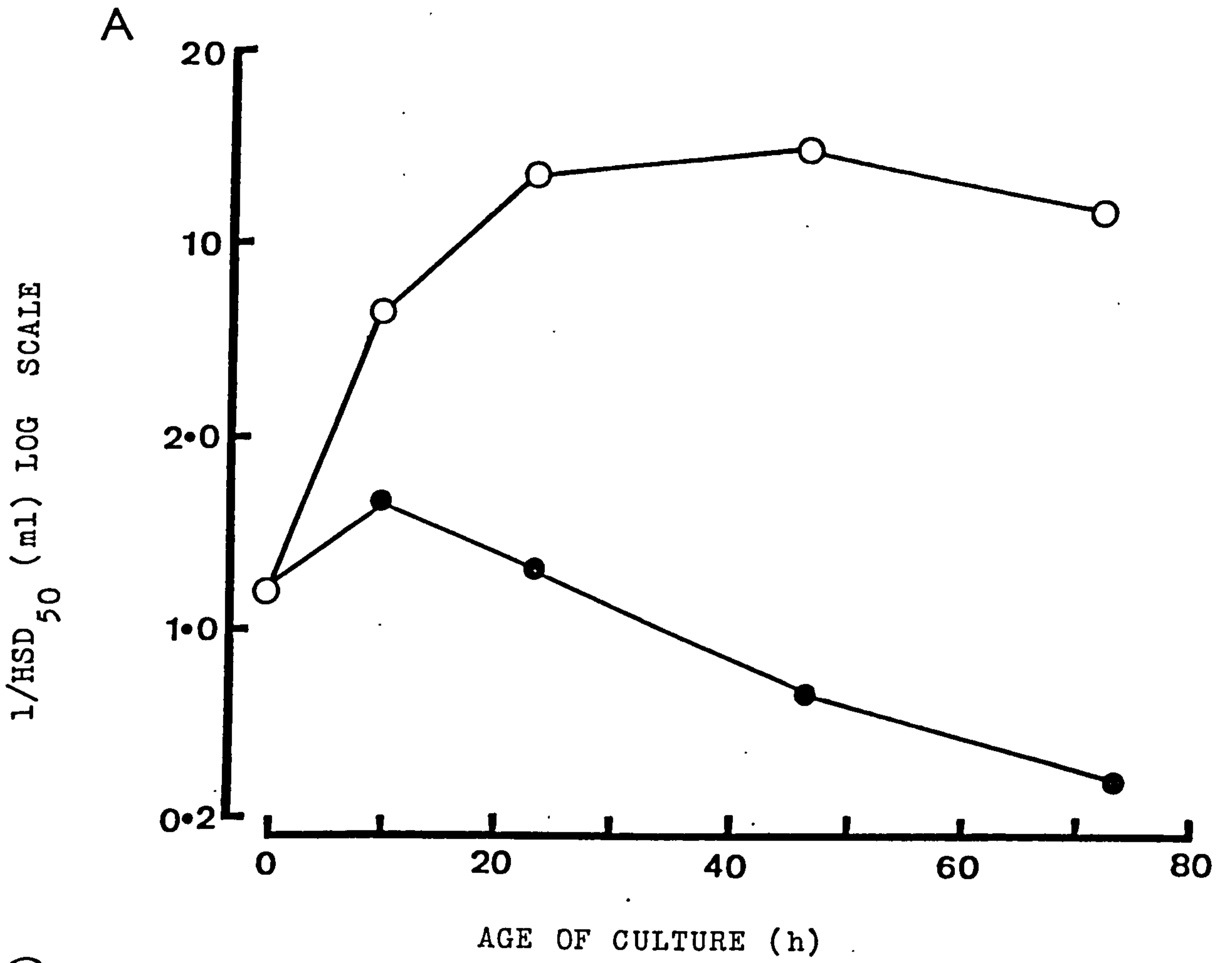


Figure 16 : Correlation between viable cell numbers and level of histamine-sensitizing activity during growth (Cells)

Data represent the reciprocal of the best-estimated HSD_{50} values and mean viable cell numbers obtained from eight similar experiments with 5% (v/v) inocula and six similar experiments with 10% (v/v) inocula.

A. Experiments with 5% (v/v) inocula

○—○ X-mode

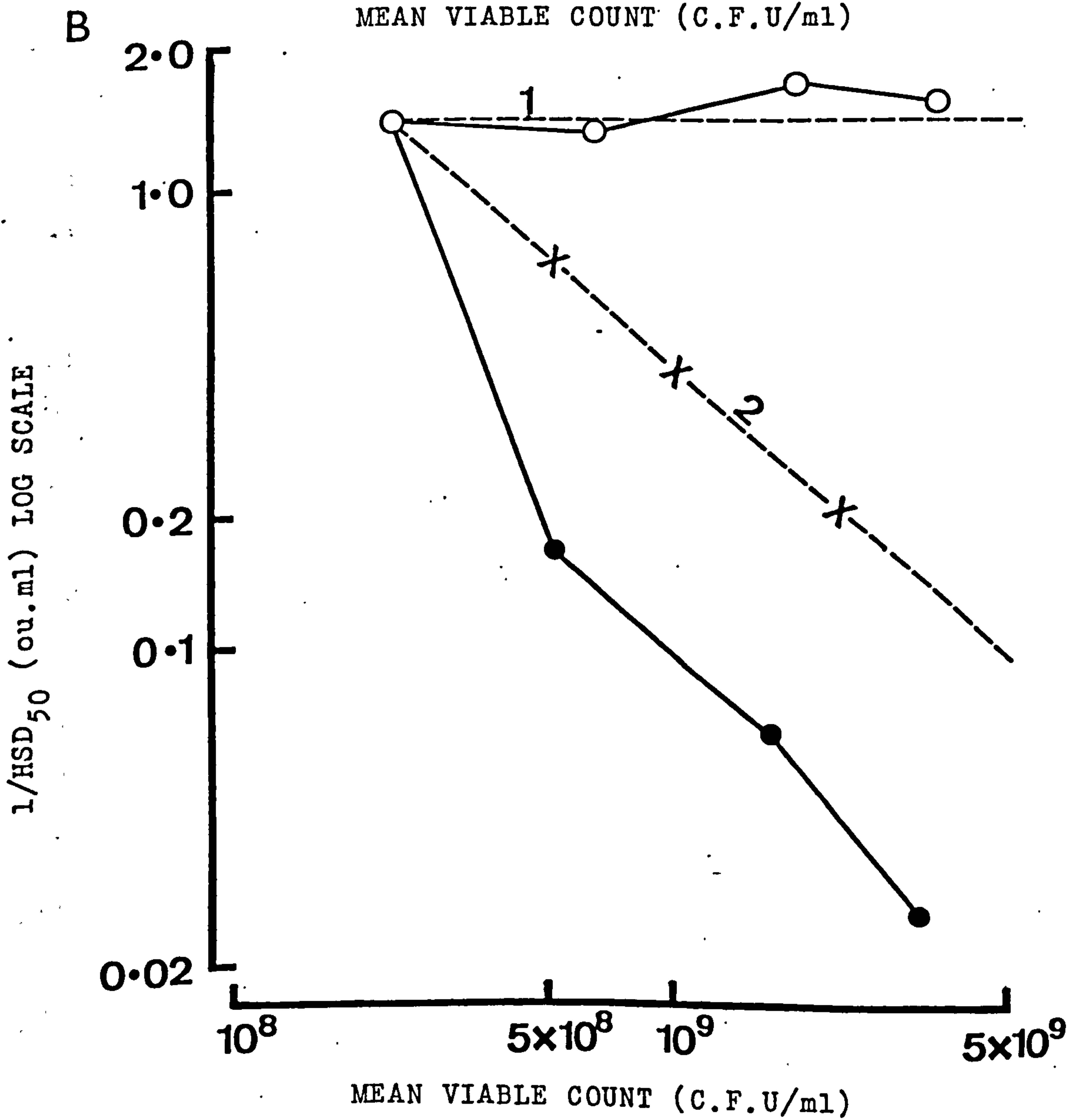
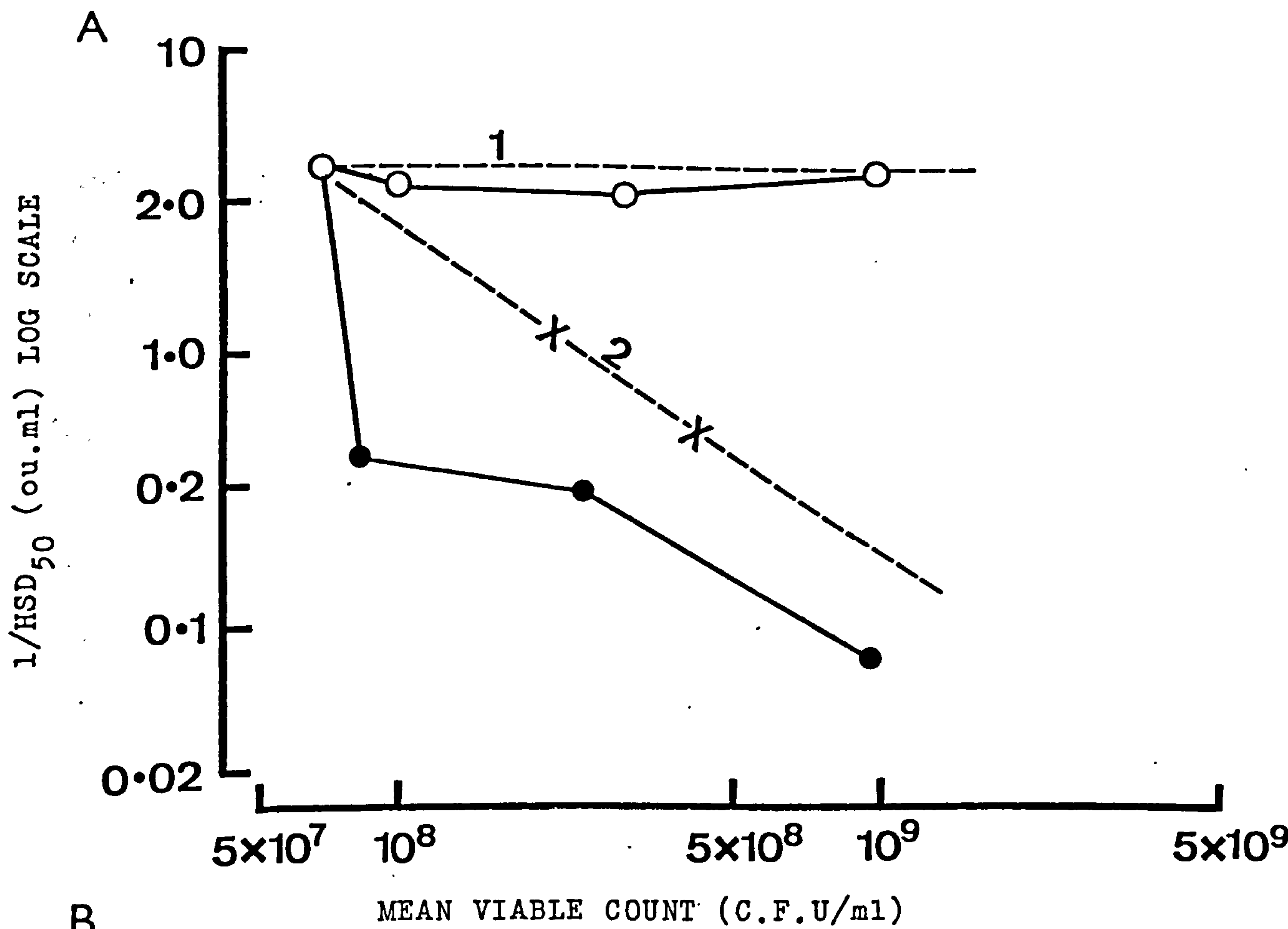
●—● C-mode

B. Experiments with 10% (v/v) inocula

○—○ X-mode

●—● C-mode

1. Theoretical level of HSF assuming a constant amount per cell during growth.
2. Theoretical level of HSF based on simple dilution among progeny cells.



in the graphs. However, the actual results obtained for the C-mode cells deviated markedly from the expected results and indicated that the loss of activity in the C-mode cells was faster than could be explained by a simple growth-dilution effect.

Figure 17 shows the correlation between the viable cell numbers and the total HSD₅₀ contained in X- and C-mode whole cultures. Line 2 predicts what would be expected if there was neither synthesis nor degradation of HSF on transfer of X-mode cells to C-medium. On the other hand, line 1 predicts the expected result if HSF was synthesized in direct proportion to viable cell numbers. The actual results indicated that in C-mode cultures the HSF appeared to be synthesized initially but after a time, this synthesis presumably ceased and the activity decreased even below the level originally present in the inoculum. With the X-mode cultures, the actual results again deviated from the expected (line 1) and showed that HSF was synthesized at a faster rate than could be accounted for by the increase in viable cell numbers.

iii. Leukocytosis-promoting activity

Changes in the levels of the LPF activity during growth of X-mode cells in X- and C-media were examined as previously described.

Both cell suspensions and whole culture samples obtained from more than a dozen similar growth experiments were assayed. In initial experiments mice were injected by the intraperitoneal

Figure 17 : Correlation between viable cell numbers and level of histamine-sensitizing activity during growth. (Whole cultures).

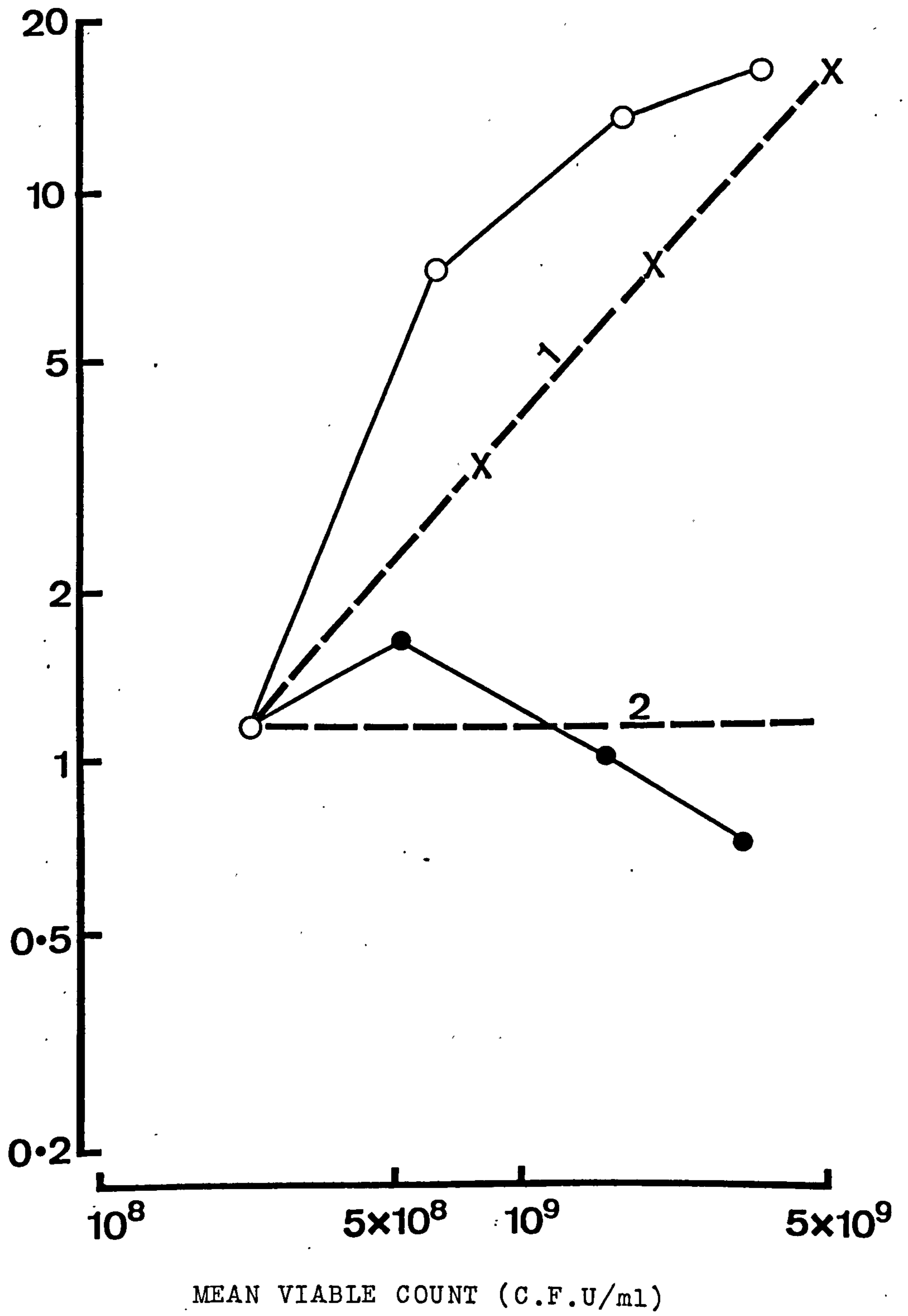
Data represent the reciprocal of the best-estimated HSD_{50} values and mean viable cell numbers obtained from three similar experiments.

○—○ X-mode

●—● C-mode

1. Theoretical level of HSF activity assuming synthesis was in direct proportion to cell numbers
2. Theoretical level of HSF activity assuming no synthesis or degradation

1/HSD₅₀ (ml) LOG SCALE



route but having demonstrated that the intravenous route was more sensitive and results more amenable to statistical analysis, the intravenous route of inoculation was used in the later part of the work. Leukocyte counts were expressed as \log_{10} .

Figures 18A and 18B show results obtained when mice were inoculated intraperitoneally with samples obtained from a typical growth experiment in which a 10% (v/v) inoculum was used. Figures 19A and 19B show the results from a similar growth experiment using the intravenous route of inoculation. With either route of inoculation the levels of activity in the X-mode cell suspensions remained relatively constant (Figures 18B and 19B). With the intraperitoneal route counts of between 20,224 and 27,669 wbc/mm^3 were obtained and between 28,380 and 32,220 wbc/mm^3 with the intravenous route. On the other hand, the level of activity in the C-mode cell suspensions decreased considerably to just above control level with the 72h samples.

A similar decrease in activity was also observed in the C-mode whole culture samples (Figures 18A and 19A). However, with the X-mode whole culture samples, the total level of activity increased initially with time, as expected, since the cell numbers were increasing. By both routes of inoculation, the LPF activity appeared to be maximal in the 24h cultures and the older culture samples showed a slight decline in the level of activities.

The loss of activity in both the C-mode whole cultures and cell suspensions did not drop below control levels.

Figure 18 : Changes in the levels of LPF responses in mice inoculated intraperitoneally.

Groups of five Ham/ICR mice were inoculated intraperitoneally with whole culture samples and cell suspensions obtained at various intervals during growth of B. pertussis strain 18334 in X- and C-media. A 10% (v/v) inoculum from a 48h X-mode culture was used.

A. Responses to whole cultures

○ — ○ X-mode

● — ● C-mode

B. Responses to cell suspensions

○ — ○ X-mode

● — ● C-mode

Bars represent the standard deviations from the mean leukocyte counts.

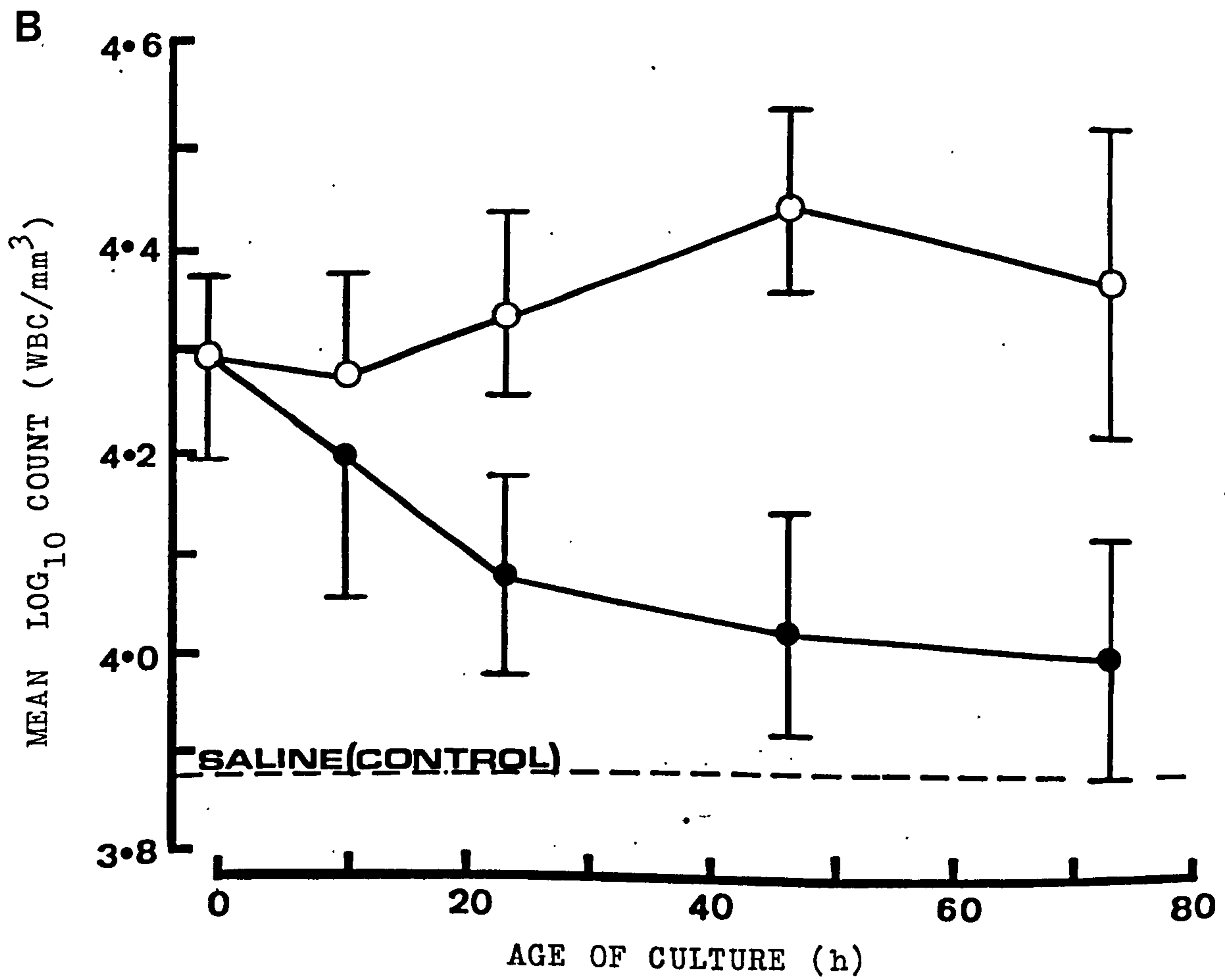
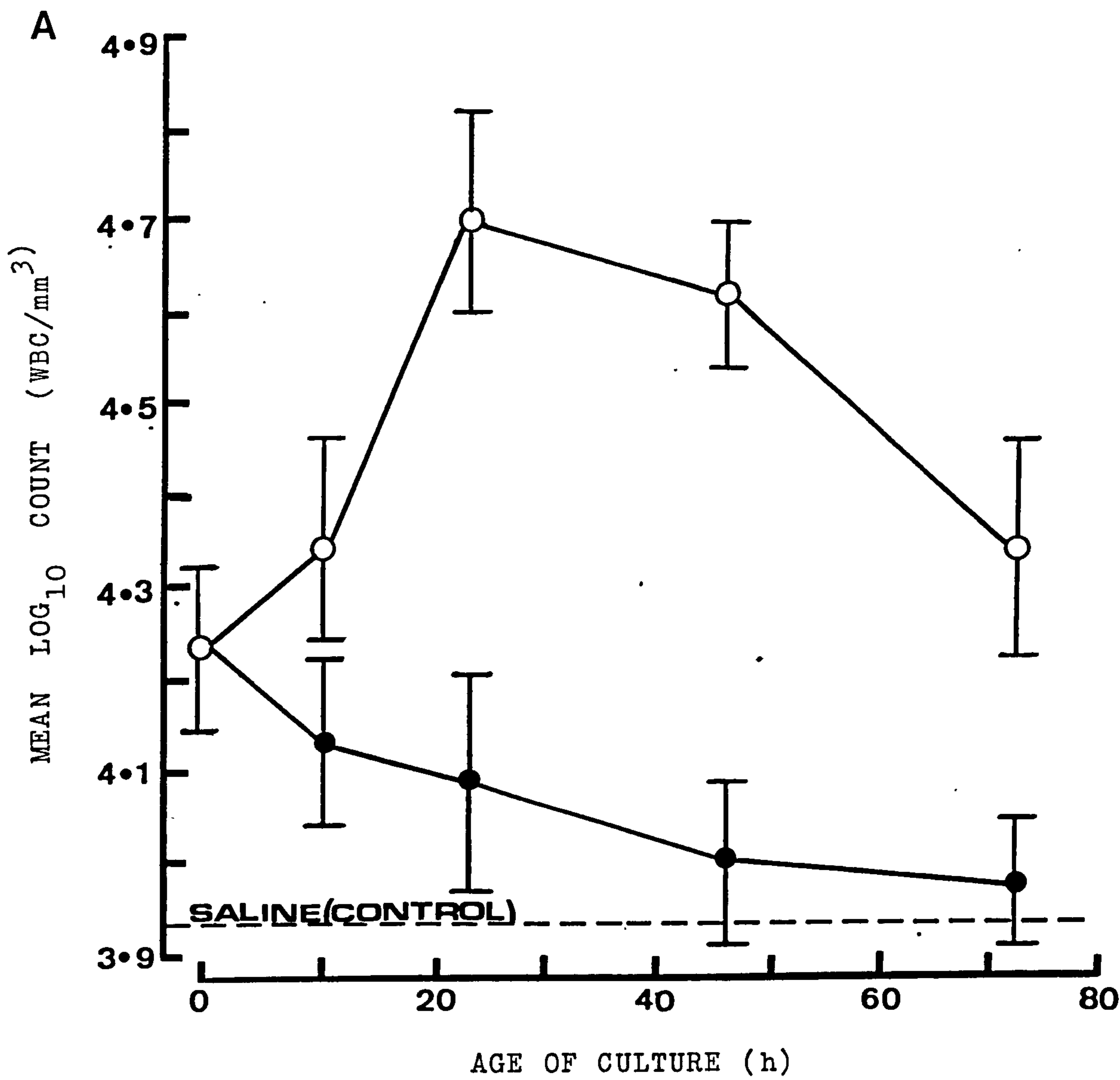


Figure 19 : Changes in the levels of LPF responses in mice inoculated intravenously.

Groups of five Ham/ICR mice were inoculated intravenously with whole culture samples and cell suspensions obtained at various intervals during growth of B. pertussis strain 18334 in X- and C-media. A 10% (v/v) inoculum from a 48h X-mode culture was used.

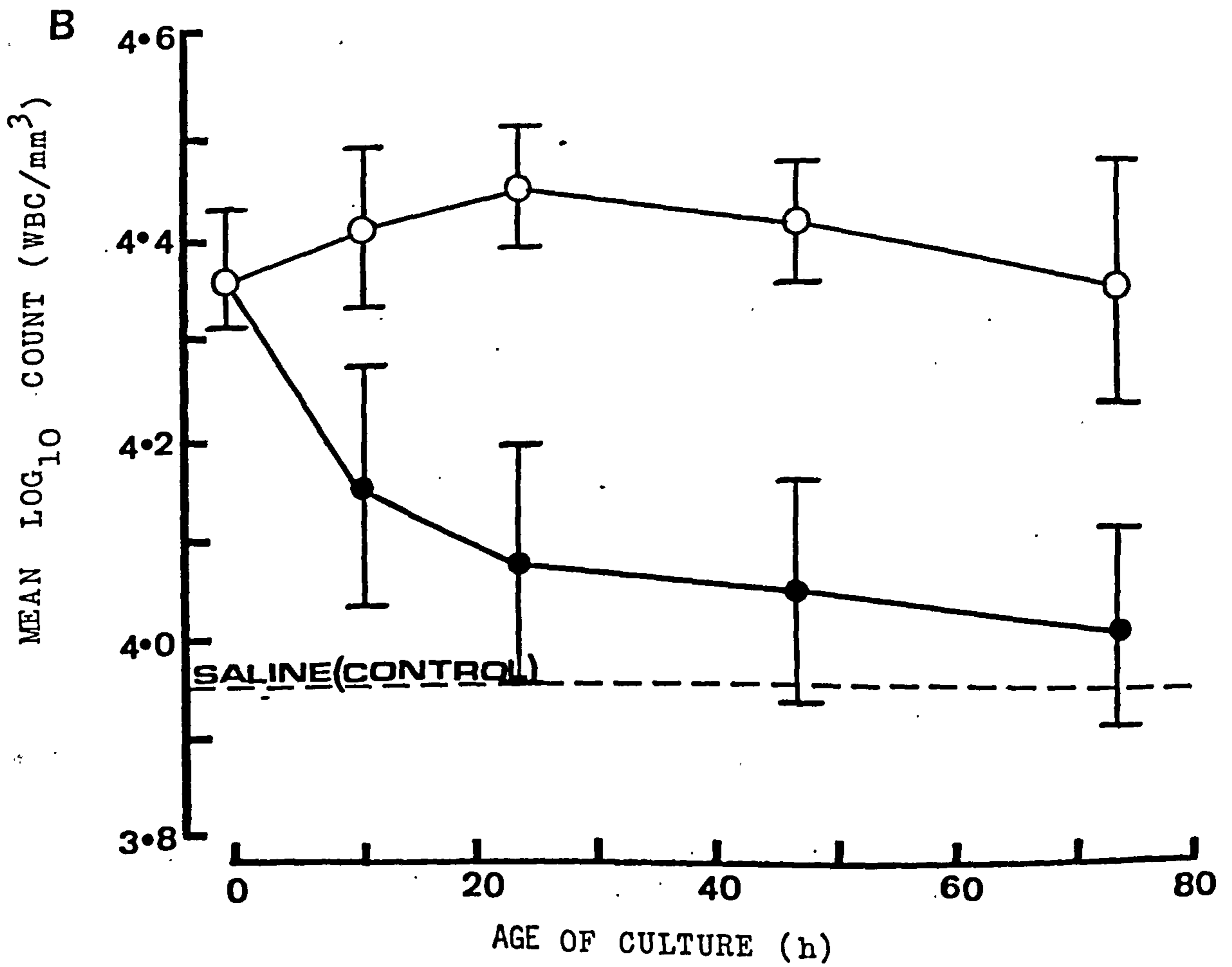
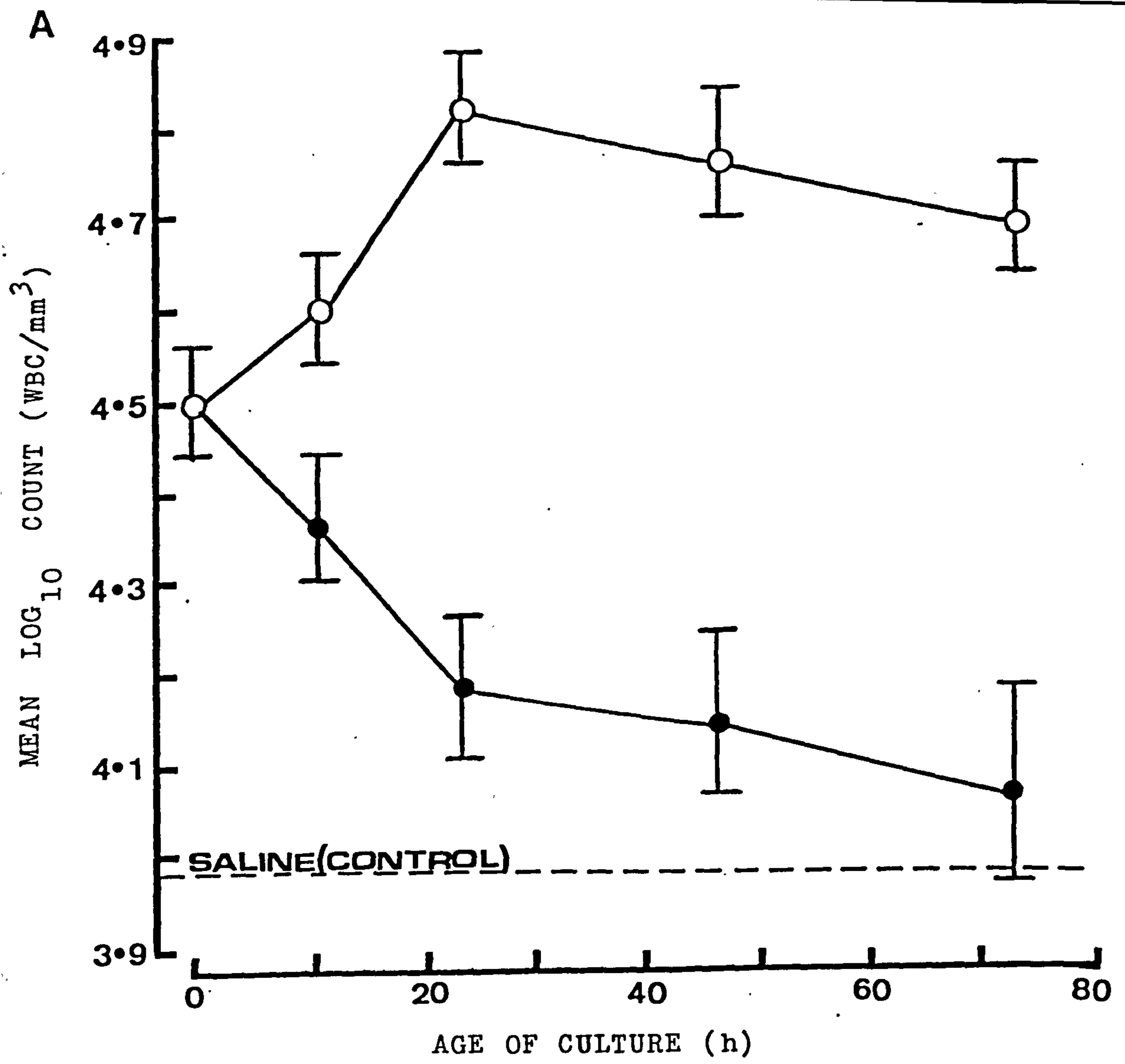
A. Responses to whole cultures.

○—○ X-mode
●—● C-mode

B. Responses to cell suspensions.

○—○ X-mode
●—● C-mode

Bars represent the standard deviations from the mean leukocyte counts.



This indicated either that not all the LPF activity was lost during one subculture in C-medium or that apart from the LPF, some other component of the cell was involved in inducing leukocytosis.

The leukocytosis-promoting potencies of the various samples of X- and C-mode cell suspensions were quantitated by the 4-point and 6-point assay methods. The 4-point assay was valid for estimating the potency of only the C-10h sample because the sample still retained some LPF activity. The potency of the C 10h sample was 20% of the potency of the X 10h sample which had an assigned value of 1.00 (Table 6). The 4-point and 6-point assays were not valid for estimating the potencies of the older C-mode samples which contained very low levels of activity (Appendices 4e and 4f). However, both assay methods were valid for estimating the potencies of the various X-mode samples which contained relatively high levels of LPF activity. The potencies of the X-mode samples, by the 4-point assay, ranged from 0.96 to 1.5 (Table 6). Comparing the potency values and the range of the 95% confidence limits, there was no significant difference between these values. Similarly, the potency values of the X-mode samples by the 6-point assay ranged from 0.93 to 0.99 (Table 7). Again based on the range of the 95% confidence limits, these values were not significantly different. These results further confirmed that the LPF potency of the X-mode cell suspensions remained relatively constant during growth. However with the C-mode cells, the expected potency, based on the observed

number of cell divisions after 10h was 0.8 (Table 6). The actual value of 0.2 indicated that the loss of the LPF was not due solely to dilution among progeny cells. Like the HSF, it also appeared to be denatured or degraded when cells, initially in the X-mode, were grown in C-medium.

iv. Heat-labile toxicity

Freshly isolated cells of B. pertussis (X-mode) possess a factor which is lethal to mice when injected intraperitoneally, or causes dermonecrosis when injected intradermally. The lethal and dermonecrotic properties have been attributed to the HLT. In this study attempts were made to determine if this HLT, known to be lost in C-mode cells (Livey et al., 1978), was lost in parallel with the other biological activities.

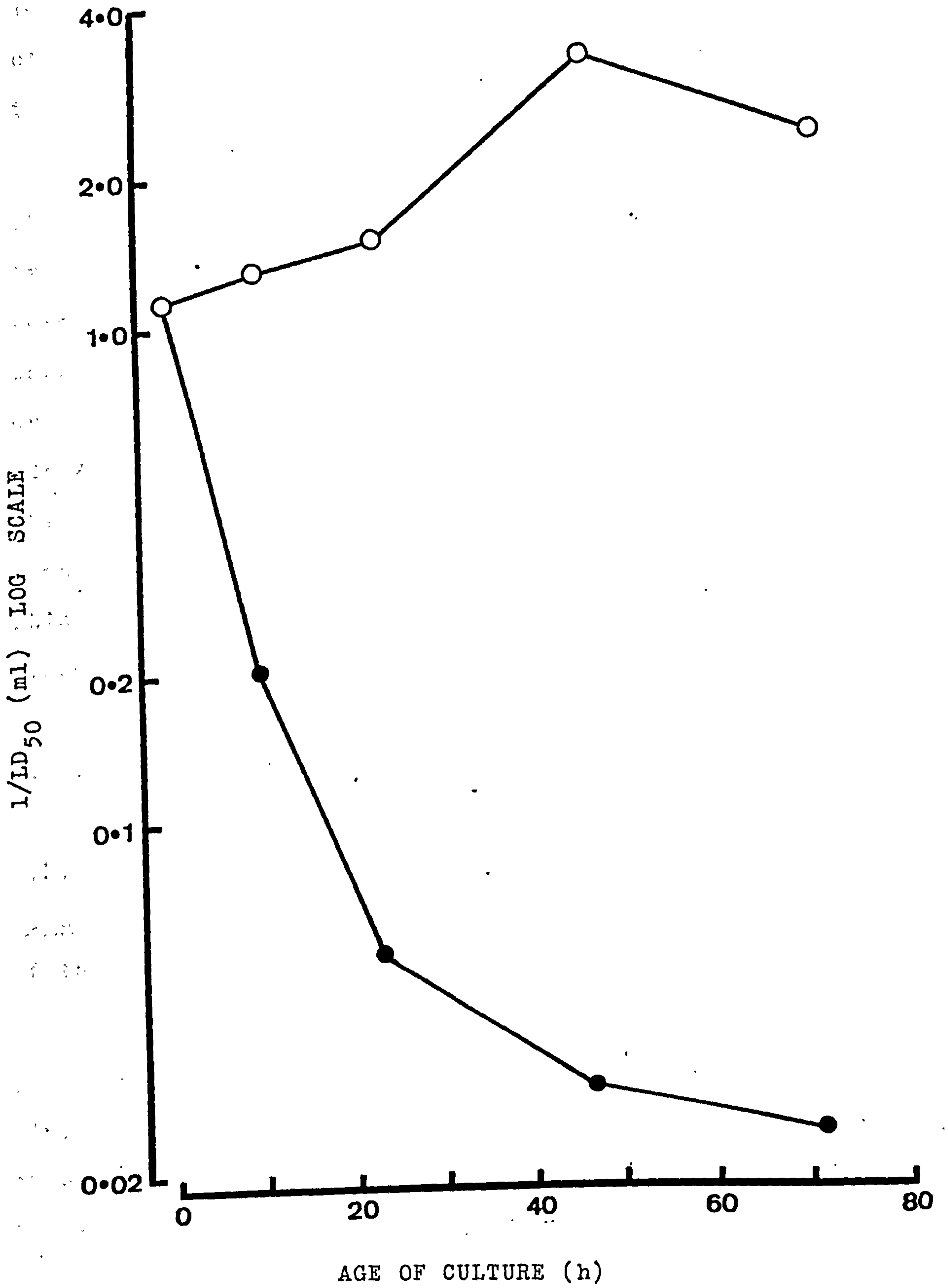
Aliquots of unheated X- and C-mode whole cultures obtained at various intervals during growth were assayed for their HLT activities by intraperitoneal injection. Results were expressed as the dose required to kill 50% of the mice inoculated (LD_{50}). The reciprocal of the best-estimated LD_{50} values from four independent experiments performed under the same conditions were plotted against time of sampling (Figure 20). It was observed that the content of the HLT in the X-mode whole culture increased with time. The increase in toxicity reached a peak after about 48h of growth and then started to decline. On the other hand, the content of the toxin in C-mode whole cultures decreased gradually during growth.

Figure 20 : Changes in the levels of heat-labile toxicity during growth of B. pertussis strain 18334 in X- and C-media (10% (v/v) inoculum).

Data represent the reciprocal of the best-estimated LD₅₀ values obtained from four similar experiments

○—○ X-mode culture

●—● C-mode culture



Further analysis of results showed that at 10h, the level of toxicity in the C-mode whole culture was only 16% of the toxicity of the X-mode culture. The level of toxicity in the C-mode culture had dropped to 1% of the X-mode toxicity after 72h of incubation (Table 10).

In the four independent experiments, a total of 300 mice were used and the individual results showed that at 10h, the levels of toxicity in the C-mode cultures were between 12.1% and 17% of the toxicity of their X-mode counterparts. By 72h, the levels of toxicity in the C-mode cultures had dropped to between 1-2% of the activity of the 72h X-mode cultures. In later experiments, it was shown that the toxicity of the X-mode cultures was lost when the samples were heated at 56°C for 30 min before assay. This confirmed the heat-labile nature of the factor responsible for the mouse-lethal toxicity.

v. Toxicity as measured by the mouse weight-gain test.

The mouse weight-gain test was recommended by the W.H.O. (1964) as a test for determining the toxicity of B. pertussis vaccines. Using this method, the toxicities of the X- and C-mode samples were compared.

Mice were weighed just before inoculation and then daily for seven days. Weighings were done at the same time each day. Three independent assays were performed with samples from similar experiments. Each assay involved the use of 140 mice and the results obtained from all three

Table 10:

Heat-labile toxicity in X- and C-mode whole cultures obtained at various intervals during growth (10% (v/v) inoculum).

Sample

LD₅₀ (ml of whole-culture). Best-estimate values from four independent experiments.

	10h	24h	48h	72h
X-mode	0.8	0.66	0.32	0.42
C-mode	5.06	15.4	23	34
Percentage activity of C-mode relative to X-mode	16%	4%	1%	1%

assays were consistent. The weight change per mouse for a typical assay is given in Appendix 6.

Results from this assay are summarized in Figures 21A, 21B and 21C. A steady gain in weight was observed in the uninoculated mice as well as in the mice injected with saline only (Figure 21A). Mice inoculated with X-mode cell suspensions (Figure 21B) and C-mode cell suspensions (Figure 21C) showed an initial weight loss at day 1 and a subsequent weight-gain. However, the rate of weight gain was faster in mice injected with the C-mode cells than with mice injected with X-mode cells.

The similar pattern of initial weight loss in the mice suggested the presence of a common toxic factor in both the X- and C-mode samples. However, the subsequent slower weight-gain in the mice inoculated with X-mode samples indicated an additional toxicity in the X-mode cell suspensions which was absent in the C-mode cell suspensions. Although the weight changes with all the X-mode samples were quite similar, the X 48h sample appeared most toxic. The pattern of subsequent weight-gain in mice inoculated with the 10h C-mode samples was much slower compared to other C-mode samples but faster than the X-mode samples.

vi. Haemagglutination activity

The haemagglutinating (HA) activities of whole cultures, culture supernatants and cell suspensions harvested at various intervals during growth in X- and C-media were estimated. At each sampling time, whole culture samples

Figure 21 : Changes in the level of toxicity of X- and C-mode cells as measured by the mouse weight-gain test

A. Weight change in control mice

●—● uninoculated
○—○ saline

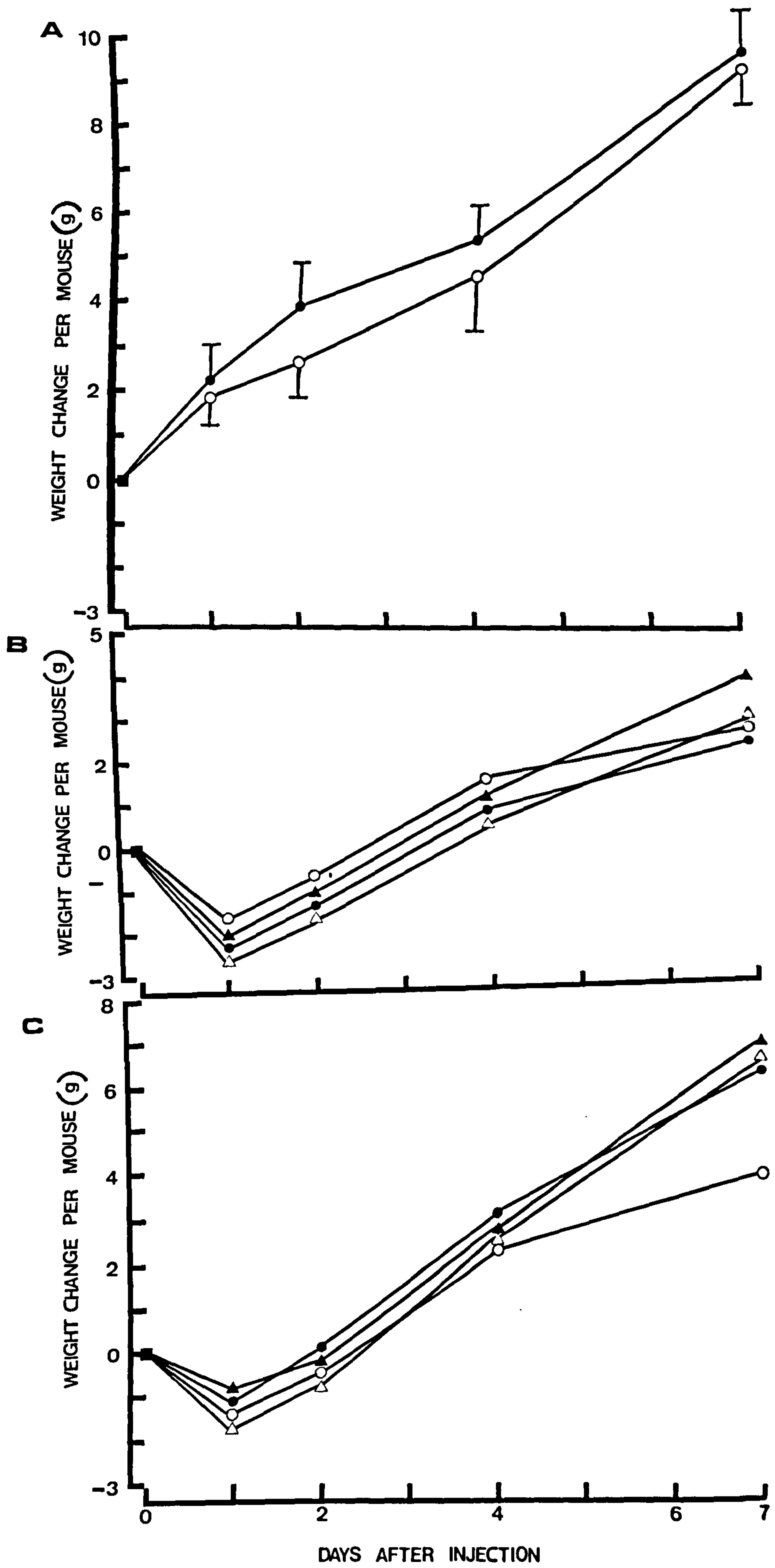
Bars represent the standard deviations from mean weight change

Figure 21 B : Weight change in mice inoculated with X-mode cell suspensions

○—○ 10h X-mode cells
●—● 24h X-mode cells
△—△ 48h X-mode cells
▲—▲ 72h X-mode cells

Figure 21 C : Weight change in mice inoculated with C-mode cell suspensions

○—○ 10h C-mode cells
●—● 24h C-mode cells
△—△ 48h C-mode cells
▲—▲ 72h C-mode cells



were centrifuged, the supernatant fluids were decanted and the bacterial cell pellets were resuspended in saline to the original volume.

Results from six independent experiments were similar and a typical result is shown in Table 11. HA activity was found to be associated with the bacterial cells during the early stages of growth in the X-medium and thereafter the level remained relatively constant. On the other hand, the culture supernatant contained no HA in the early stages of growth but showed increasing titres after 24h. With the growth in C-medium, there was no activity detected at any sampling time. It thus appeared that the original activity in the inoculum, which was mainly in the supernatant rather than cell-associated, once diluted in C-medium was no longer detectable and no further HA was synthesized in detectable amounts.

vii. Agglutinability

Possible changes in cell surface agglutinogens during modulation were examined. This was done by determining the titres of specific and unabsorbed X- and C-mode antisera against X- and C-mode cell suspensions harvested at various intervals during growth. Results are shown in Figures 22 and 23.

The titres of unabsorbed anti-X serum against X-mode cells stayed constant at 4096 throughout growth. The titres of unabsorbed anti-X serum against C-mode cells showed a slight drop from 4096 to 2048 by 10h, decreased further to 512 by 24h and remained unchanged (Figure 22).

Table 11: Haemagglutinating activities of X- and C-mode whole cultures, supernatant and cell suspensions harvested at various intervals during growth

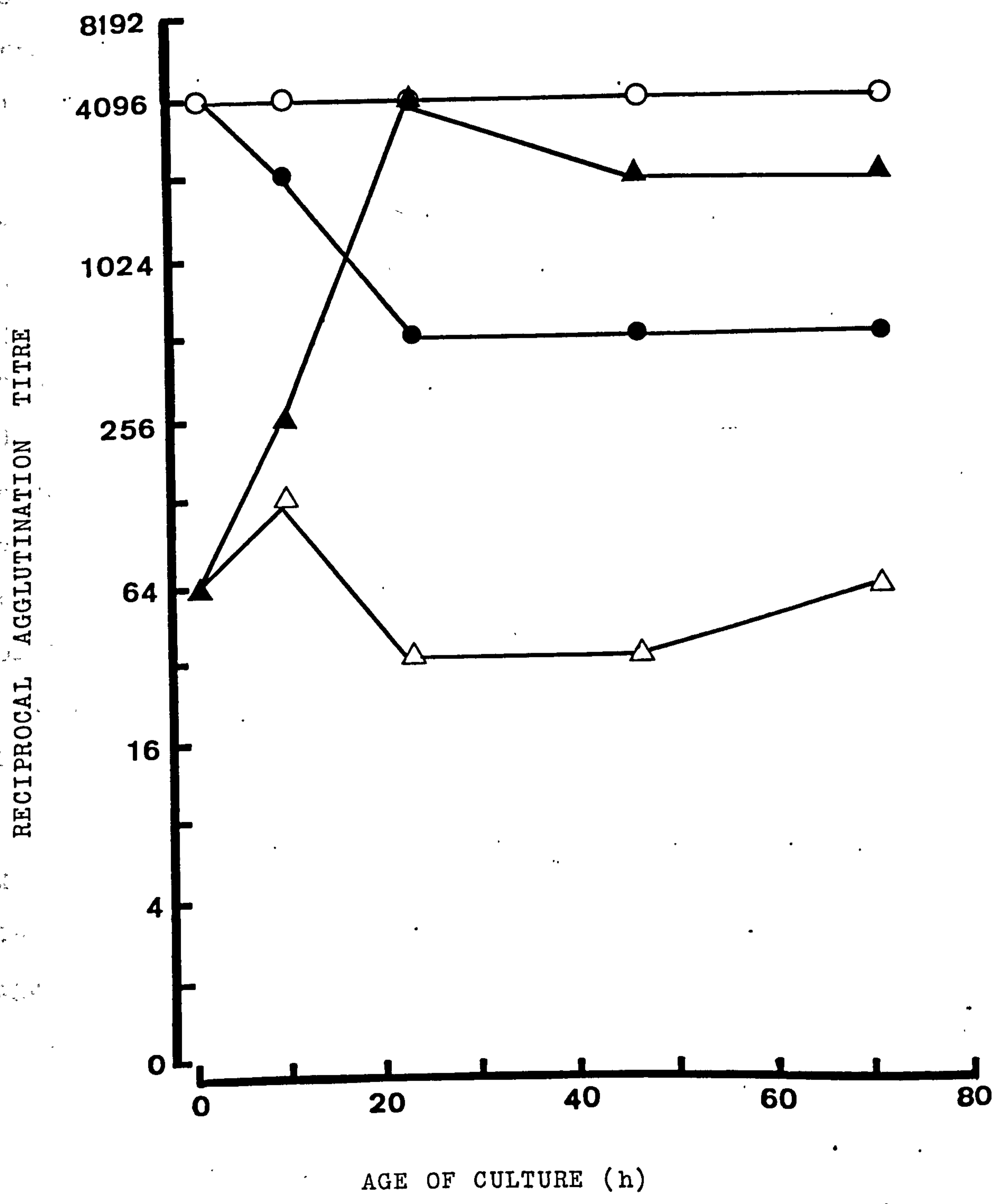
Sample	Haemagglutination titres*		
	Whole culture	Supernatant	Cell suspension.
Inoculum (X-mode) (48h)	16	16	4
X-mode (10h)	8	0	16
X-mode (24h)	16	4	8
X-mode (48h)	16	8	8
X-mode (72h)	16	16	8
C-mode (10h)	0	0	0
C-mode (24h)	0	0	0
C-mode (48h)	0	0	0
C-mode (72h)	0	0	0

* reciprocal of highest dilution of samples showing complete agglutination of the erythrocytes.

Figure 22 : Changes in agglutinability with unabsorbed antisera

A 10% (v/v) inoculum from a 48h X-mode culture of B. pertussis strain 18334 was used in the growth experiment

- anti-X serum against X → X cells
- anti-X serum against X → C cells
- △—△ anti-C serum against X → X cells
- ▲—▲ anti-C serum against X → C cells



With the unabsorbed anti-C serum against X-mode cells, the titres showed a slight but not significant increase from 64 to 128 by 10h, then decreased to 32 by 24h and thereafter remained relatively constant. However the titres of unabsorbed anti-C serum against C-mode cells increased from 64 to 256 by 10h and further increased to 4096 by 24h and stayed relatively unchanged.

Results obtained using specific antisera are shown in Figure 23. The titre of specific anti-X serum against X-mode cells stayed relatively constant at 4096 throughout growth. However, the titre of specific anti-X serum against C-mode cells decreased from 2048 to 1024 by 10h and further decreased to 64 by 24h after which it stayed relatively constant. With the specific anti-C serum against C-mode cells, the titres increased from 4 to 64 by 10h, increased further to 256 by 24h and remained relatively unchanged. On the other hand, the titres of the specific anti-C serum against X-mode cells showed a slight increase from 4 to 8 by 10h, then dropped to 4 by 24h and remained constant.

These results indicated that after 10h of growth of X-mode cells in C-medium, there was no appreciable change in the surface agglutinogens but by 24h, the X-specific agglutinogens appeared to have been lost.

3. Envelope proteins

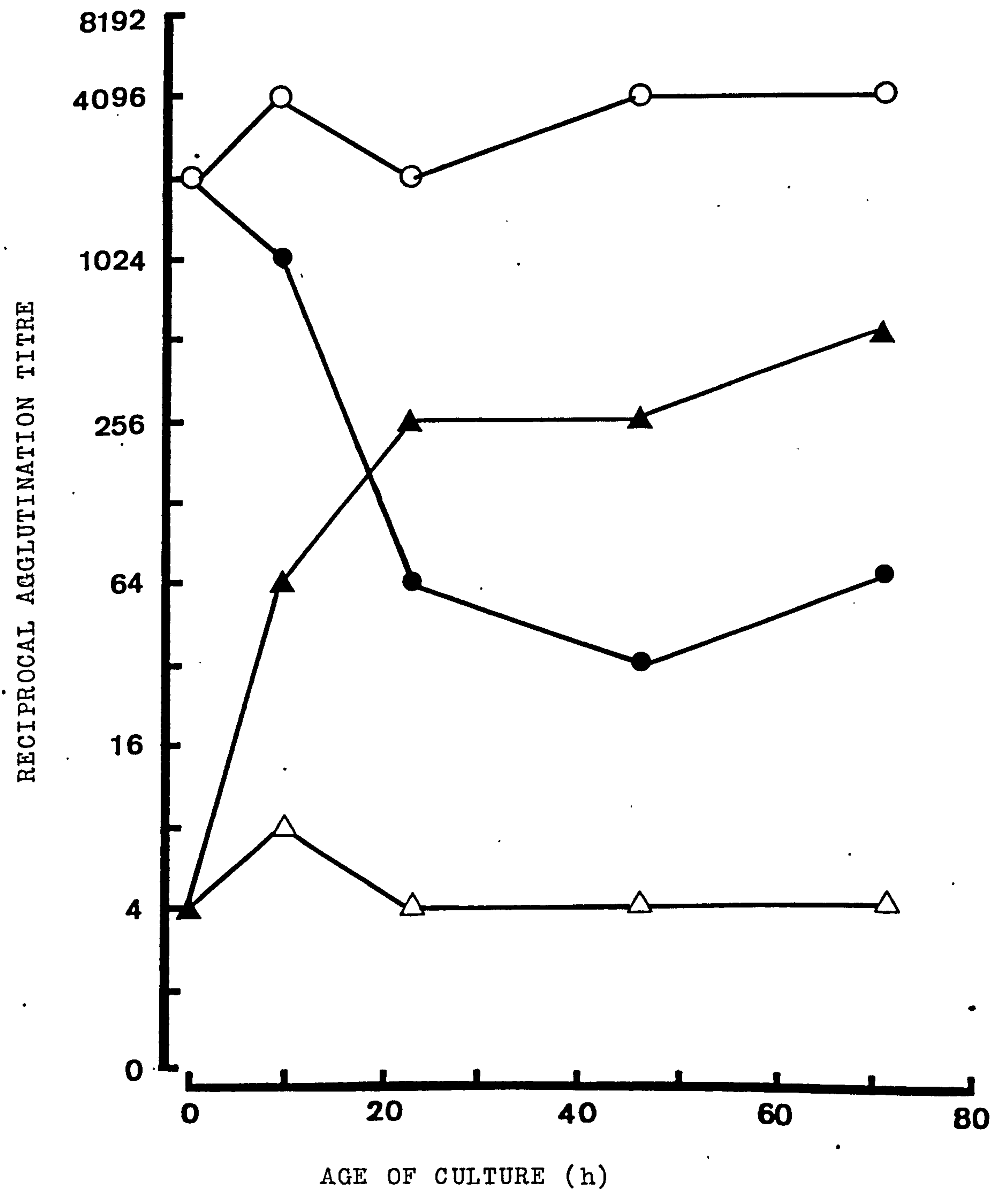
i. Changes in electrophoretic profiles

The various immunological and pathophysiological activities of B. pertussis, except the heat-labile toxicity,

Figure 23 : Changes in agglutinability with specific (absorbed) antisera

A 10% (v/v) inoculum from a 48h X-mode culture of B. pertussis strain 18334 was used in the growth experiment

- anti-X serum against X → X cells
- anti-X serum against X → C cells
- △—△ anti-C serum against X → X cells
- ▲—▲ anti-C serum against X → C cells



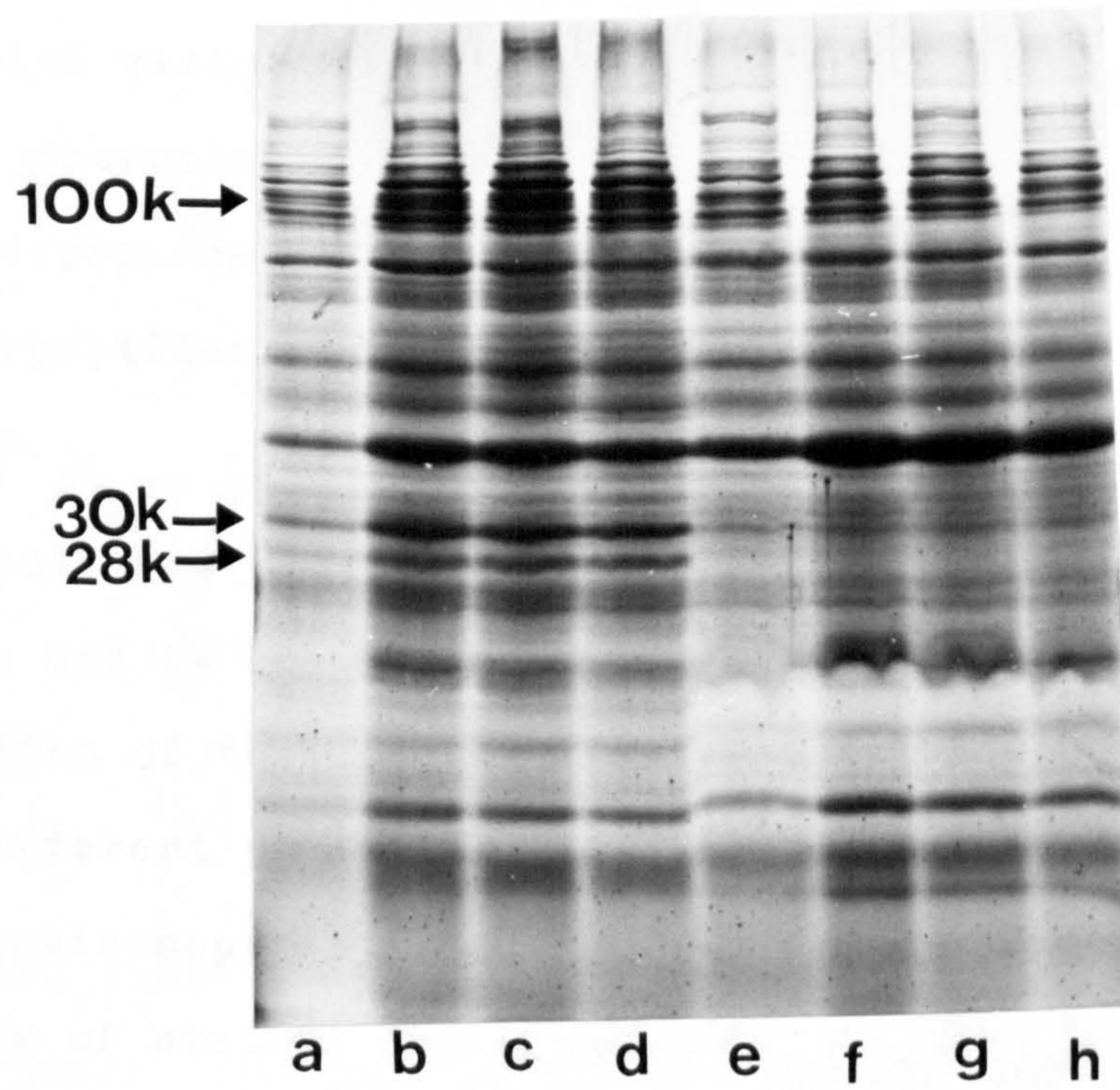
have been reported to be associated with the cell envelope proteins of the bacterium (Munoz, Ribí and Larson, 1959; Morse and Morse, 1970; and Munoz, 1971). The presence of two major gel lines appeared to correlate with the presence of various activities such as the PA and the HSF activities (Wardlaw, Parton and Hooker, 1976). Therefore, polyacrylamide gel electrophoresis of envelope polypeptides was used to follow the modulation process in this study. In initial experiments X- and C- mode whole cell suspensions were compared and it was found that the difference in the two major envelope polypeptide bands were clearly visible in whole-cell preparations. Since the preparation of cell-envelopes was time consuming, whole-cell suspensions were used in all subsequent experiments.

Comparison of X- and C-mode cells, obtained from more than twelve growth experiments, in which strains 18334 and 134 were used, revealed that the electrophoretic patterns of the polypeptide bands were consistently similar except that three polypeptide bands which were present in the all the X-mode samples were absent in the C-mode samples (Plate 1). From the molecular weights of marker proteins run side by side with the samples, the molecular weights of the three bands missing in the C-mode cells were estimated at about 28,000, 30,000 and 100,000 daltons.

The absence or reduction of the three bands in the 10h C-mode cells, suggest that these polypeptides were lost in parallel with the various activities, i.e. by degradation and not by simple growth-dilution.

Plate I. SDS-polyacrylamide gel electrophoresis of envelope polypeptides of *B. pertussis* grown in X- and C- media. (X \longrightarrow C modulation).

- a. X10h
- b. X24h
- c. X48h
- d. X72h
- e. C10h
- f. C24h
- g. C48h
- h. C72h



ii. Time of onset of synthesis of 28k and 30k polypeptides

As earlier stated the presence of the 28k and 30k polypeptide bands in the X-mode cells were correlated with the presence of PA and HSF activities (Wardlaw et al., 1976). In the present study it was observed that as early as 10h during growth of X-mode cells in C-medium, the two polypeptide bands had either been greatly reduced or had disappeared in parallel with various activities. These findings stimulated efforts to determine if there was a transient synthesis of the two polypeptides in C-medium or whether the synthesis of the polypeptides was switched off immediately upon inoculation.

Initially the experimental approach was to grow X-mode cells in X- and C- Hornibrook media with added ^{14}C glutamate. The estimation of the incorporation of labelled glutamate into the different protein components of the cells involved electrophoretic separation of proteins in the samples, densitometry of stained gels, slicing of gels and radioactive counting. However, there was very little or no uptake of labelled glutamate by the cells. This probably was due to the fact that the casamino acids in Hornibrook medium contained high levels of glutamate which competed with the ^{14}C glutamate. Thus in later experiments, X- and C- Stainer and Scholte defined media were used. Since B. pertussis is known to prefer glutamate to other amino acids as a source of nitrogen, the original level of glutamate in Stainer and Scholte X- and C-media was reduced and an increased amount of ^{14}C glutamate was added to ensure

an adequate uptake of the label. Also, the original level of proline in the media was increased as a secondary source of nitrogen. With this modification much better uptake of labelled glutamate by the X- and C-mode cells was obtained.

Figures 25A and 25C respectively show the densitometer traces of stained gels and the graph of the uptake of labelled glutamate in the X-mode cells; Figures 25B and 25D show the results for the C-mode cells. Comparing the densitometer traces of X- and C-mode gels (Figure 24) it was observed that while the 28k and 30k bands were present in the X-mode samples only remnants of these bands were present in the C-mode. In addition, one other band with a molecular weight of approximately 100k which was present in the X-mode gels was found to be greatly reduced in the C-mode gels.

When the densitometer traces and the graphs of the glutamate uptake were compared, the labelled glutamate was found to be incorporated into nearly all the polypeptide components of X-mode cell in proportion to their protein content. However, there was a protein component with a molecular weight of approximately 25,000 where there was little or no uptake of labelled glutamate (arrow, Figure 25A). With the C-mode cells, the labelled glutamate, in general, was incorporated into the bands in proportion to their protein contents as shown by the densitometer traces. However, in the region of the 28k and 30k polypeptides there

Figure 24 : Comparison of densitometer traces of protein profiles of B. pertussis strain 18334 grown in Stainer and Scholte X-medium (top) and Stainer and Scholte C-medium (bottom)

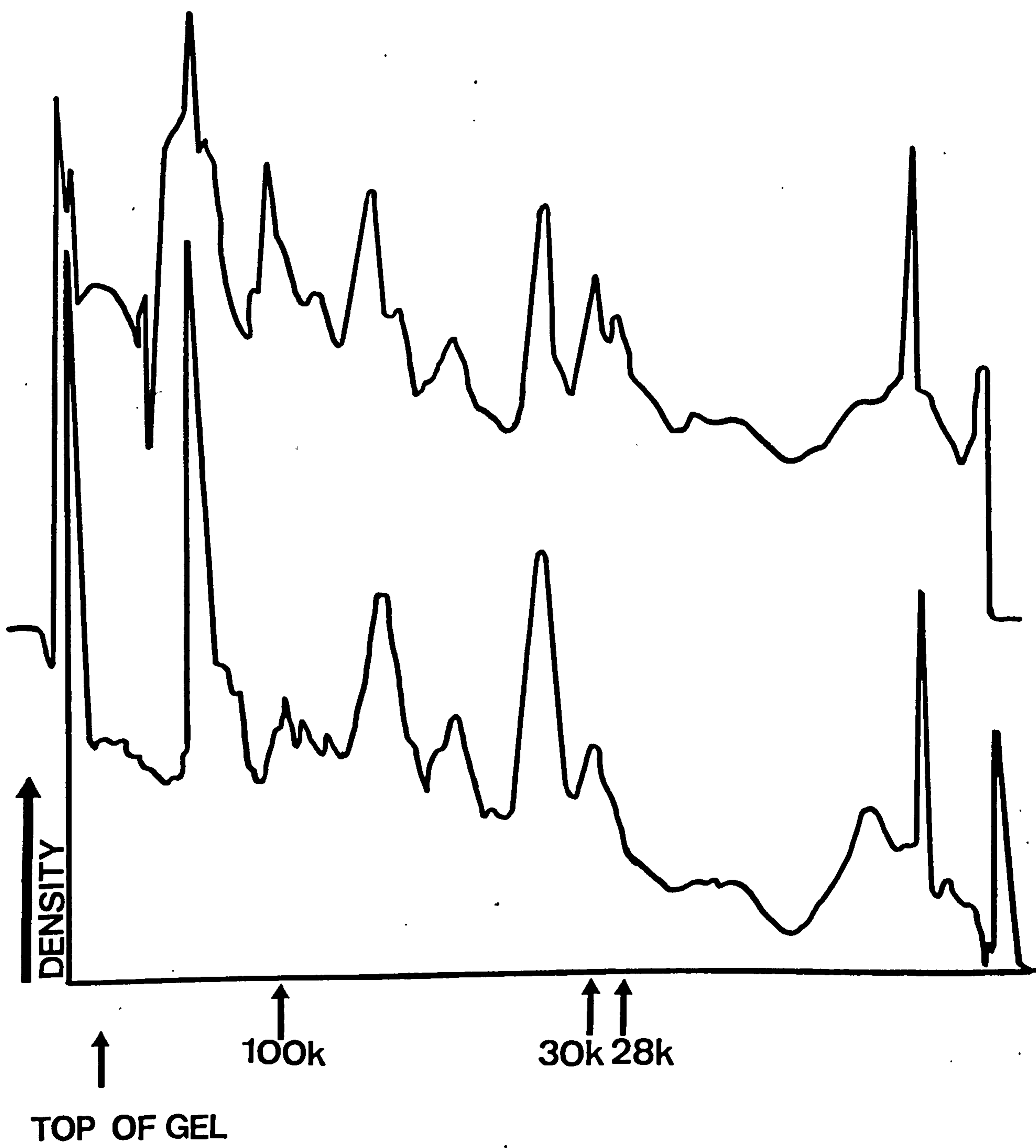


Figure 25 : Comparison of densitometer traces of protein profiles and incorporation of ^{14}C glutamate into cell proteins of B. pertussis strain 18334 grown in Stainer and Scholte X- and C-media.

A and C.

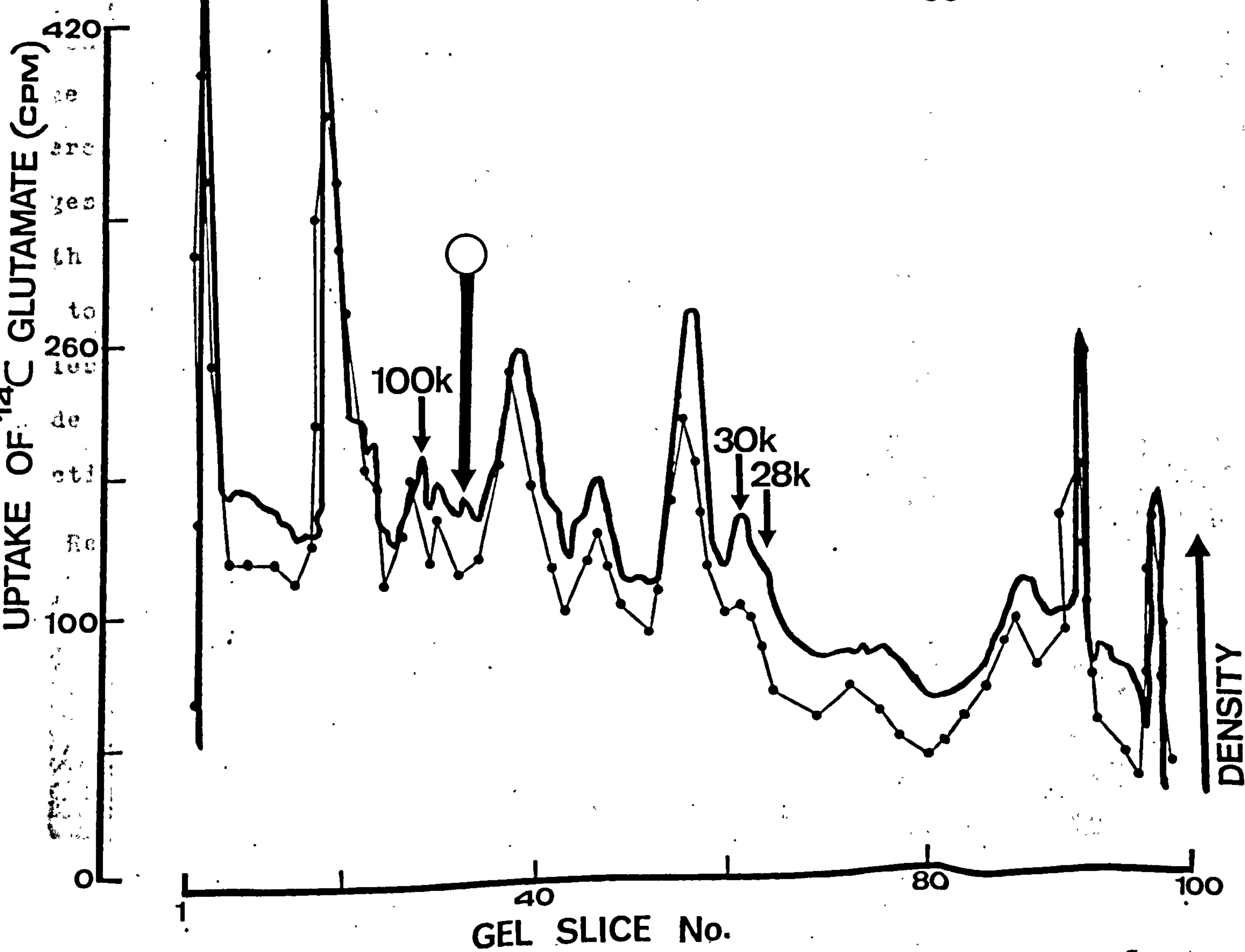
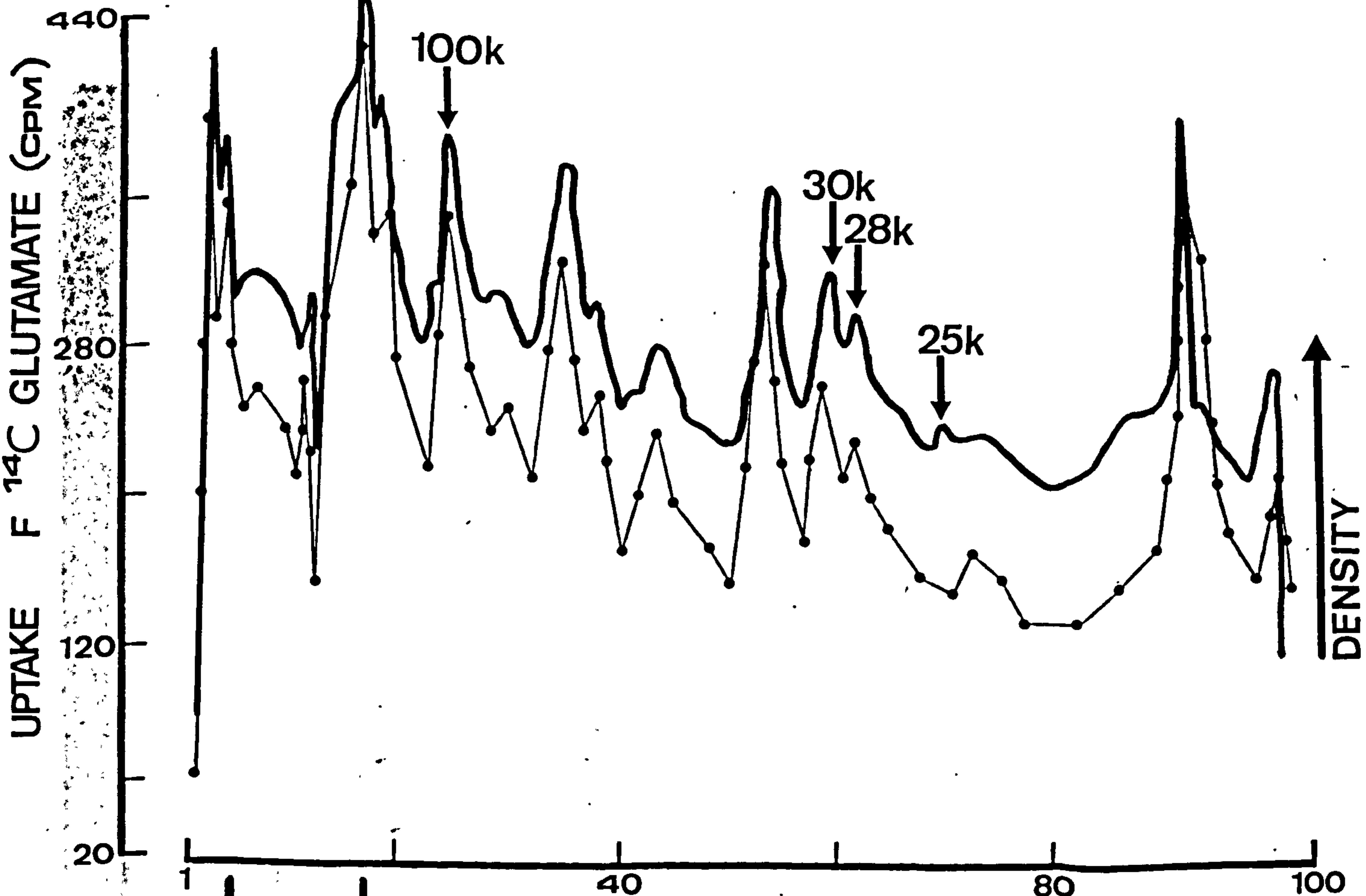
A. ————— Densitometer traces of cells grown in X-medium.

C. ●————● Incorporation of ^{14}C glutamate into cells grown in X-medium

B and D.

B. ————— Densitometer traces of cells grown in C-medium

D. ●————● Incorporation of ^{14}C glutamate into cells grown in C-medium.



was very little uptake of labelled glutamate even though remnants of these bands were clearly visible in the densitometer traces. Also there was no glutamate uptake in the region of one of the minor high molecular weight polypeptides (arrow, Figure 25B). This is not the same band as the 100k band which is major in X and minor in C. There appeared to be incorporation of ^{14}C into the 100k band in proportion to its peak height; even though it is a minor component in C-mode.

4. Correlation of changes in the immunological and pathophysiological activities during growth

One of the main aims of this project was to determine if the various activities were lost at the same or at different rates during modulation. Thus having followed changes in the levels of the individual activities during growth of X-mode cells in X- and C- media, attempts were made to correlate the loss of some of these activities. Samples obtained at various intervals during growth of X-mode cells in X- and C-media were assayed for a number of activities.

Results from a number of similar experiments did not differ significantly and Figures 26A, 26B and 26C show the levels of HSF, LPF and HLT activities in whole culture samples obtained from a typical growth experiment. The total levels of these activities in the X-mode cultures increased simultaneously with cell numbers reaching a peak

Figure 26 : Correlation of changes in the levels of histamine-sensitizing activity, leukocytosis-promoting activity and heat-labile toxicity. (10% (v/v) inoculum)

A. Changes in HSF activity in whole cultures.

○—○ X-mode
●—● C-mode

B. Changes in LPF activity in whole cultures

○—○ X-mode
●—● C-mode

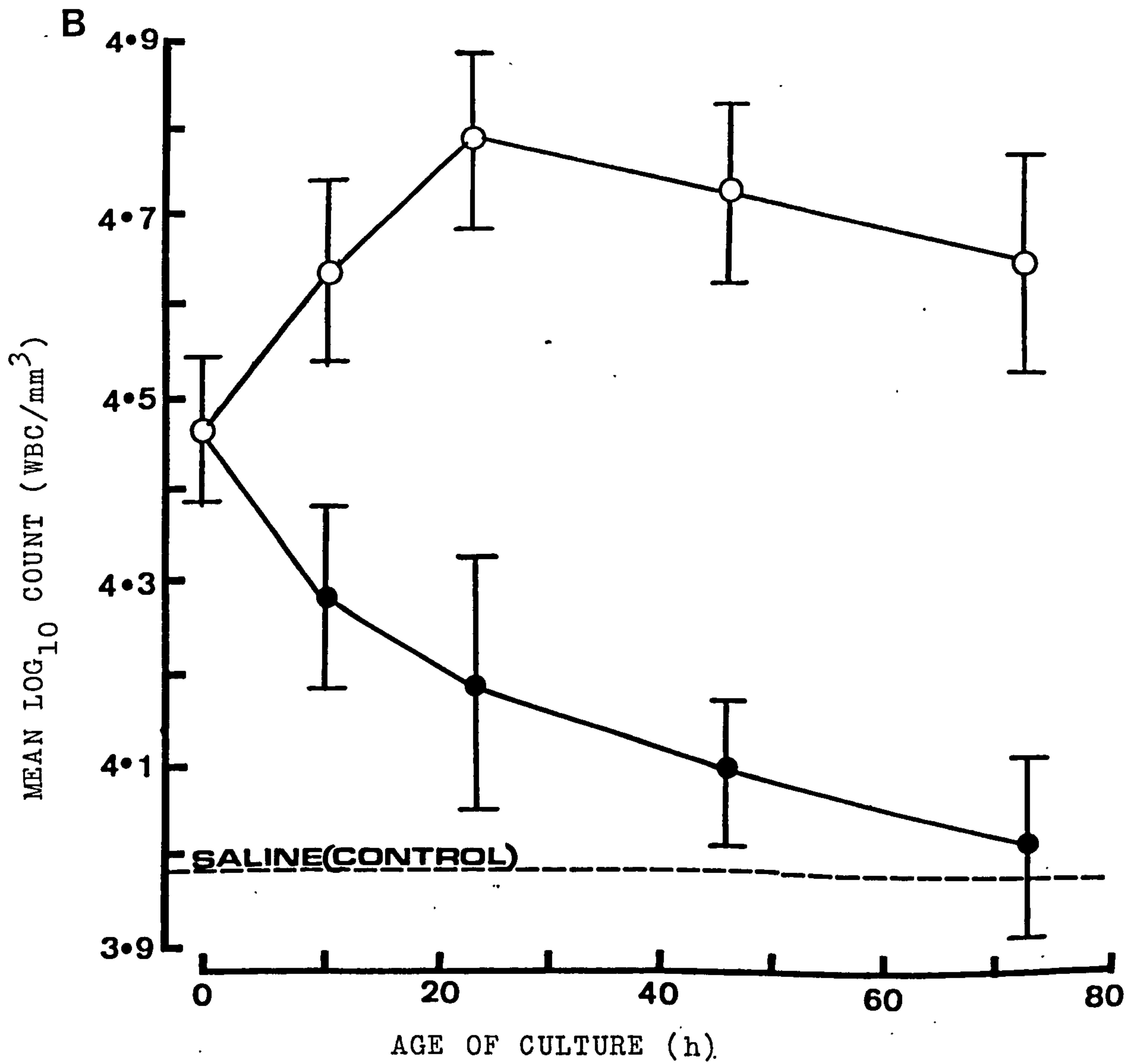
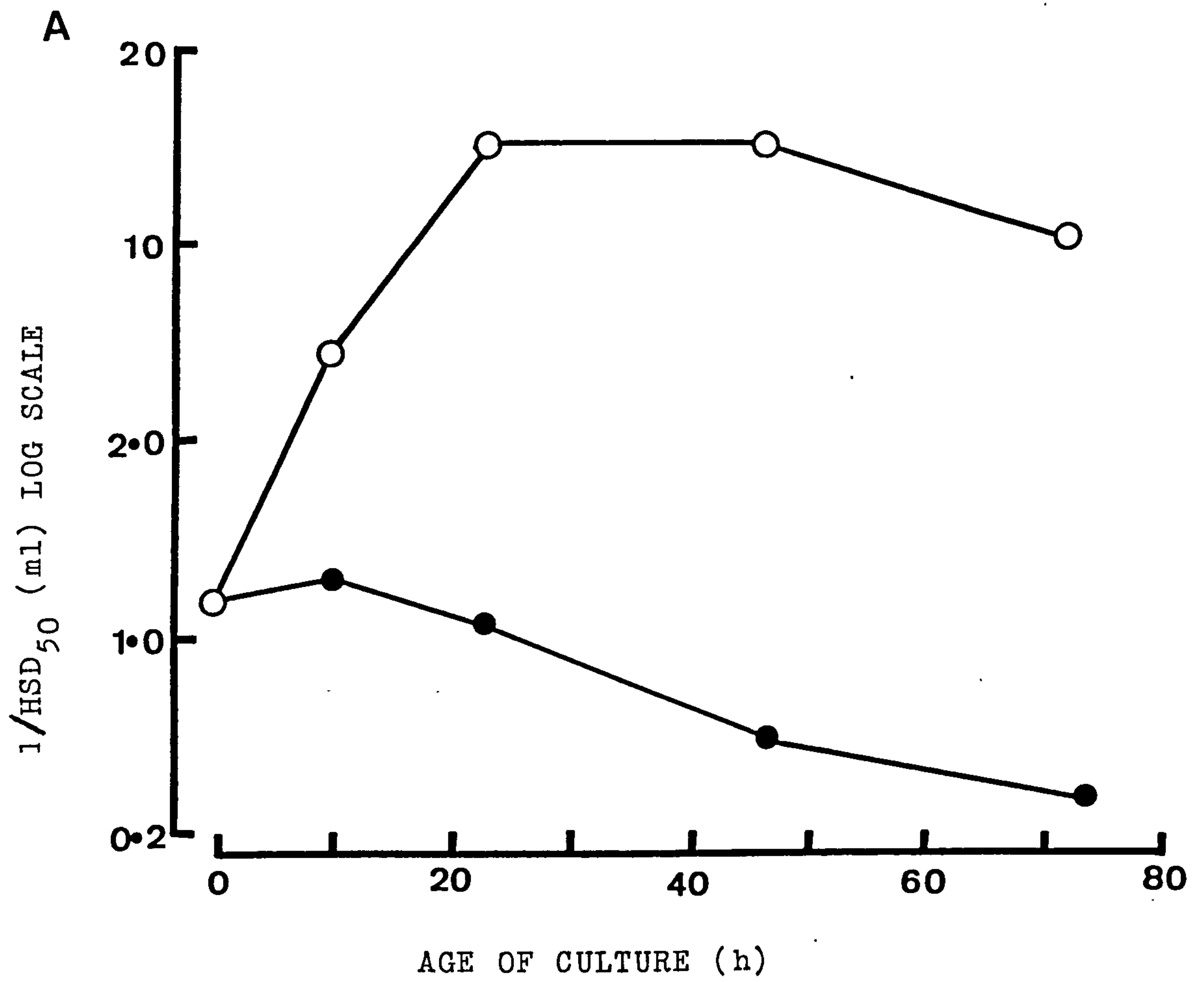
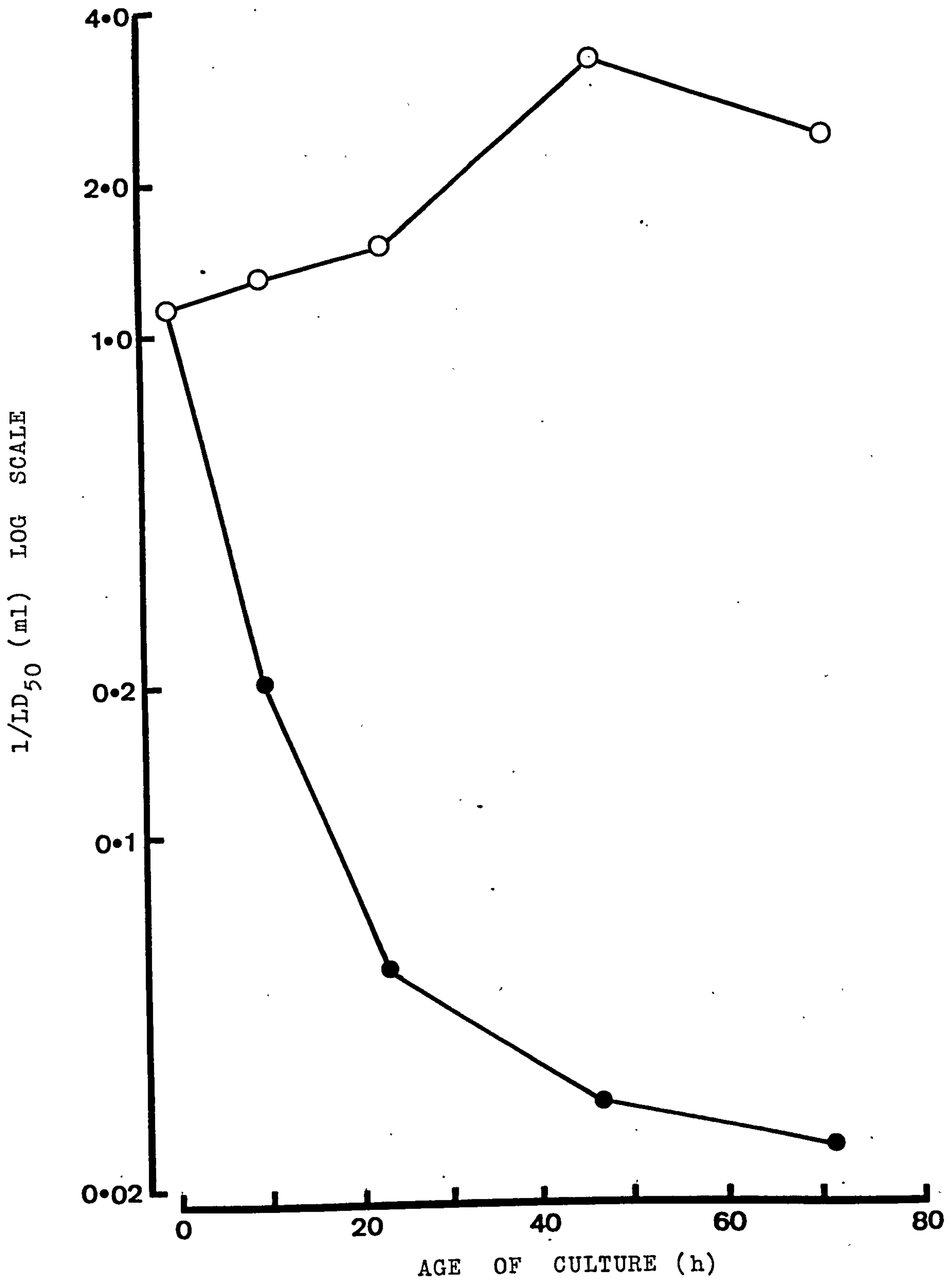


Figure 26C : Changes in HLT activity in
whole cultures.

○ — ○ X-mode

● — ● C-mode



between 24h-48h and then started to decline. However, the levels of these activities in the C-mode whole cultures decreased gradually from the time of inoculation. With the levels of HSF and LPF activities in the cell suspensions, Figures 26D and 26E show that the level of these activities in X-mode cells remained relatively constant during growth but decreased gradually from time of inoculation in the C-mode cells. The results indicated that during growth of X-mode cells in C-medium, the loss of the HSF, LPF and HLT activities in whole cultures, and the HSF and LPF activities in cell suspensions, appeared to follow the same pattern. This suggested that these activities were apparently lost at about the same rate during modulation.

The best-estimated potency values for the various activities, obtained from between four to a dozen experiments done under the same conditions, were calculated for X- and C-mode samples. The percentage loss of the HSF, LPF and HLT activities in the C-mode cells and whole cultures are shown in Tables 12A and 12B. At 10h there was a two-fold difference between the levels of the LPF and HSF activities in the C-mode cells.

However, with the best-estimated potency values from four independent mouse protection tests, the percentage loss of the protective activity in the C-mode cells at different stages of growth, was greater than the values for the other three mentioned activities. These observations suggested that while the two (HSF and LPF) activities were lost at about the same rate during modulation, the mouse

Figure 26D : Changes in HSF activity in cells

○—○ X-mode

●—● C-mode

Figure 26E : Changes in LPF activity in cells

○—○ X-mode

●—● C-mode

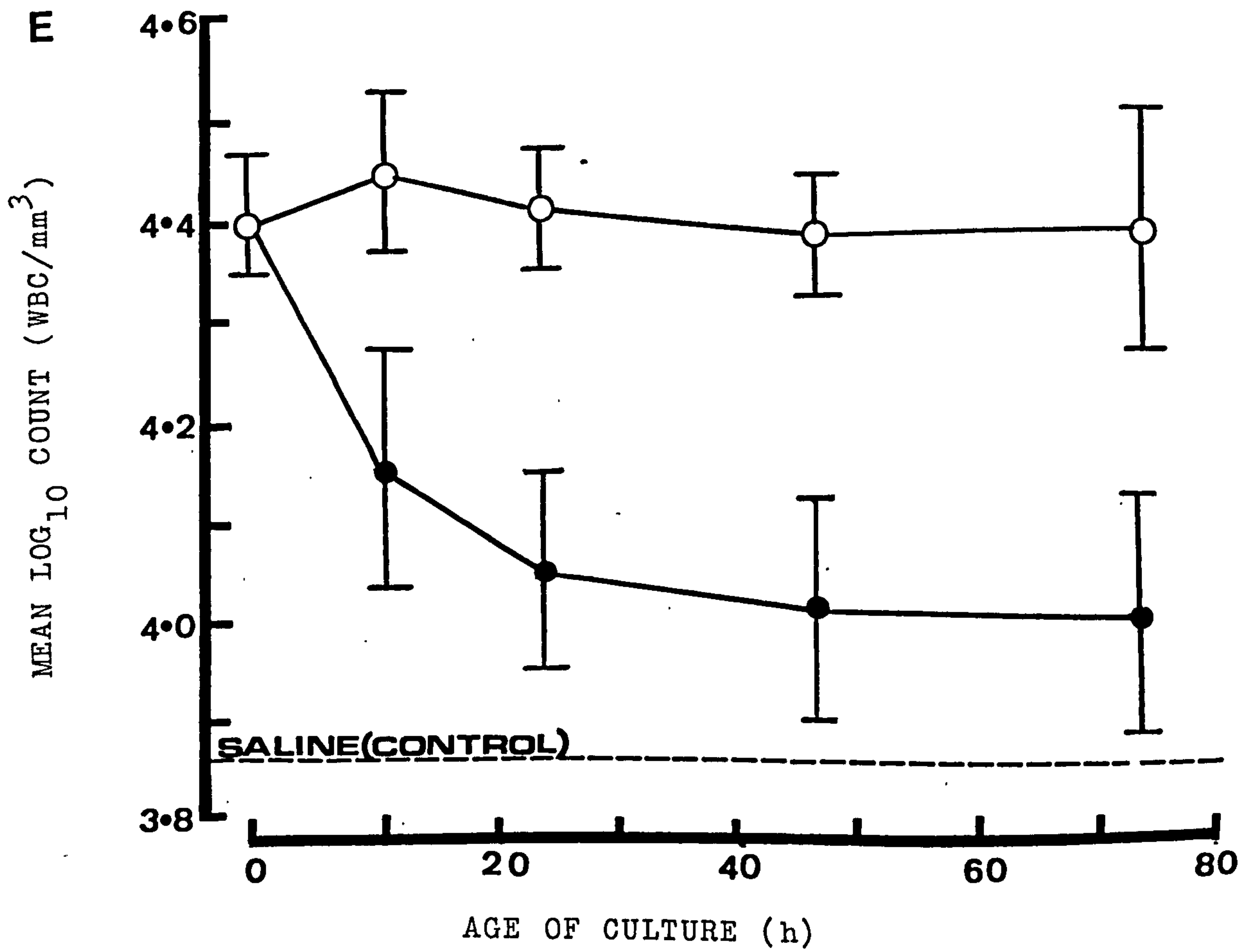
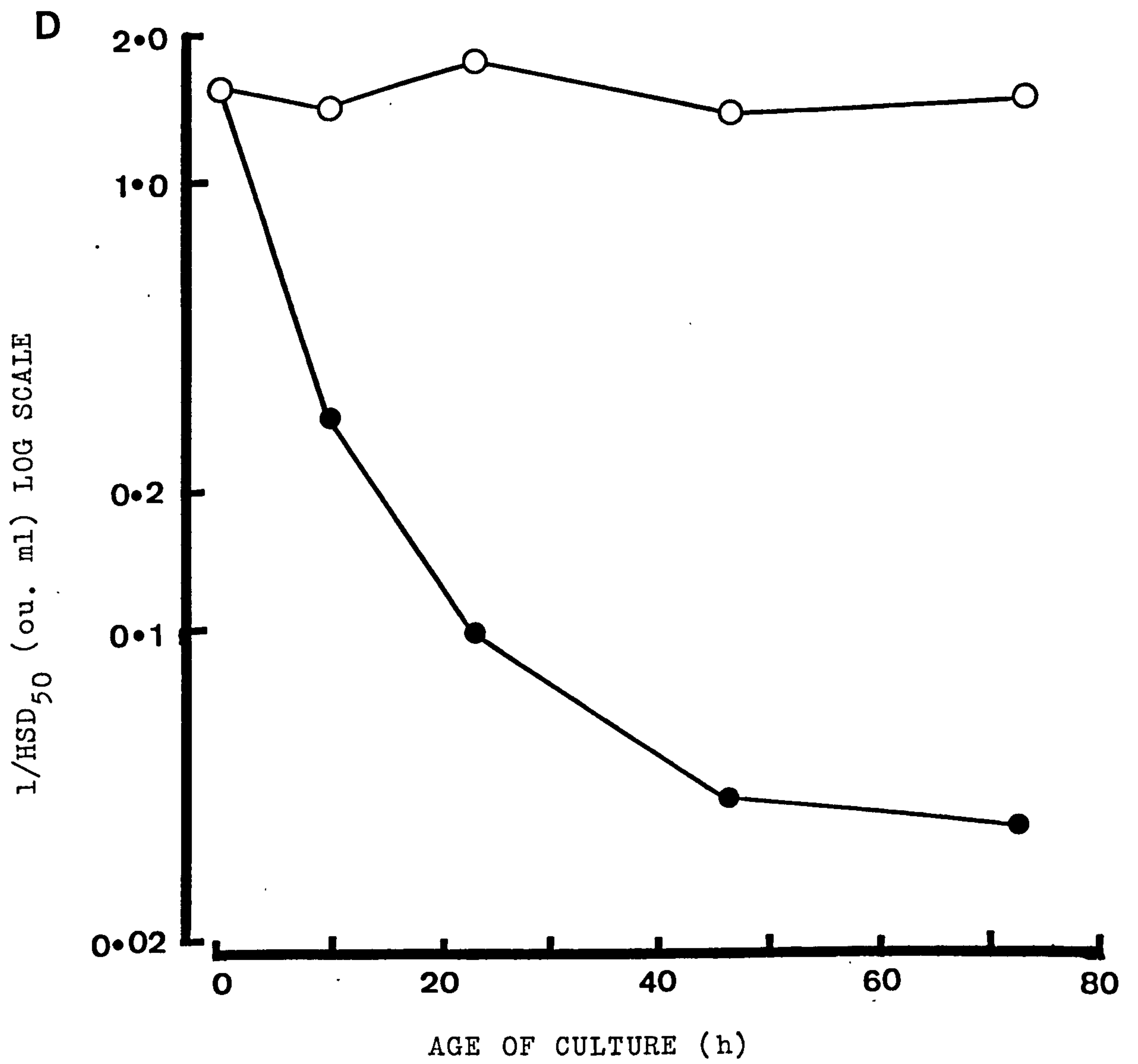


Table 12A: Correlation of loss of activities in cell suspensions after inoculation of X-mode cells into C-medium

Activity of C-mode cell suspensions as a percentage of the corresponding X-mode cell suspensions.

	10h	24h	48h	72h
Mouse-protective activity	* 2%	N.T.	0.5%	N.T.
Histamine-sensitizing activity	* 11% + 12%	4% 10%	2% 2%	2% 1%
Leukocytosis-promoting activity	* 20%	N.T.	N.T.	N.T.
	* 10% (v/v) inoculum			
	+ 5% (v/v) inoculum			
	N.T. = not tested			

Table 12B:

Correlation of loss of activities in whole
cultures after inoculation of X-mode cells
into C-medium

Activity of C-mode whole cultures as a percentage
of the corresponding X-mode whole cultures

	10h	24h	48h	72h
Histamine-sensitizing activity	* 23%	10%	5%	3%
Heat-labile toxicity	* 16%	4%	1%	1%
	* 10% (v/v) inoculum			

protective activity appeared to be lost much more rapidly. Furthermore, the residual activities still present in the C-mode cells by 72h indicated that the HSF and LPF activities were not completely lost during a single subculture of X-mode cells in C-medium with 5%-10% inoculum.

5. Distribution of the HSF and LPF activities between cells and culture supernatants.

A number of experiments were performed to determine the distribution of the HSF and LPF activities between the cells and the culture supernatants during growth. These experiments were carried out with a view to determining if the loss of activities in the C-mode cells was due to liberation of the activities into the culture supernatant and, if so, to further ascertain if activities in the supernatant were degraded faster than in the cells or vice versa. At various intervals during growth, aliquots of X- and C-mode cultures were centrifuged, the supernatant fluid was decanted and the cell pellets obtained were resuspended in saline to the original volume. The supernatant fluids, cell suspensions and unfractionated culture samples were detoxified by heating and assayed for LPF and HSF activities.

Results from the various experiments were similar and a typical LPF result is shown in Figures 27A and 27B. With the X-mode samples, most of the LPF activity in early

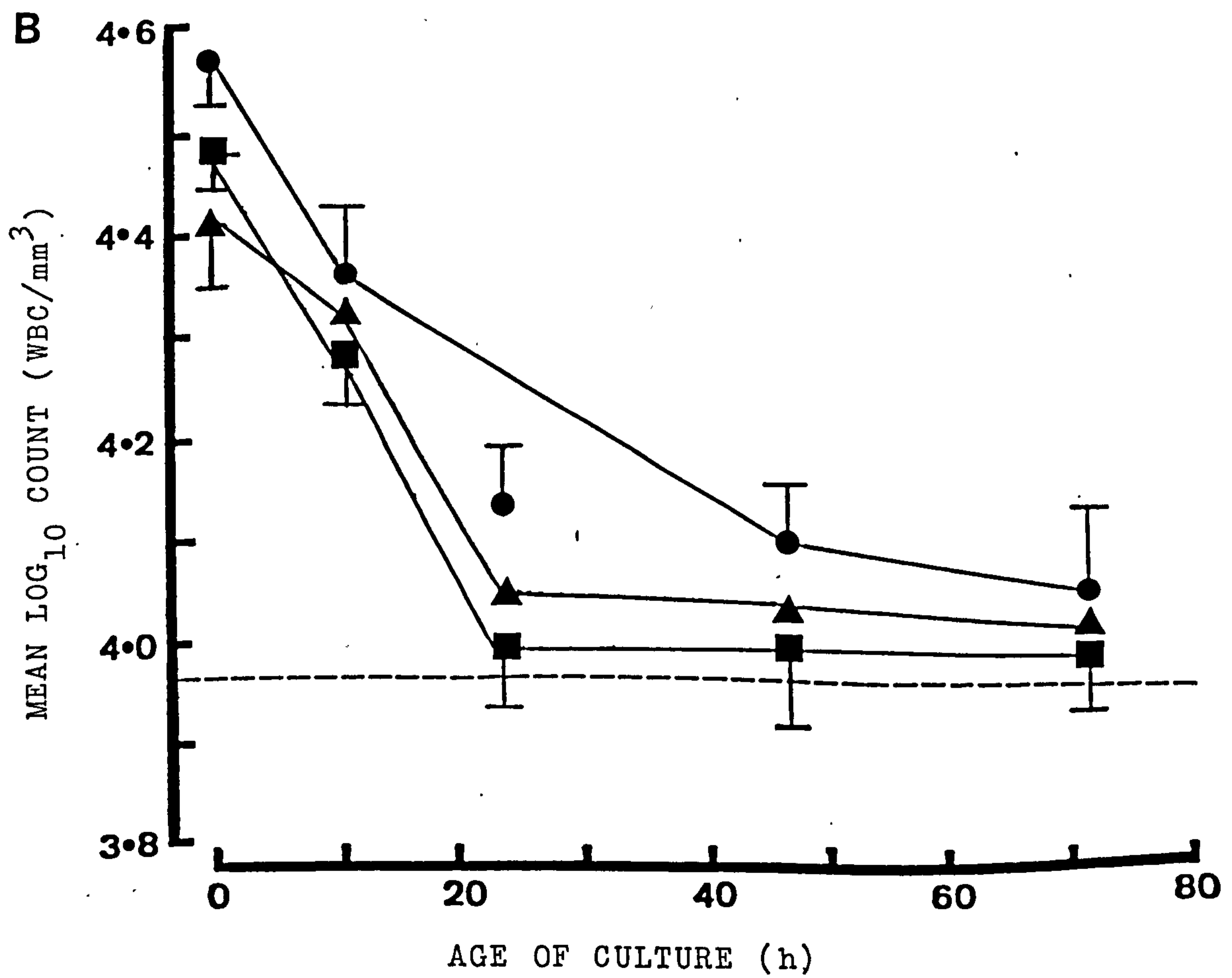
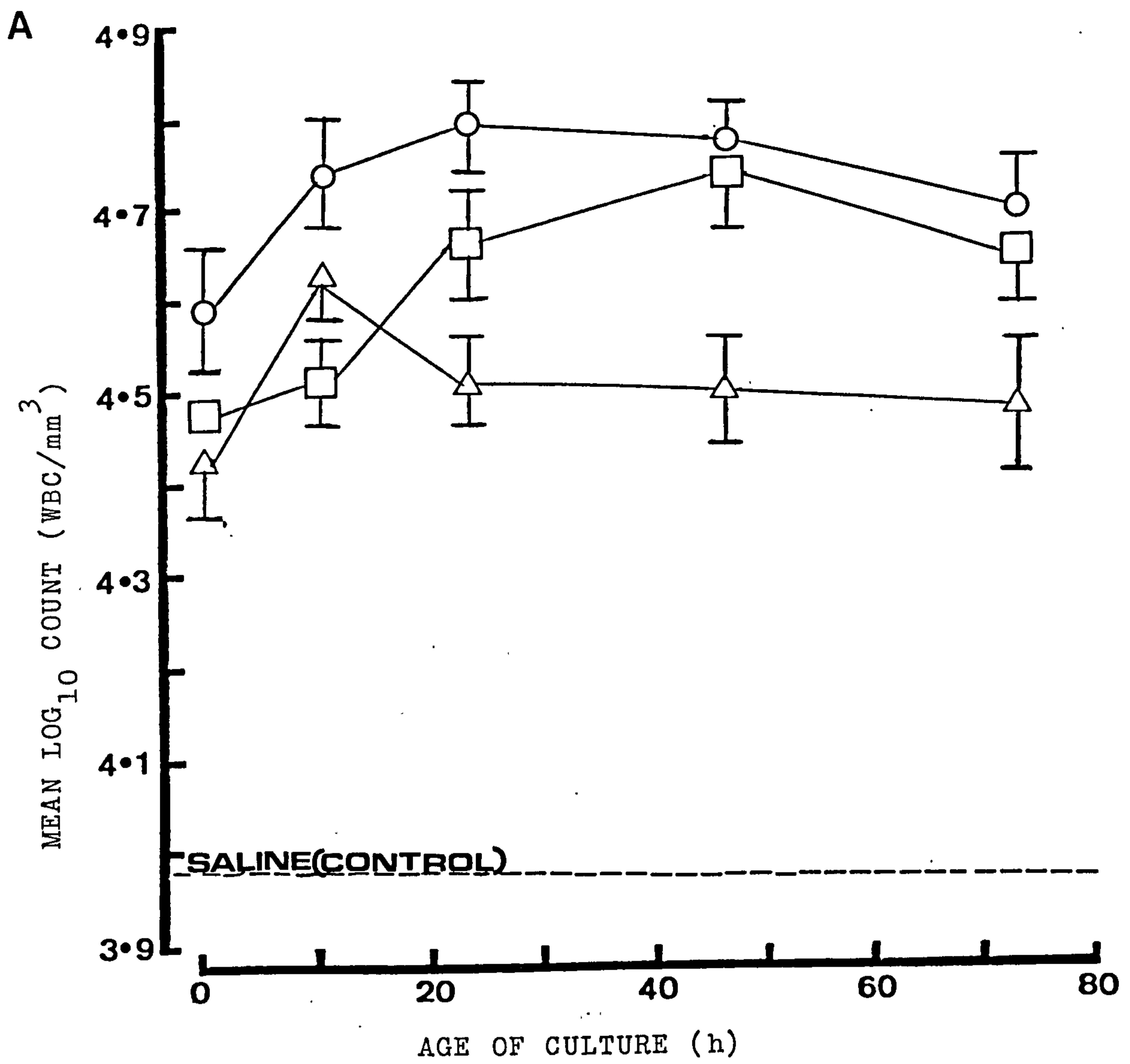
Figure 27 : Distribution of LPF activity between cells and culture supernatant during growth of X-mode B. pertussis strain 18334 in X- and C-media

A. Growth in X-medium

○—○ whole culture
□—□ supernatant
△—△ cells

B. Growth in C-medium

●—● whole culture
■—■ supernatant
▲—▲ cells



stages of growth was associated with the cells, with little activity in the culture supernatant. However, after 10h of growth the level of activity began to increase in the supernatant but decreased in the cells after an apparent initial increase. This suggested that during the log phase of growth, LPF was liberated from the cells into the culture supernatant. The total level of activity in the whole culture samples increased steadily, reached a peak at about the end of log phase and started to decline as found previously (Figures 18A, 19A and 26B). With the C-mode samples, the levels of LPF activity in the whole cultures, cell suspensions and culture supernatants showed a progressive decline from the time of inoculation (Figure 27B). By 24h, activity in the cells had been greatly decreased and there was no evidence that the LPF was released into the supernatant since the total level of activity in the whole culture and supernatant also showed a decline.

With the HSF a typical result (Figures 28A and 28B) showed that the level of activity in the cell suspensions was only slightly higher than the levels in the culture supernatant by 10h of growth of X-mode cells in X-medium. But at the later stages of growth, most of the activity was present in the supernatant while the levels in the cells declined after an initial increase. This indicated that, like the LPF the HSF was excreted by the cells into the extracellular fluid during the log phase of growth.

Figure 28 : Distribution of histamine-sensitizing activity between cells and culture supernatant during growth of X-mode B. pertussis strain 18334 in X- and C-media

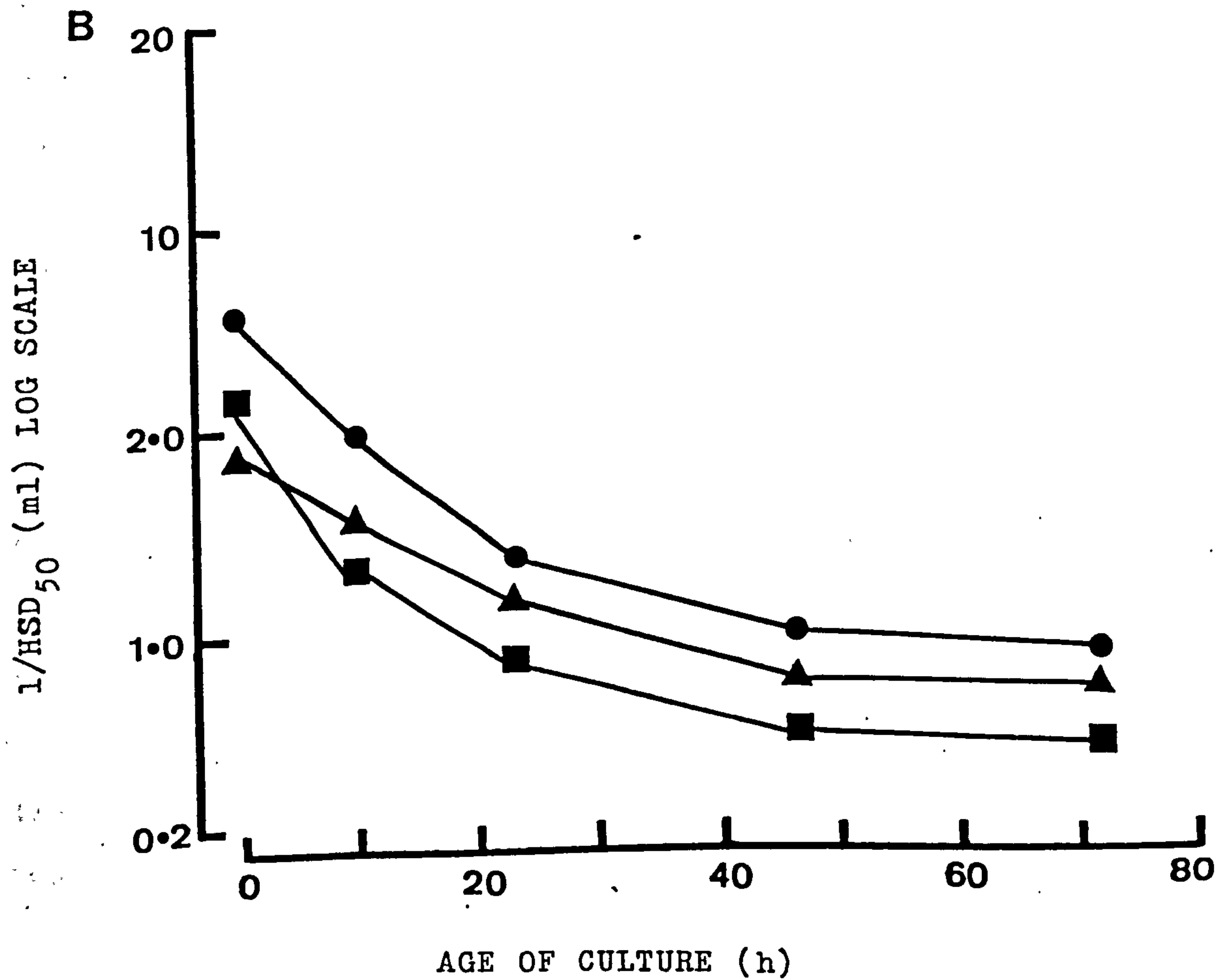
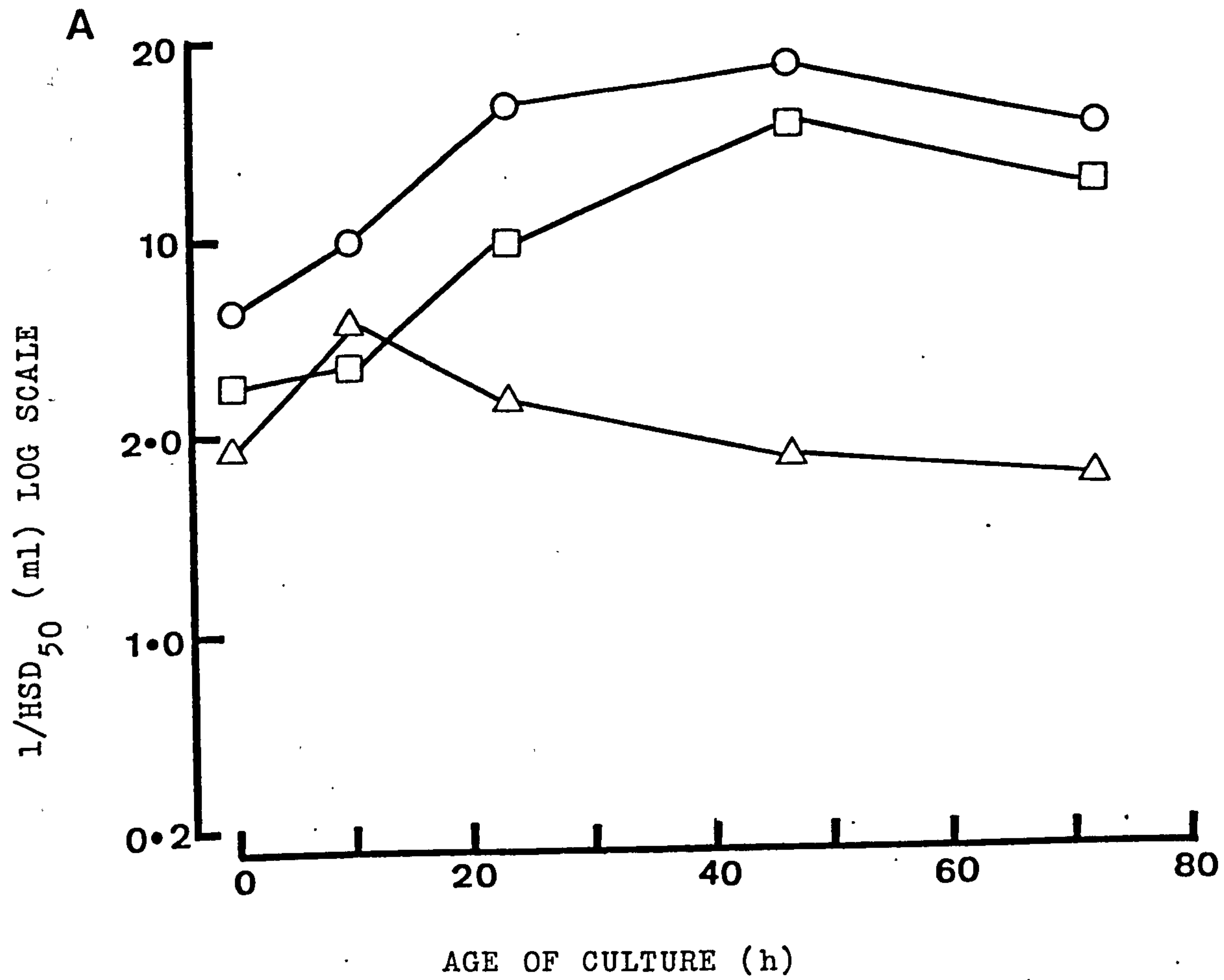
HSD₅₀ values are given as ml of whole cultures, supernatants and cell suspensions

A. Growth in X-medium

○—○ whole culture
□—□ supernatant
△—△ cells

B. Growth in C-medium

●—● whole culture
■—■ supernatant
●—● cells



The total level of HSF activity in the whole culture increased in parallel with cell numbers (Figure 28A). With the C-mode samples the original activity from the inoculum was detected initially in the cells and whole cultures. But this activity was lost gradually in the whole culture, cell suspension and supernatant during growth (Figure 28B).

One discrepancy observed in the results was that the levels of both the HSF and LPF activities in the X-mode cell suspensions showed an initial increase by 10h then decreased thereafter. Previous experiments had shown that the level of activities in the cell suspensions, of equal opacity units, but harvested at various times during growth, was constant. (e.g. Figures 13A, 14A, 18B and 19B). Thus since at every sampling time, the number of cells increased, the levels of activities in the cell suspensions were expected to increase in parallel with cell number. However, an initial increase then a decrease in the level of activities in the cell suspensions was found. A possible explanation for this could be that the samples were stored frozen before thawing and heating at 56°C for 30 minutes. There may have been some loss of activities either due to direct denaturation or possibly due to proteolysis by intracellular enzymes released by freezing and thawing and active during the thawing period prior to heating.

To investigate the possible effect of freezing and thawing prior to heating, the experiment was repeated. Samples of whole culture, supernatant, and cell suspensions

obtained in this experiment were heated immediately after sampling, preserved by the addition of 0.1mg/ml thiomersal and stored at 4°C. A week later, X-mode samples were assayed for their HSF activities.

Results for the whole culture and supernatant samples were similar to those obtained in previous experiments. Figure 28C shows that the total level of HSF activity in the culture increased in parallel with cell numbers. The level of activity in the supernatant was relatively low at 10h but thereafter it increased rapidly up to 48h. However, with the cell suspensions there was a steady increase in the level of activity during growth. This differed markedly from the previous results where the levels of activity in the cell suspensions at first increased then decreased (Figure 28A).

The level of activities in whole cultures, supernatant samples and cell suspensions in this experiment were higher than the levels of activity obtained in the previous experiments (Figures 28A). This suggested that freezing and thawing of the samples prior to heating allowed some degrading or denaturing effect on the B. pertussis components responsible for its pathophysiological activities.

In conclusion, it has been shown that during growth in X-medium, cells synthesized LPF and HSF. Initially these factors were bound to the cell but as growth progressed a proportion of the activity was liberated into the extracellular fluid where it accumulated and accounted for most of the activity in the culture.

Figure 28 : Distribution of histamine-sensitizing activity between cells and culture supernatant during growth of X-mode B. pertussis strain 18334 in X- and C-media

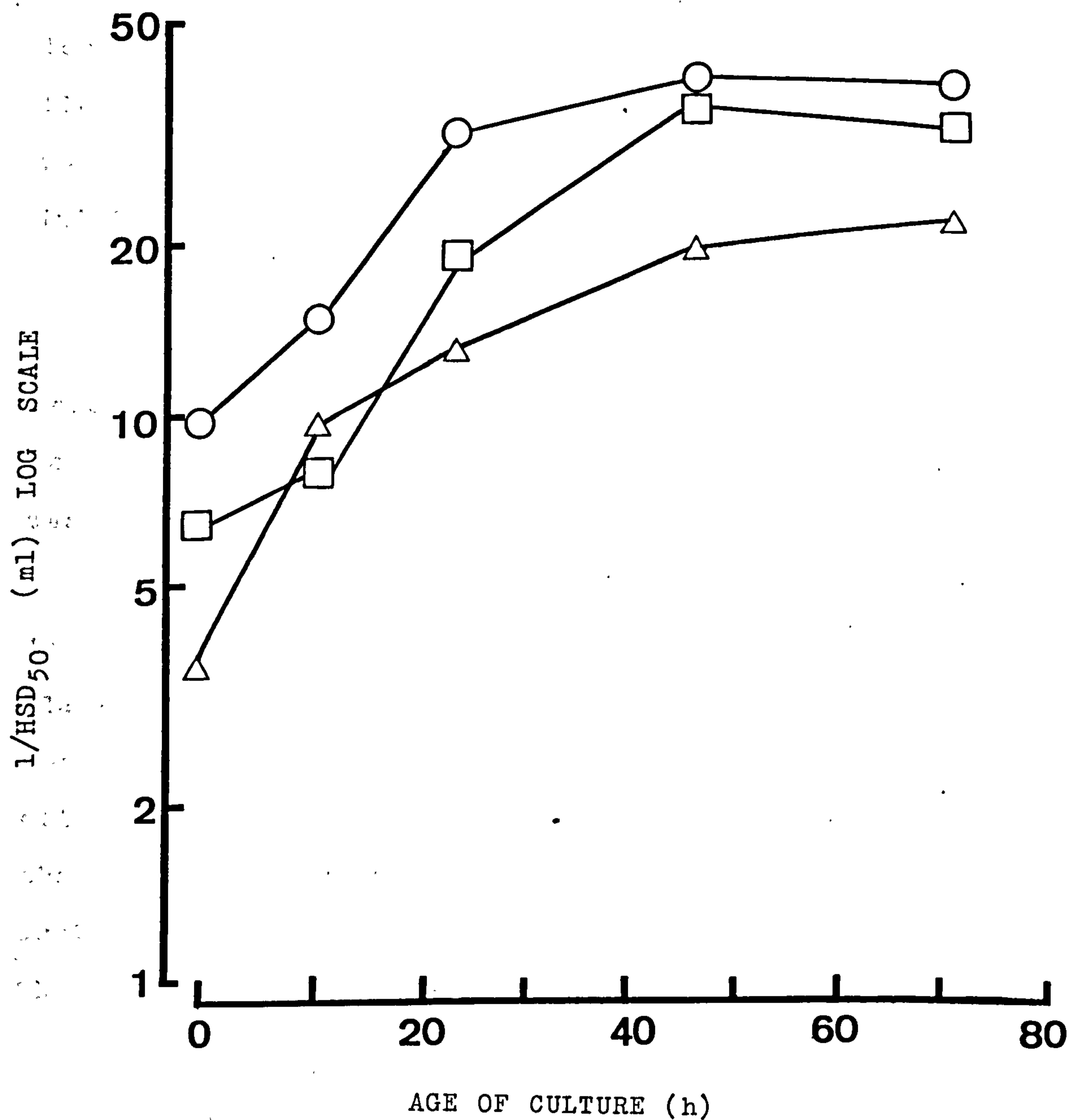
HSD₅₀ values are given as ml of whole cultures, supernatants and cell suspensions

C : Growth in X-medium

○—○ whole culture

□—□ supernatant

△—△ cells.



The LPF was apparently not synthesized during growth in C-medium but there appeared to be a transient increase in the histamine sensitizing ability of the whole culture during the early stages of growth. Evidence suggested that the loss of activities in the C-mode cells was not due solely to liberation of activities into the extracellular fluid but also to the denaturation or degradation of the activities present initially in both the cells and the extracellular fluid.

Subsection B : C to X modulation

Towards the end of this study, the reverse process of modulation (i.e. C to X) was examined to check if modulation is freely reversible as stated by Lacy (1960). However this was not examined in the same detail as for the X to C modulation.

X- and C-media were inoculated with cells grown for 48h in C-medium (C-mode cells). As a control, another set of flasks with X- and C-media were inoculated with cells grown for 48h in X-medium (X-mode cells). Cultures were harvested after 48h and the cell suspensions obtained were assayed for the levels of LPF, HSF and HLT activities. Changes in surface agglutinogens and envelope polypeptides were also examined. The experiment was repeated three times.

1. Growth characteristics

The optical densities of culture samples obtained from the above experiments were monitored at various intervals during growth. The changes in optical densities observed in the three different experiments were similar. One set of

results is shown in Figure 29. The growth of X- or C-mode cells in C-medium had a consistently higher optical density than the growth of X- or C-mode cells in X-medium. These results were similar to the previous observations with the X to C modulation.

2. Immunological and pathophysiological activities.

i. Histamine-sensitizing activity, leukocytosis-promoting activity and heat-labile toxicity

Cell suspensions obtained from 48h cultures of C-mode cells inoculated into X- and C-media and of X-mode cells inoculated into X- and C-media, were assayed for the above activities. Assay methods and analysis of results were done as in previous experiments.

The best-estimated values of the histamine-sensitizing potencies from two similar experiments are given in Table 13. After 48h, C-mode cells grown in X-medium had 66% of the activity of X-mode cells grown in X-medium whereas C-mode cells grown in C-medium had only 2.4% of the activity of X-mode cells grown in X-medium. (Table 13). Thus C-mode cells appeared to regain HSF activity when grown in X-medium; though the regain of this activity was apparently slower than the loss in X to C modulation (Figures 13A and 14A and Tables 9A and 9B). In X to C modulation, activities were lost faster than can be explained by dilution effect presumably because denaturation or degradation was occurring.

Figure 29 : Changes in optical density during growth of B. pertussis strain 18334 in X- and C-media.

10% (v/v) inocula of 48h X- and C-mode cultures were used.

○—○ X → X culture

△—△ C → X culture

●—● X → C culture

▲—▲ C → C culture

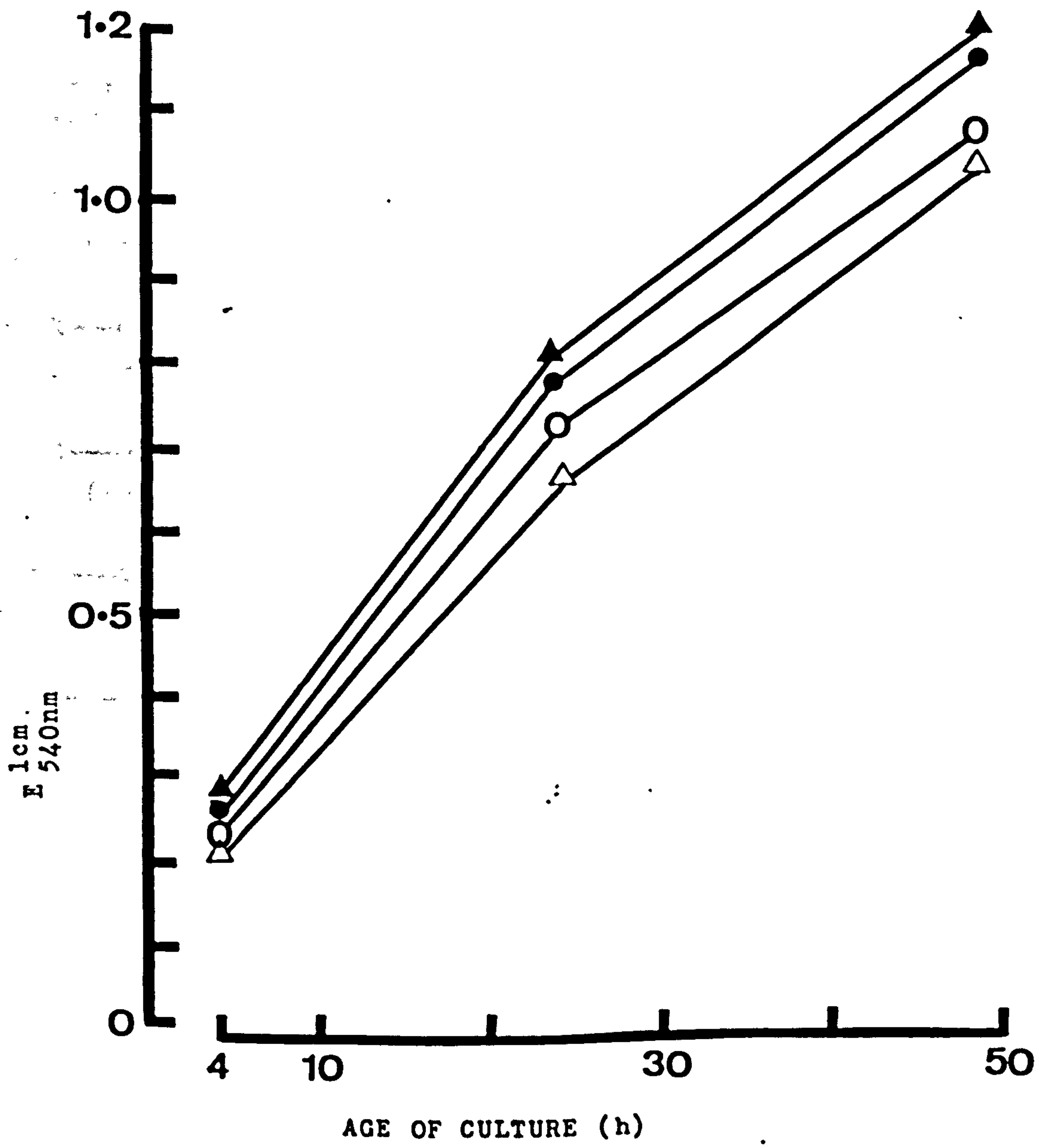


Table 13: Histamine-sensitizing activity
in cells during X to C and
C to X modulation.

Sample	Relative potencies (and 95% confidence limits)		Best-estimate relative potency values
	Experiment 1	Experiment 2	
Standard pertussis vaccine	1.00	1.00	1.00
X → X cells (48h)	1.4 (0.63-3.6)	0.63 (0.37-1.1)	0.81 (0.51-1.3)
X → C cells (48h)	0.007 (0.000-0.05)	0.02 (0.002-0.08)	0.01 (0.003-0.06)
C → C cells (48h)	0.02 (0.004-0.05)	0.02 (0.002-0.08)	0.02 (0.007-0.06)
C → X cells (48h)	0.63 (0.27-1.3)	0.50 (0.28-0.84)	0.53 (0.34-0.83)

Results of the LPF assay showed that X- or C-mode cells grown in C-medium had very low levels of detectable activity compared to X-mode cells grown in X-medium. On the other hand, C-mode cells grown in X-medium had almost as high a level of activity as X-mode cells grown in X-medium (Figure 30). This again suggested that the LPF activity was regained when C-mode cells were grown in X-medium and that modulation was freely reversible. The actual leukocyte counts obtained are tabulated in Appendix 7.

The same trend of results as for the HSF and LPF assays were obtained when samples were assayed for heat-labile toxicity. The potency values obtained from a typical experiment (Table 14) indicated that X- or C-mode cells grown in C-medium had 22% and 16% respectively of the levels of activity of X-mode cells grown in X-medium. Also C-mode cells grown in X-medium regained HLT activity and had 77% of the activity of X-mode cells grown in X-medium. As with the HSF, the potency values and 95% confidence limits indicated no significant difference in the level of toxicity when either C-mode or X-mode cells were grown in X-medium for 48h.

These results suggested that the HSF, LPF and HLT were regained and restored to almost normal levels after a single subculture of C-mode cells in X-medium.

ii. Agglutinability

Cell suspensions harvested at various intervals during growth of C-mode cells in X-medium were tested for possible

Figure 30 : Levels of LPF activity in X- and C-mode cells

Cells were harvested after 48h growth of B. pertussis strain 18334 in X- and C-media (10% (v/v) inoculum).

8 ou.ml of each cell suspension was injected per mouse.

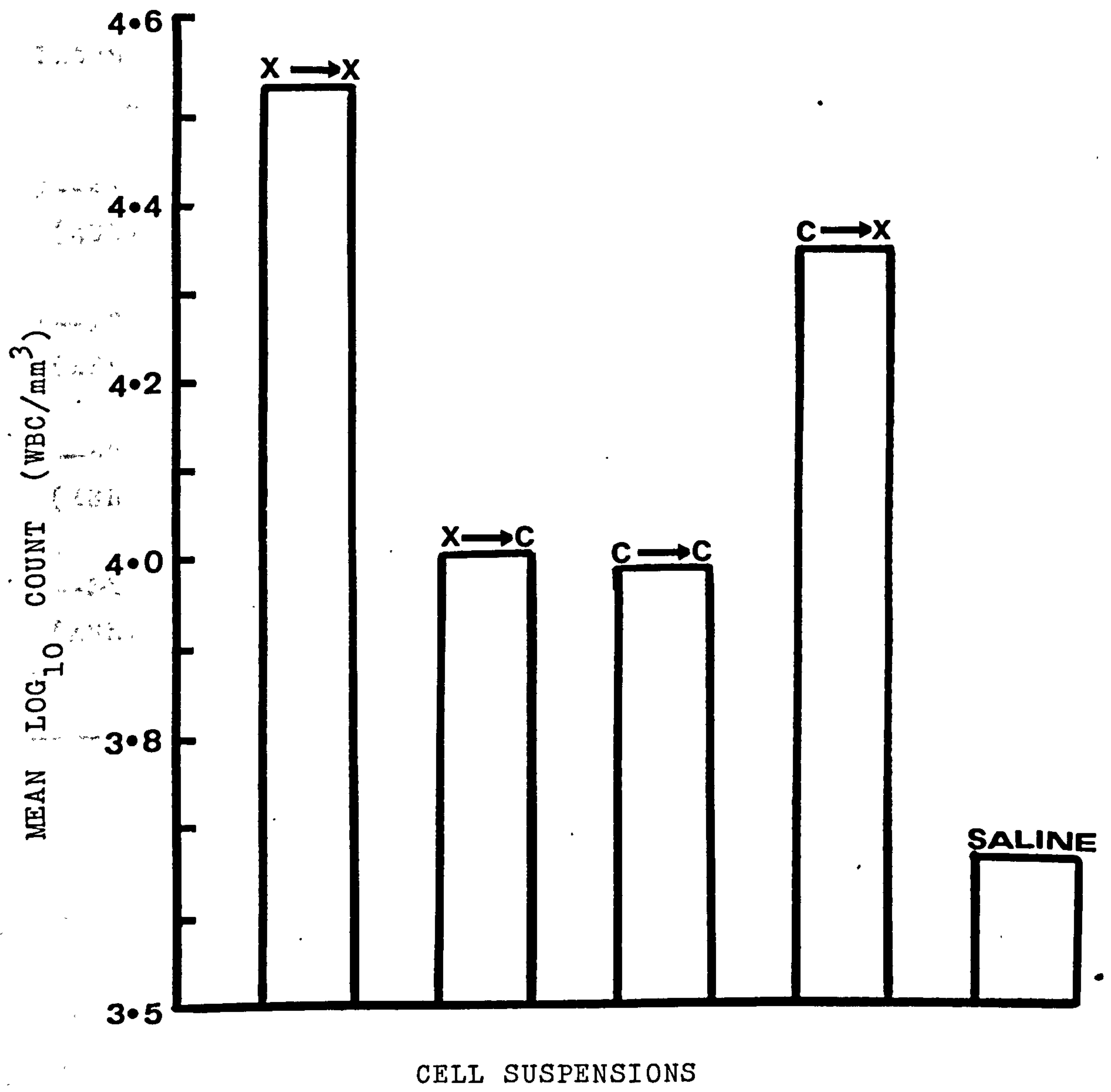


Table 14:

Heat-labile toxicity in cells
during X to C and C to X
modulation

Sample	Relative potency (and 95% confidence limits)	LD ₅₀ (ou.ml)
Inoculum (X-mode) (48h)	1.00	0.38
X → X (48h)	0.74 (0.42 - 1.3)	0.52
X → C (48h)	0.16 (0.093 - 0.28)	2.4
C → C (48h)	0.12 (0.067 - 0.21)	3.2
C → X (48h)	0.57 (0.32 - 0.99)	.67

changes in their surface agglutinogens. Samples from two similar experiments were tested against specific and unabsorbed X- and C-mode antisera for their agglutinability. Results from the two experiments were not significantly different.

Figure 31A shows the results of one of the assays using unabsorbed antisera. The titre of anti-X serum against the inoculum cells (C-mode cells) was initially low but as the C-mode cells grew in X-medium the titre increased gradually until about 24h after which it stayed constant. On the other hand, the titre of anti-C serum against the inoculum cells (C-mode cells) was high but as the cells grew in X-medium, it decreased and remained relatively constant after 24h.

With the specific antisera (Figure 31B), the titre of the anti-X serum against C to X cells showed only a slight increase for up to 48h and remained unchanged. However, the titre of the anti-C serum against C to X cells decreased significantly within 24h and stayed constant.

The increase in the titres of specific and unabsorbed anti-X sera for C to X cells and the decrease of the titre of the anti-C sera for C to X cells during growth indicated that surface agglutinogens were regained. This appeared to have occurred within 24h of inoculation of C-mode cells into X-medium. In previous results, the loss of the agglutinogens during X to C modulation was also observed to occur within 24h of growth of X-mode cells in C-medium.

Figure 31 : Changes in agglutinability with unabsorbed and absorbed antisera.

A 10% (v/v) inoculum from a 48h C-mode culture of B. pertussis strain 18334 was used in the growth experiment.

A. Agglutination with unabsorbed antisera.

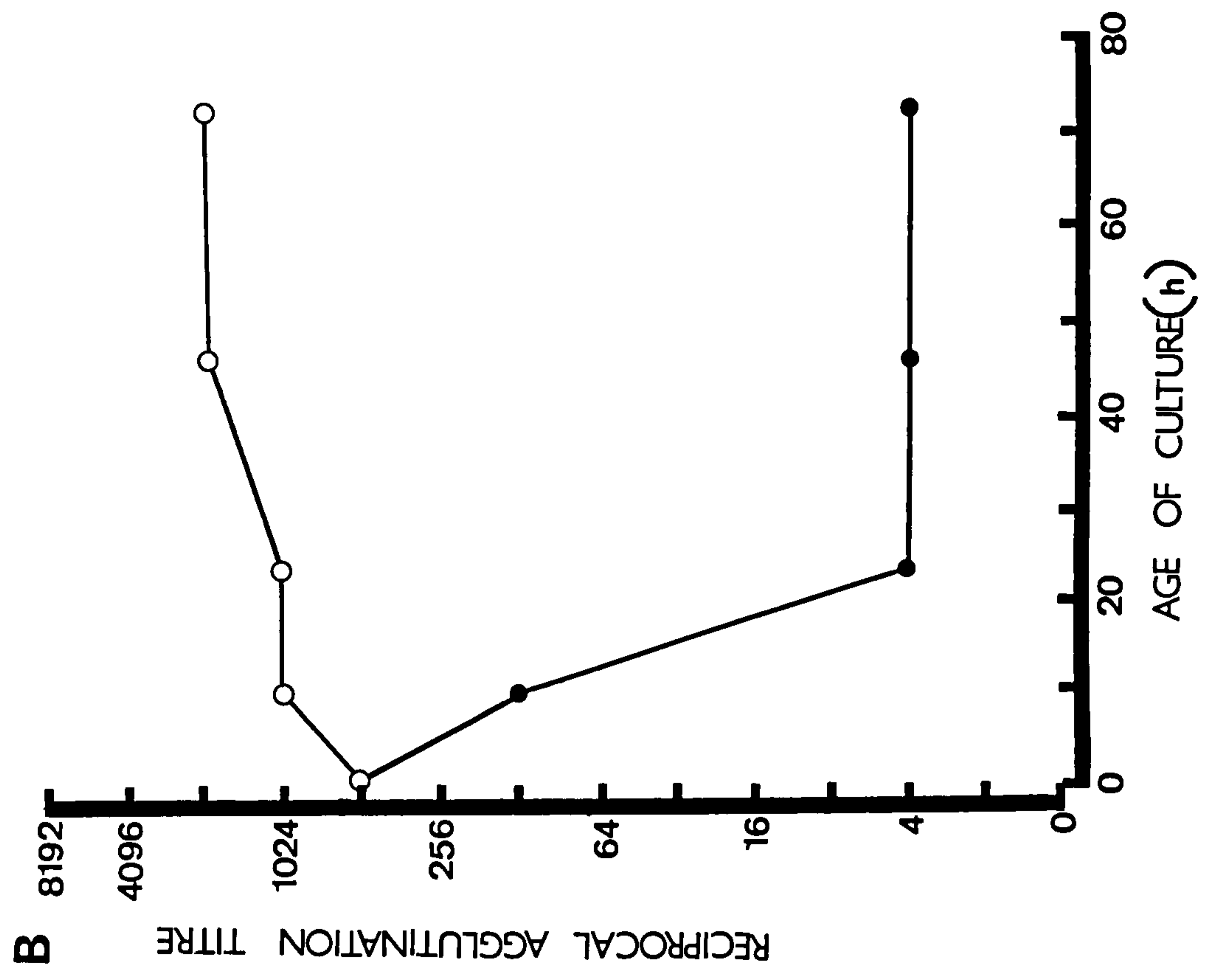
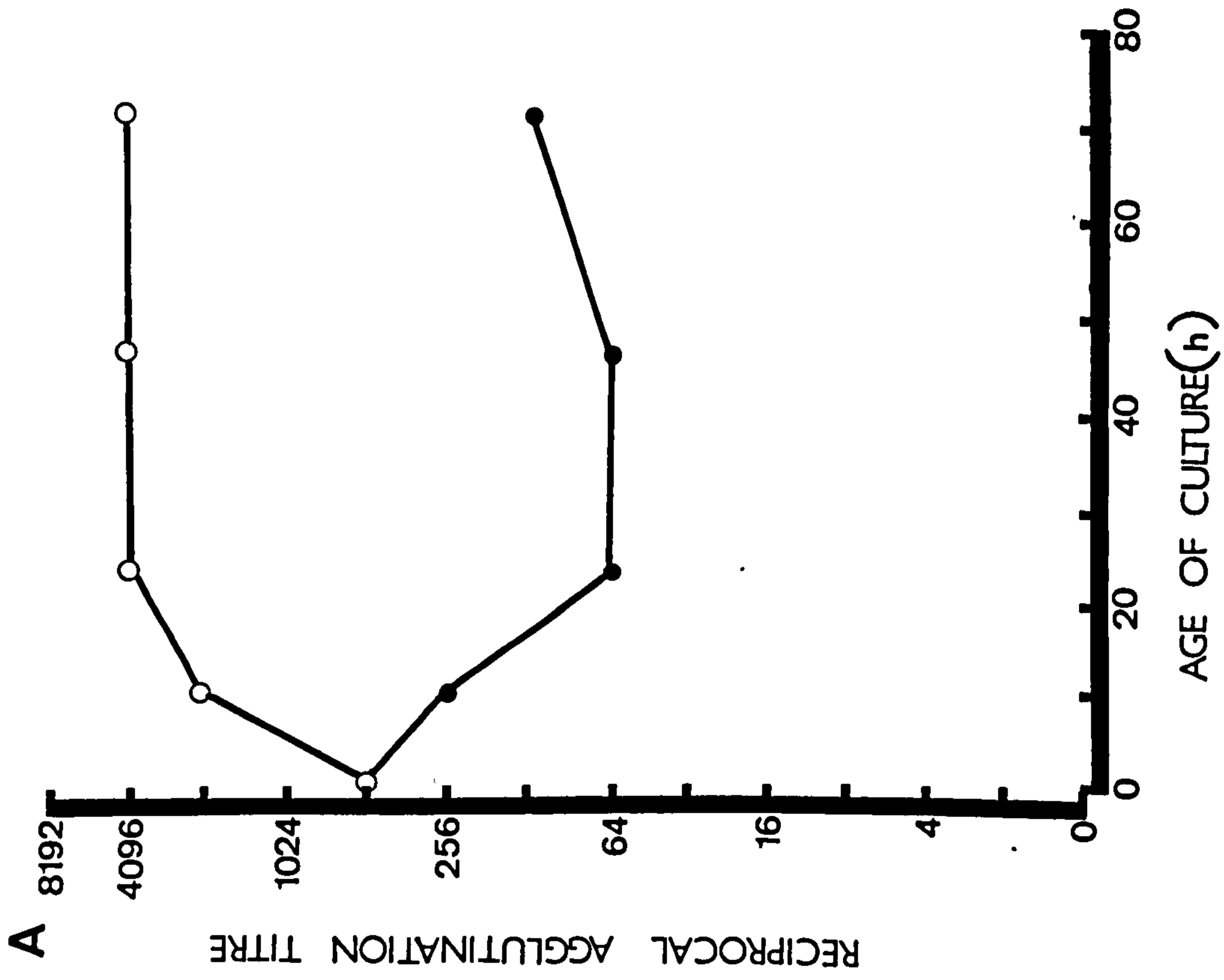
○—○ anti-X serum against C → X cells

●—● anti-C serum against C → X cells

B. Agglutination with absorbed antisera

○—○ anti-X serum against C → X cells

●—● anti-C serum against C → X cells



3. Changes in cell-envelope proteins

During the growth of C-mode cells in X-medium and X-mode cells in C-medium, the protein profiles of cell suspensions obtained were analysed by the SDS-polyacrylamide electrophoretic method. Results revealed that polypeptides with molecular weights of 28k, 30k and 100k were absent in X-mode cells grown in C-medium. These polypeptides however were present in C-mode cells grown in X-medium (Plate 2). Thus these polypeptides were regained during the reverse process of modulation. This gain of envelope proteins correlated with the gain of other immunological and pathophysiological activities during C to X modulation.

SECTION 3 : MECHANISMS OF ANTIGENIC MODULATION

During modulation from the X-mode to the C-mode, the various immunological and pathophysiological activities associated with the X-mode cells were lost and during the change from C-mode to X-mode these activities were regained. In an attempt to explain the mechanism of these changes the roles of a number of different factors in modulation were examined.

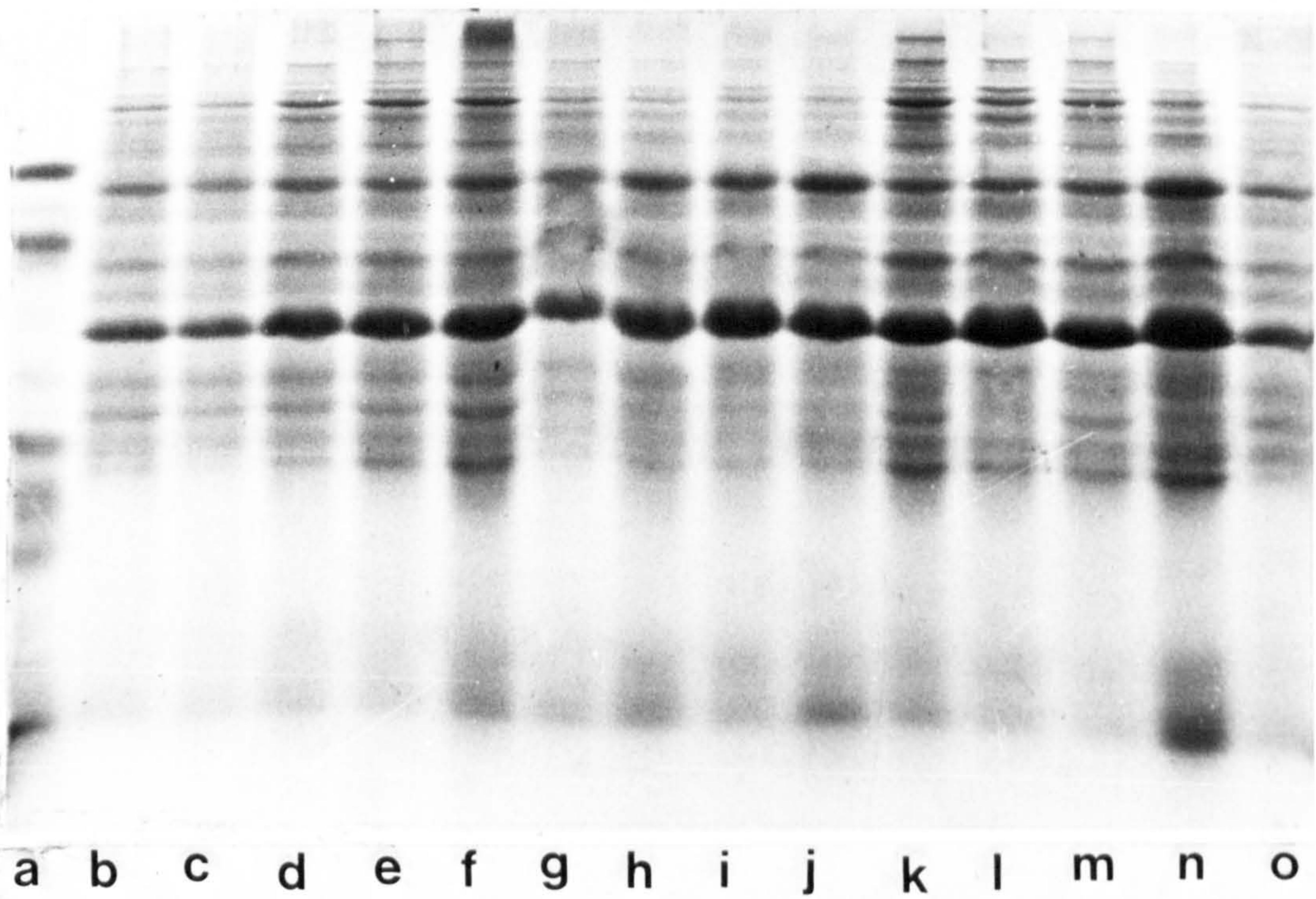
Plate 2.

SDS-polyacrylamide gel electrophoresis
of envelope polypeptides of *B. pertussis*
grown in X- and C-media (X→C and
C→X modulation).

- a. standard proteins
- b. inoculum
- c. X10h
- d. X24h
- e. X48h
- f. X72h
- g. C10h
- h. C24h
- i. C48h
- j. C72h
- k. X→X 48h
- l. X→C 48h
- m. C→X 48h
- n. C→C 48h .
- o. inoculum

100k →

30k →
28k →



1. Specificity of the magnesium sulphate requirement

In the present study, X to C modulation was induced by replacing the NaCl content of the growth medium with an equivalent amount of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. In order to understand the mechanism of modulation, it was necessary to examine the possible roles of NaCl and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in modulation.

Preliminary experiments involved growing X-mode cells in X-medium and in Hornibrook medium with neither NaCl nor $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as an added ingredient. In later experiments, growth of X-mode cells in C-medium was included as a further control. Two phase I strains of B. pertussis, 18334 and D30042 were used. Both strains grew well in the medium with neither added NaCl nor $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. In all the experiments, cells were harvested after 48h and both cell suspensions and whole culture samples were assayed for their HSF activity.

Tables 15A and 15B show the HSD_{50} values obtained. With strain 18334, there was a significant drop in the level of HSF activity in both the cell suspensions and whole culture samples of cells grown in Hornibrook medium with neither added NaCl nor $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The percentage levels of activity in the cultures and cell suspensions relative to the level of activity in X-mode cultures and cell suspensions was 31% and 22% respectively. The loss of activity represented a three-fold and four-fold difference in the whole cultures and cell suspensions respectively.

Table 15A:

Histamine-sensitizing activity in whole culture and cell suspensions of *B. pertussis* strain 18334 grown in medium without added NaCl or MgSO₄.

Growth-medium	Whole culture		Cell suspensions	
	HSD ₅₀ (in ml)	% activity relative to X-mode	HSD ₅₀ (in ml)	% activity relative to X-mode
X-medium	0.27	100	0.41	100
Hornibrook medium - no added NaCl or MgSO ₄	0.82	31	1.90	22

Table 15B:

Histamine-sensitizing activity in cell suspensions of *B. pertussis* strain D30042I grown in medium without added NaCl or MgSO₄.

Growth medium	Cell suspensions	
	HSD ₅₀ (in ou.ml)	% activity relative to X-mode
X-medium	0.30	100
C-medium	2.38	13
Hornibrook medium without added NaCl or MgSO ₄	0.62	48

With strain D30042, only the cell suspensions were assayed for activity. However, similar results were obtained as with strain 18334. Cells grown in Hornibrook medium with neither added NaCl nor $MgSO_4 \cdot 7H_2O$, had only 48% of the activity of cells grown in X-medium. This indicated a two-fold difference in activity.

Cells grown in C-medium had only 13% of the activity of cells grown in X-medium.

In both strains, growth in Hornibrook medium with neither added NaCl nor $MgSO_4 \cdot 7H_2O$ resulted in a decrease in the level of HSF activity, though not to the same level as when cells were grown in C-medium. This suggested that although high levels of $MgSO_4 \cdot 7H_2O$ plays a major role in the induction of modulation, the lack of NaCl also may be important.

2. Proteolytic activities of X- and C-mode cultures

During X to C modulation, activities decreased faster than could be explained by dilution amongst the progeny cells. One possible explanation was that the protein components responsible for these activities were degraded by proteolytic enzymes released from the cells. Therefore the proteolytic activities present in X- and C-mode cultures were estimated. In the assay, azocoll and azocaesin were used as substrates while pronase and subtilisin respectively were used as standard proteolytic enzymes.

Assays were done on samples of X- and C-mode whole cultures and culture supernatants obtained at various intervals during growth. As controls, heated (100°C for 30 mins.) whole cultures and supernatants as well as samples of uninoculated X- and C-media were tested.

Concentration of proteases in the samples were to be estimated from standard curves obtained with pronase and subtilisin and their respective substrates (Figures 32A and 32B). However, there was no significant difference between the values obtained for any of the test samples and the controls. Thus it was concluded that proteolytic activity was not detectable in either the X- or C-mode cultures.

Later attempts to estimate the intracellular levels of activity using X-preserved cells also showed no detectable activity. In conclusion, it appeared that the loss of activities during modulation was probably not due to proteolytic degradation of envelope components or cell components released into the culture medium.

3. Cyclic AMP levels during modulation

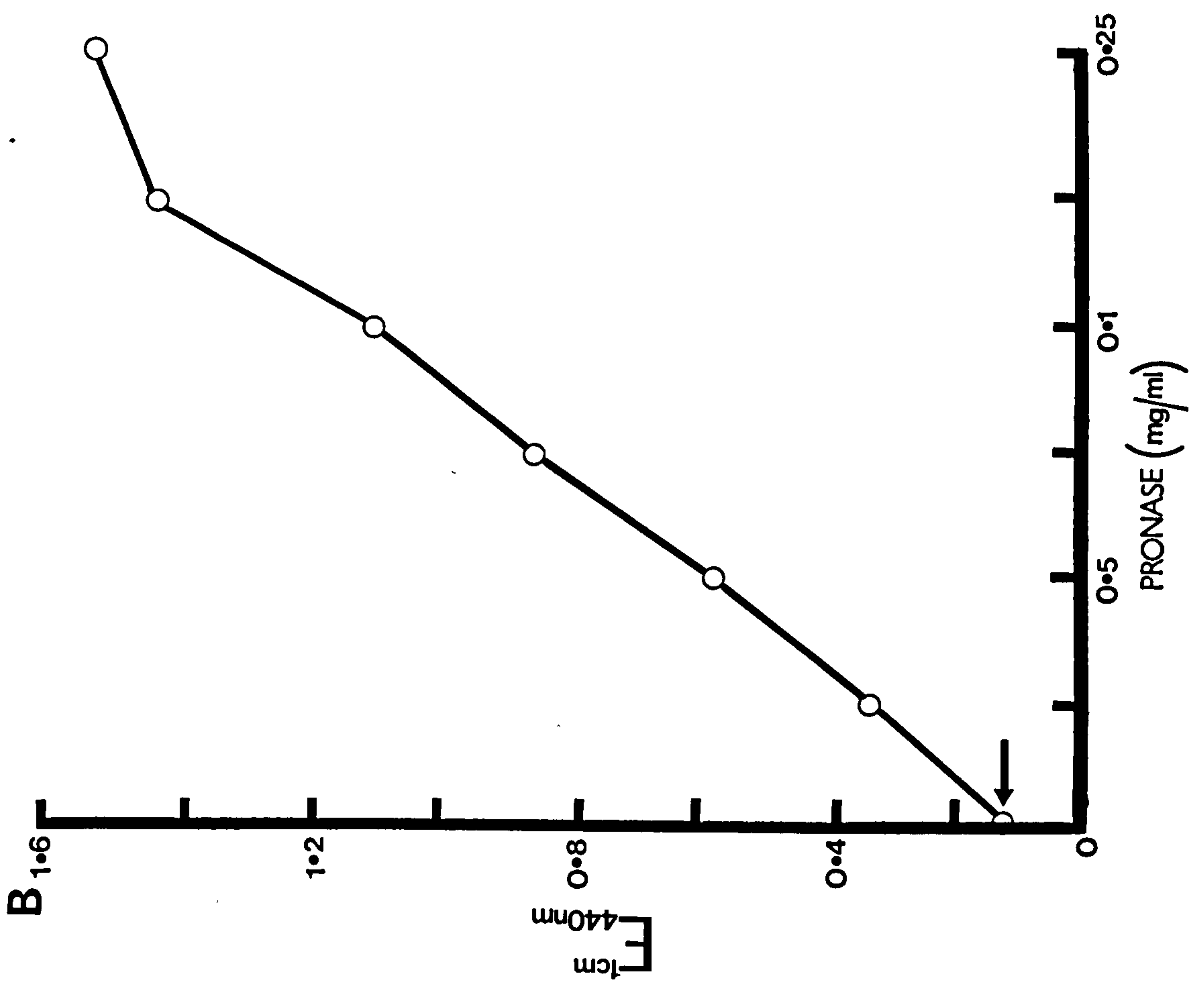
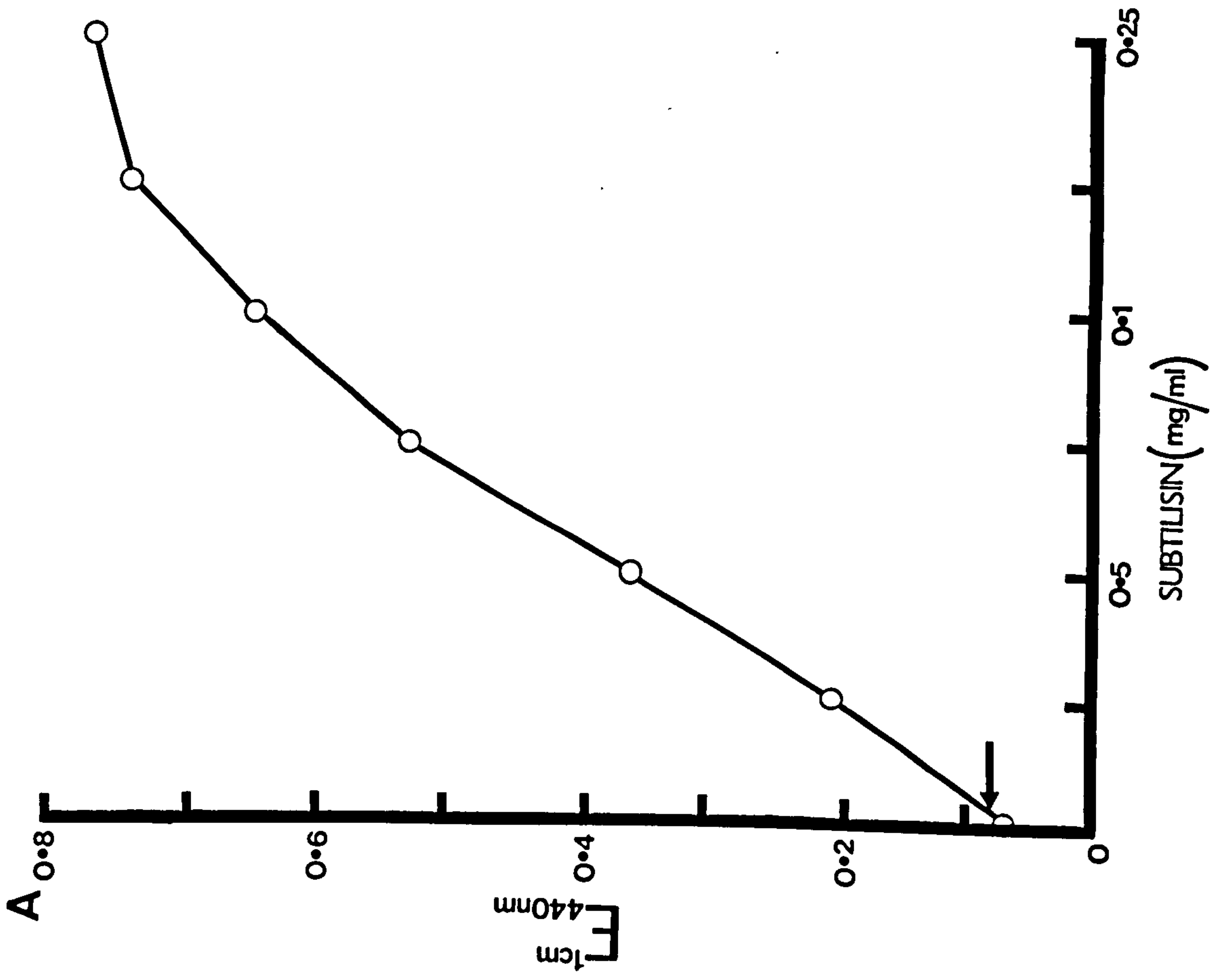
Recently, Parton and Durham (1978) reported the absence of adenylate cyclase activity and cyclic AMP in C-mode cultures of B. pertussis. The role of cyclic AMP in the regulation of gene expression in some microorganisms has been clearly established. Thus efforts were directed towards investigating the possible role of cyclic AMP in antigenic modulation in B. pertussis.

Figure 32 : Estimation of proteolytic activity.
(Standard curves).

A. Digestion of azocasein by subtilisin.

B. Digestion of azocoll by pronase.

Arrow in both graphs indicate value
obtained for the X- and C-mode
samples.



Total cyclic AMP in X- and C-mode cultures harvested at various intervals were estimated.

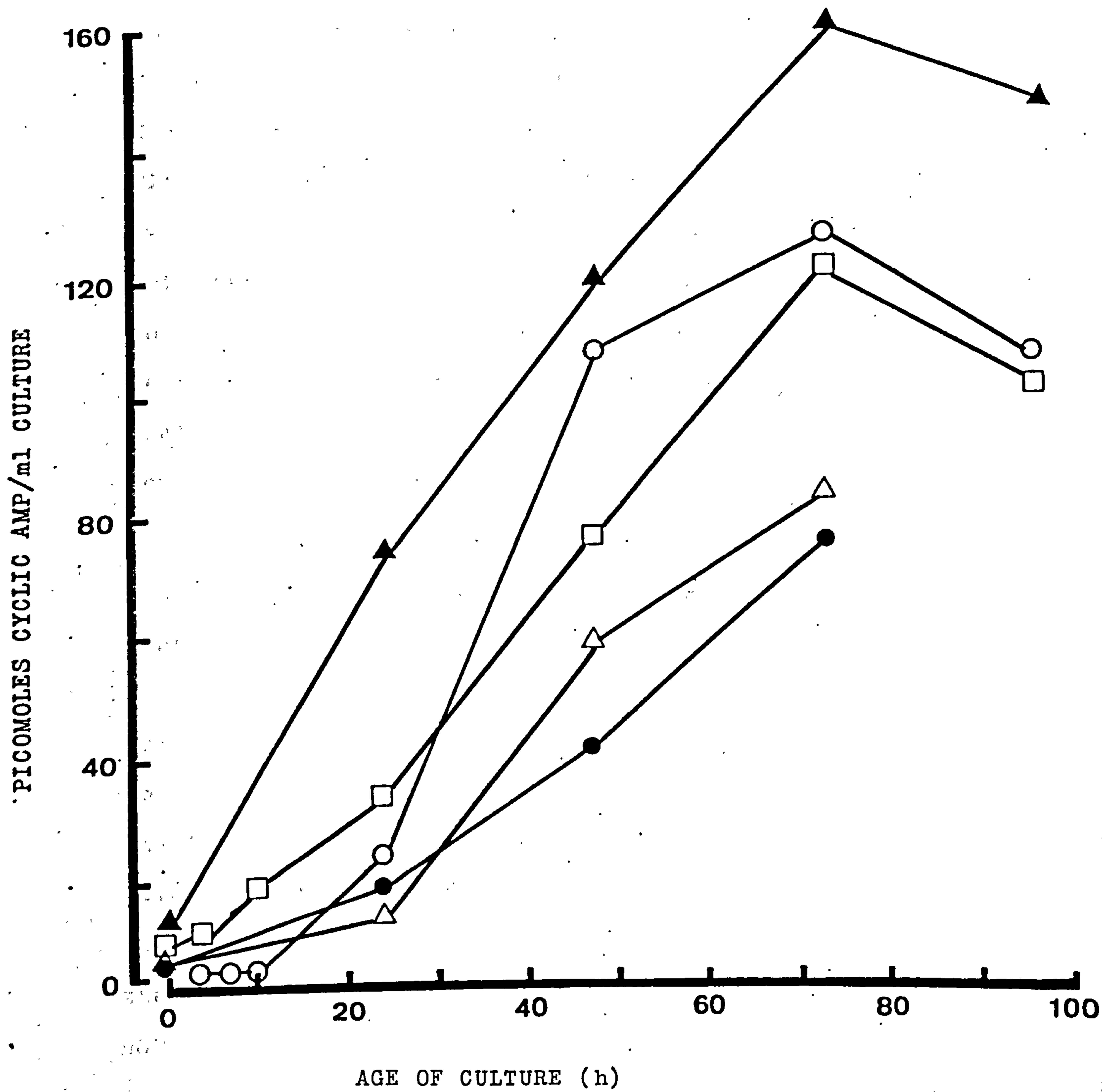
Figure 33 shows the levels of cAMP in X-mode cultures obtained from five similar experiments. In all the experiments, there was a steady increase in cAMP during growth. In the three experiments where the sampling time was extended to 96h, it was observed that the increase in cAMP reached a peak at about 72h and thereafter declined. In two other experiments where samples were taken earlier than 10h during growth, it was observed that the levels of cAMP in the cultures did not show any marked increase until after about 8h of incubation, although these low levels were at the limits of detection. Strains 134 and D30042 produced higher levels of cAMP than strain 18334 though the significance of this observation is unknown. In all the experiments, there was no detectable cAMP ($< 2.5\text{pmol/ml}$ of undiluted culture) in any of the samples taken from C-mode cultures of the three strains after 10h of incubation.

Thus, during growth in X-medium cAMP was synthesized by the cells whereas there appeared to be no synthesis at any time during growth in C-medium. Also the initial cAMP content of the X-mode inoculum appeared to be rapidly destroyed by growth in the C-medium.

In later experiments, X-mode cells were grown in X- and C-media to which was added $5\mu\text{g/ml}$ of bovine liver catalase, known to contain an activator of B. pertussis

Figure 33 : Changes in cyclic AMP levels in whole cultures during growth of B. pertussis strains in X-medium.

- experiment 1. (18334)
- experiment 2. (18334)
- △—△ experiment 3. (18334)
- ▲—▲ experiment 4. (D30042)
- experiment 5. (134).



adenylate cyclase. Samples of whole cultures were obtained at various intervals during growth and assayed for their cAMP levels. With the X-mode culture samples, there was an approximately two-fold increase in the levels of cAMP formed when compared to the levels in cultures grown in X-medium without added catalase. On the other hand, even with a source of adenylate cyclase activator, there was still no detectable cAMP in the C-mode samples (Table 16). This result further suggested that there was apparently no synthesis of cAMP by the C-mode cells. If there had been even low activity of adenylate cyclase, it is possible that the presence of catalase might have enhanced cAMP formation to detectable levels.

Samples obtained from these experiments were further assayed for their HSF activity. It was observed that despite the two-fold enhancement in the levels of cAMP in the X-mode cultures, the levels of HSF activities in cultures grown in the presence or absence of catalase did not alter significantly (Table 16). Thus it appeared that the level of HSF activity was not directly related to the level of cAMP in the X-mode cultures. In both the C-mode cultures grown in the presence or absence of catalase, there was a rapid decrease with time in the levels of HSF activity.

Changes in cAMP levels during the reverse process of C to X modulation were also investigated. X- and C-media were inoculated with 48h C-mode liquid culture of strain D30042 containing no detectable cAMP. Growth of X-mode

Table 16: Effect of catalase on cyclic AMP levels and histamine-sensitizing activity in cultures of *B. pertussis* strain 18334 grown in X- and C-media

Sample	Total cyclic AMP pmol/ml culture	HSD ₅₀ (ml)
Inoculum (X-mode, 48h)	51.0	0.41
X→X 24h	30.6	0.47
X→X + catalase 24h	53.5	0.53
X→C 24h	< 2.5 *	2.20
X→C + catalase 24h	< 2.5 *	2.64
X→X 48h	45.9	0.41
X→X + catalase 48h	76.5	0.35
X→C 48h	< 2.5 *	5.58
X→C + catalase 48h	< 2.5 *	5.58

* limit of detection

cells in X-medium was set up in parallel as control.

Results depicted in Figure 34 shows that the levels of cAMP in cultures of X-mode cells grown in X-medium increased rapidly between 10h and 24h and thereafter showed only a further slight increase to 48h.

With growth of C-mode cells in X-medium the level of cAMP showed a similar pattern of a steady increase during growth, though there was an initial lag period during which cAMP was undetectable in the culture. There was no cAMP detected at any time during growth of C-mode cells in C-medium.

Results from earlier experiments indicated that lost immunological and pathophysiological activities were regained during C to X modulation. These observations coupled with the fact that cAMP was also regained during the reverse process of modulation, prompted attempts to determine the possibility of stimulating a faster restoration of the activities by enhancing the cAMP levels during C to X modulation. C-mode cells were grown in normal X- and C-media and in X- and C-media with added catalase (5 μ g/ml). As a control X-mode cells were also grown in normal X-medium and X-medium with added catalase. Whole culture samples obtained at various intervals during growth were assayed for their cAMP content and HSF activities. Table 17 shows that there was no detectable cAMP in any whole culture samples of C-mode cells grown in C-medium and correspondingly, there were very low levels of HSF activities. When C-mode cells were grown in normal X-medium for 8h, there was no detectable cAMP though there

Figure 34 :

Changes in cyclic AMP levels in whole cultures during growth of X-mode and C-mode B. pertussis strain 18334 in X-medium.

○ — ○ X → X culture
● — ● C → X culture

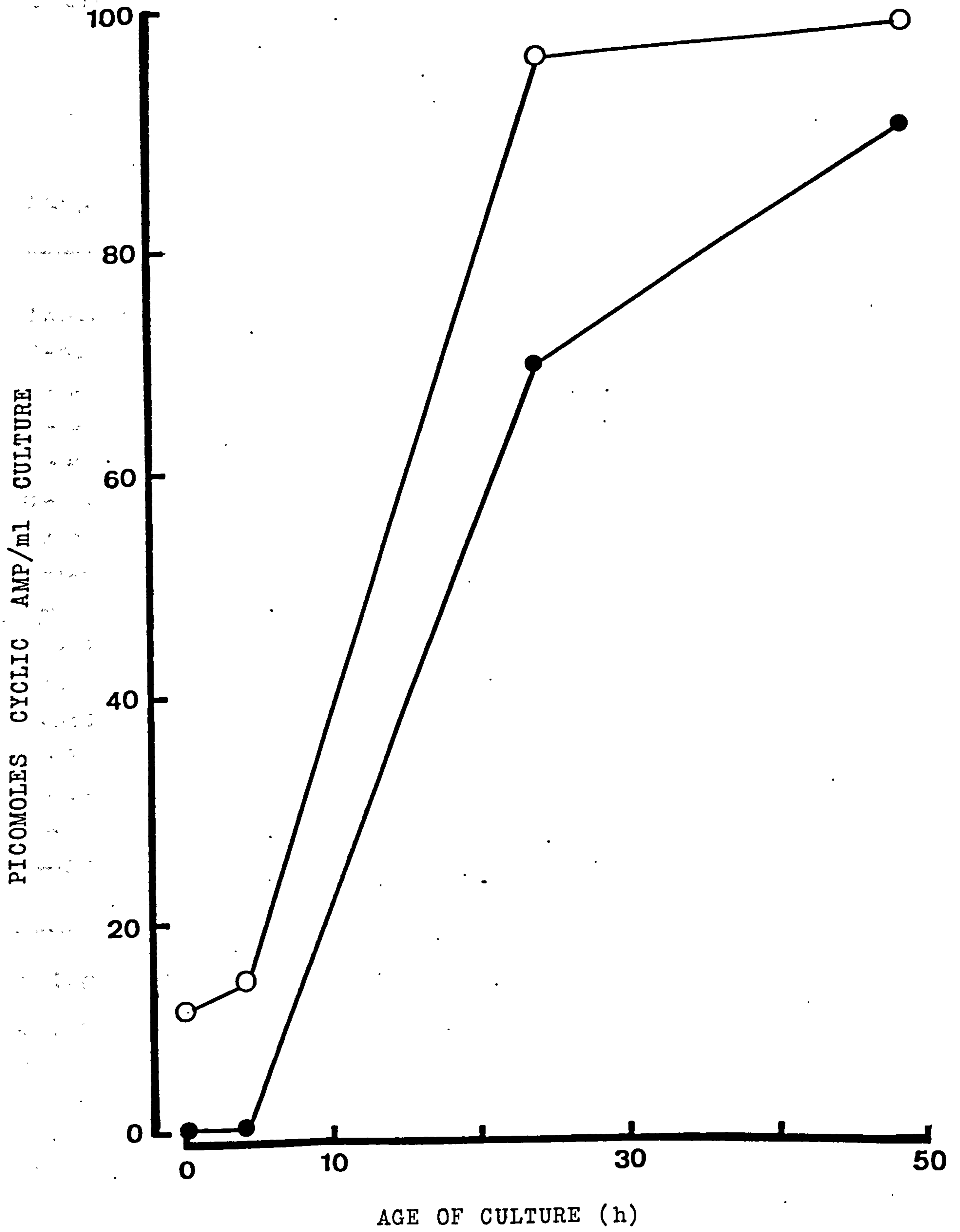


Table 17: Effect of catalase on the levels of cyclic AMP and histamine-sensitizing activity in cultures of *B. pertussis* strain D30042I in X- and C-media

Sample	Total cyclic AMP pmol/ml culture	HSD ₅₀ (ml)
Inoculum		
(C-mode, 48h)	< 2.5 *	6.08
C → C 8h	< 2.5 *	6.42
C → C + catalase 8h	< 2.5 *	6.01
C → C 24h	< 2.5 *	5.98
C → C + catalase 24h	< 2.5 *	5.98
C → X 8h	< 2.5 *	4.64
C → X + catalase 8h	5.1	2.18
C → X 24h	68.25	0.84
C → X + catalase 24h	112.4	0.31
Inoculum		
(X-mode, 48h)	7.12 +	0.61
X → X 8h	10.25	0.49
X → X + catalase 8h	27.5	0.26
X → X 24h	97.6	0.28
X → X + catalase 24h	163.0	0.16

* limit of detection

+ 1/10 dilution

was a slight but insignificant increase in the level of HSF activity when compared to the level of activity in the C-mode inoculum. On the other hand, when C-mode cells were grown in X-medium with added catalase for 8h, low levels of cAMP were detected and there was a corresponding increase in the levels of HSF activity. Thus there appeared to be some relationship between the level of cAMP and the rate at which the HSF activity was regained.

In conclusion the synthesis of cAMP during the log phase of growth of X- or C-mode cells in X-medium appeared to parallel the increase in the levels of various activities.

The lack of cAMP in C-mode cultures accompanied the lack of these activities. Furthermore, when formation of cAMP was enhanced by the addition of an activator, restoration of HSF activity also appeared to be enhanced. The enhancement of cAMP levels in cultures may have accelerated the gain in HSF activity during the C to X change.

DISCUSSION

SECTION 1 : DEVELOPMENT OF AN LPFASSAY METHOD

Recent fractionation studies have provided substantial evidence for the relationship between certain of the immunological and pathophysiological activities associated with X-mode B. pertussis. For example, Morse and Morse (1976) and Lehrer et al., (1976) reported that the histamine-sensitizing ability and lymphocytosis-promoting ability were different manifestations of a single protein component of the cell. The loss of HSF in C-mode cells was reported by Wardlaw et al., (1976). If a single component were responsible for the HSF and LPF activities then the LPF would also be lost in C-mode cells. Since there was no previous report on whether the LPF was present or absent in C-mode cells, this was investigated in the present work. Results indicated clearly that the LPF activity was lost from C-mode cells during modulation.

In order to determine more precisely the time sequence of loss of each of the immunological and pathophysiological activities during modulation and to further elucidate the relationship, if any, between them, it was necessary to quantitate the potencies of these activities associated with the cells at various intervals during growth.

At the beginning of the present study, suitable assay methods were available for determining the potencies of the PA, HSF and HLT; but there was no method available for measuring the LPF potency of B. pertussis preparations.

In LPF studies by various workers, male or female mice of different strains have been used. There has been no report of any work done to compare the effect of sex of mouse or strain of mouse on LPF response. Since different strains of mice vary greatly in their response to HSF (Munoz and Bergman, 1968), the possible effect of sex and strain of mouse on LPF response was investigated in the present study. Male mice were found to be slightly more responsive to LPF than female mice. This was an unexpected result since the female mice were smaller and weighed less than male mice of the same age. It would have been expected therefore that the females getting a greater dose per unit weight, would have had a greater response than the males.

With regards to mouse strain there was no significant difference between the responses of Ham/ICR and NIH mice to LPF. These were the only mice conveniently available for the present work. It is possible that differences would have been detected if more strains of mice had been investigated.

Further experiments were performed to examine the LPF response in mice of different ages since it has been reported that age was an important factor in histamine-sensitization. In certain strains of mice, such as the CFW, age does not seem to play a marked role in histamine-sensitization but in other strains it has a profound effect on the response (Bergman and Munoz, 1964). In this project there was no significant difference observed in

the LPF responses of the various ages (3 weeks to 20 weeks) of Ham/ICR and NIH mice tested. This was also an unexpected result for the smaller 3-week old mice, received a much higher dose per unit body weight than the 20 week old mice. Doubling of the dose of B. pertussis preparations significantly increased the leukocytosis in mice of the same age. However, an approximately two-fold difference in body weight, as found in mice of the different ages, appeared not to have any significant effect on LPF response.

Another factor of considerable importance in the LPF response in mice is the route of inoculation of the pertussis sample. Both the intraperitoneal and intravenous routes of inoculation have been used by various workers. Morse (1965) and Finger, Emmerling and Plager (1972) reported that the intravenous route of inoculation stimulated leukocytosis more rapidly and to a greater extent than the intraperitoneal route. The subcutaneous route was reported to be ineffective. The finding in this study comparing the intravenous and intraperitoneal routes of inoculation agreed with the views of these workers. However, whereas Morse (1965) reported a two-fold difference in LPF response between the two routes of inoculation, a four-fold difference was obtained in the present work. This discrepancy may not be significant because of the differences in the doses of samples, strains of mice and assay methods used.

Having determined the optimal conditions for an LPF assay, later experiments were directed towards quantitating the

LPF potency of the X- and C-mode samples. Earlier workers had quantitated the LPF potency of B. pertussis in different ways. Ishida (1968) based his measurements of LPF potency on the effect of LPF on body weight of mice. He stated that after intraperitoneal injection with B. pertussis samples, the effect of endotoxin/heat-labile toxin on decrease in body weight was observed on the second day after inoculation while the effect of LPF on further decrease in body weight was manifested from the third or fourth day after inoculation. He therefore proposed D_0D_4 as an absolute unit of LPF for the body weight-decreasing toxicity. This stood for the dose with which the body weight of mice did not increase nor decrease during a time period from the second to the fourth day after intraperitoneal injection.

Sato and Arai (1972) arbitrarily defined one unit of LPF activity as the amount of a sample which when injected into mice gave rise to a total leukocyte population of $32,000/\text{mm}^3$. They showed a linear relationship between the logarithmic leukocyte count and logarithmic LPF dose and further added that the magnitudes of variance of logarithmic numbers of leukocyte counts at different doses were on the same level. However, Kurokawa, Ishida, Asakawa and Iwasa (1978), did not accept this LPF unitage proposed by Sato and Arai (1972) on the basis that it was an arbitrary potency unit. Instead, these workers proposed the applicability of the parallel line assay method for the determination of LPF potency, based on total peripheral leukocyte count. They tentatively

proposed one LPF unit as the amount of a reference diphtheria-pertussis-tetanus combined vaccine equivalent to one histamine-sensitizing unit. The histamine-sensitizing potency of this vaccine had earlier been estimated at 5HSU/ml. They estimated the LPF potency of other samples by comparing them against the standard vaccine using a 6-point assay method.

The method used in the quantitation of LPF potency in this study was similar in many respects to that used by Kurokawa et al., (1978). Using the cumulative percentage frequency of the leukocyte counts, these workers showed that the arithmetic leukocyte counts were not normally distributed but that logarithmically transformed leukocyte counts were normally distributed. However, in this project, both the arithmetic and logarithmic leukocyte counts were shown to be at least approximately normally distributed by the rankit method. Further analysis on the homogeneity of the variances obtained for arithmetic and logarithmic leukocyte counts revealed a high degree of heterogeneity in the variances of the arithmetic leukocyte counts whereas a satisfactory degree of homogeneity was obtained with logarithmically transformed leukocyte counts. This result was in accord with the findings of Sato and Arai (1972) and Kurokawa et al., (1978) who stated that the deviations from variance homoscedasticity were insignificant only after the logarithmic transformation of leukocyte counts.

Using the 6-point assay method attempts were made, in this study, to estimate the LPF potency of the C-mode

samples relative to their X-mode counterparts. These attempts did not succeed because of the very low potency of the C-mode preparations leading to non-parallelism of the dose-response curves. However, with the 6-point assay, there was parallelism between the LPF dose-responses of the various X-mode samples when they were compared to the LPF dose-response of a standard X-mode preparation. These X-mode samples had very high levels of LPF activity. Thus the 6-point assay appeared to be valid only when the LPF potency of preparations with high levels of activity were being estimated. Estimation of the potency of preparations containing low levels of LPF activity by the 6-point assay method were found to be invalid. Although Kurokawa et al., (1978) did not attempt to quantitate the LPF potency of preparations with very low levels of activity, they found the 6-point assay was valid when they estimated the LPF potency in preparations with apparently high levels of activity.

As a further attempt to quantitate the LPF potency of C-mode samples, the 4-point assay method was used. It was observed that only the 10h C-mode samples which still retained moderate levels of LPF activity, showed parallelism to the 10h X-mode sample.

There was no parallelism between the dose-responses of the older C-mode samples and those of their X-mode counterparts. Again the 4-point assay appeared not to be suitable for estimating the LPF potency of preparations with very low levels of activity. However, if in future

work, the LPF potency of the standard X-mode preparations were reduced, it might be possible to use the 4-point and 6-point assay methods to estimate the LPF potency of the older C-mode samples.

SECTION 2 : CULTURAL CHANGES DURING MODULATION

The growth rates of B. pertussis in different culture media have been examined by several workers. Rowatt (1957) stated that the mean generation time, measured turbidmetrically, for a number of virulent strains of B. pertussis grown in Cohen and Wheeler's medium was between 2.3-2.8h. She also reported that alteration of the concentration of some of the medium constituents led to an increase or decrease in the mean generation time. With 0.5% (w/v) glutamate the mean generation time was approximately 5h ; with 1% (w/v) glutamate the mean generation time was increased to 8h or more.

In stationary cultures of B. pertussis 18323 in Cohen and Wheeler's medium, the generation time was 18.5h but with shaking, the generation time was reduced to 5h (Csizer, Pusztai and Joó, 1970). Other workers (Csizer, Szammet, Niedermayer, Zsidai, Auber and Joó, 1977) further reported that the doubling time of their cultures in modified Cohen and Wheeler medium was 5h. However, their estimations were made from photometric measurements. By plate counts of viable cell numbers at various intervals during growth of B. pertussis strain 18334, in modified Hornibrook medium,

the generation time for X- and C-mode cultures obtained in this study was approximately 6-7h. Although there were differences in the culture medium used and the method of estimation of cell numbers, the result is comparable with those of the latter two groups of workers. It also falls within the range of values reported by Rowatt (1957) when different concentrations of glutamate were used.

The growth pattern observed in this study indicated that despite a slight lag which occurred when X-mode cells were inoculated into C-medium, the logarithmic phase of growth in both X- and C-media occurred between 10h and 48h after inoculation. There was no significant difference in this pattern of growth when either a 10% (v/v) or 5% (v/v) inoculum was used, though growth of cultures with 10% (v/v) inocula was sometimes faster.

Although both the X- and C-mode cultures were found to grow at about the same rate, the viable counts of the C-mode cultures were consistently slightly less than those of the X-mode culture at any time during growth. Although the difference was not very significant in this study, Lacey (1960) reported a similar observation, that the number of viable cells of B. pertussis was slightly but significantly less on the medium with magnesium sulphate.

Despite the slightly lower viable counts, it was observed in this study that the C-mode cultures consistently had a higher optical density than their corresponding X-mode cultures at all sampling times during growth.

When C-mode cultures were inoculated into X- and C-media, it was observed that the resulting X-culture had a lower optical density than the corresponding C-cultures. This suggested that the C-medium might be responsible for this increase in optical density. Davis, Dulbecco, Eisen, Ginsberg and Wood (1967) stated that growth of some bacteria in the presence of high levels of certain salts or sugars resulted in the shrinkage of the cells and an increase in their refractive index which lead ultimately to an increased light scattering. Thus it is possible that transfer of X-mode cells to C-medium led to shrinkage of the cells although it might be expected that this would occur instantly. Another possible explanation for the higher optical density of the C-mode cultures could be due to the fact that bacterial cell walls may undergo radical changes in response to relatively small changes in the growth environment (Ellwood and Tempest, 1972). During modulation changes are known to occur in the cell envelope proteins (Parton and Wardlaw, 1975). It is possible that these changes or other alterations in the cell wall composition could alter the light scattering properties of the cell.

The loss, during antigenic modulation of the protective antigen (PA) the histamine-sensitizing factor (HSF), the heat-labile agglutinogens, the heat-labile toxin (HLT) and adenylate cyclase activity has been well documented. In addition the present study showed that the leukocytosis-promoting factor (LPF) was also lost.

One of the primary aims of this investigation was to determine the time sequence of the loss of these activities. Thus, it was necessary to monitor the levels of activities in the X- and C-mode samples at various intervals during growth.

Comparing the potency values obtained in this study, for the various C-mode cells with their X-mode counterparts, it was observed that by 10h and 48h during modulation of B. pertussis strain 18334, the C-mode cells had only 2% and 0.5% respectively of the mouse protective activity present in the X-mode cells. These results, obtained in the present investigation, were not significantly different from the findings of Wardlaw et al., (1976) who also induced modulation by the use of magnesium sulphate and reported that after 48h, C-mode cells of strain 18334 had only 4% of the mouse-protective activity of their X-mode counterparts. These workers further examined modulation in four other strains of B. pertussis and reported that 48h C-mode cells had between 1.6%-16% of the mouse protective activity of the 48h X-mode cells.

Holt and Spasojevic (1968) also grew B. pertussis in the presence of high concentrations of magnesium sulphate and reported that the C-mode cultures they obtained had less mouse-protective activity than the X-mode cultures although data suitable for calculating relative potencies were not reported.

Wardlaw et al., (1976) also investigated the loss of the HSF activity in the C-mode cells of B. pertussis strain 18334. They showed that by 48h during modulation C-mode cells had only 3% of the HSF activity present in 48h X-mode cells. Livey, Parton and Wardlaw (1978) reported that growth of B. pertussis in the presence of magnesium sulphate led to a decrease in the amounts of HSF activity of the cells. From a number of experiments, they stated that their 65h C-mode cells had only 0.4%-2% of the level of activity present in 65h X-mode cells. In the present investigation, using 5% (v/v) or 10% (v/v) inocula, it was found that 48h C-mode cells had only about 2% of the HSF activity of their X-mode counterparts. Though the previous workers used a 1% (v/v) inoculum of the same age of culture and strain as used in this study, the results obtained from the three investigations did not differ significantly. The present study also revealed that the loss of HSF activity from the C-mode whole culture was slower than the rate of loss of the activity in the cells. The activity in 10h C-mode whole culture was 23% of the level of activity in the 10h X-mode culture whereas the C-mode cells, after 10h, contained about 11% of the activity in X-mode cells. This suggests that the HSF activity in the supernatant persists longer than the activity associated with the cells. The HSD₅₀ of 10h C-mode whole culture showed an initial increase from the HSD₅₀ of the original inoculum, thus suggesting that the HSF may be transiently synthesized by the C-mode cells in the initial stages of growth and released into the medium. However, there was evidence from other experiments to show that envelope

components which seem to be associated with the pathophysiological activities were not synthesized or not incorporated into the cells during modulation. The initial increase in the level of HSF activity in 10h C-mode whole culture could be explained by the fact that HSF might be synthesized and liberated into the extracellular fluid rather than be incorporated into the cellular substance. This liberation of HSF into the extracellular fluid explains the slower loss of the HSF activity from the whole culture than from the cells alone.

Although it was not possible to estimate the LPF potency of older C-mode cells, it was shown that at 10h during modulation, the C-mode cells had only about 20% of the LPF activity present in the 10h X-mode cells.

When the loss of the HLT activity was examined in this study, it was observed that by 10h and 48h during modulation C-mode whole cultures had only 16% and 1% respectively of the level of toxicity present in 10h and 48h X-mode whole cultures. Although the loss of the HLT activity in the C-mode cells was not examined in as much detail as for the whole cultures, results showed that at 48h during modulation, the C-mode cells had 22% of the level of toxicity contained in the X-mode cells of the same age. Thus, there appeared to be a faster rate of loss of toxicity in the whole cultures than in the cell suspensions. Since these results were obtained from two different experiments, and little is known about the distribution of toxicity between cells and culture supernatant at various stages during growth, it will be

necessary to repeat these experiments to ascertain the significance of this difference. Other workers who looked at the loss of HLT activity during modulation reported that after 65h, the average toxicity of C-mode lysates was 4.9% of that in X-mode lysates (Livey et al., 1978).

The exact location of the HLT in the cell is still not clear. Some reports claimed that it was located in the cytoplasm (Munoz, Ribi and Larson, 1959) while others reported that it was a cell envelope constituent (Yoshida et al., 1955 cited by Billaudelle et al., 1960). The majority of opinion presently appears to support the cytoplasmic location of the toxin. If this is true, then evidence from this study indicated that the process of modulation in B. pertussis produced changes in the cell which were not restricted to the cell envelope. This was earlier discussed by Livey et al., (1978).

The toxicity of the X- and C-mode cells as measured by the mouse weight-gain test was also examined in this study. Mice inoculated with X- or C-mode cells showed a similar initial weight loss. However, the subsequent weight gain was faster in mice inoculated with the older C-mode cells than in mice inoculated with the X-mode and 10h C-mode cells. These observations indicated the presence of a common toxic factor in both the X- and C-mode cells which was responsible for the similar initial weight loss. Kurokawa et al., (1978) reported that early appearing toxicity resulting in weight loss in mice inoculated with B. pertussis samples was attributable to endotoxin or HLT. However, since heated cell

suspensions were used in this investigation, the effect of HLT would have been eliminated. The indication therefore was that the endotoxin present in both X- and C-mode cells was responsible for the initial weight loss. Also some workers have reported the presence of a factor in B. pertussis which was responsible for slow weight-gain in mice inoculated with B. pertussis. Pittman and Cox (1965) reported that this slow weight-gain factor was the same as the LPF while Iwasa et al., (1966) referred to the factor as late-appearing toxicity (LAT). On further purification, these latter workers showed that the LAT had properties similar to LPF.

Thus it appears that the X- and C-mode cells had similar levels of lipopolysaccharide which were responsible for the initial weight loss when injected into mice. However, in addition to LPS, it appears that the X-mode cells and 10h C-mode cells had the factor responsible for late-appearing toxicity. This was absent in the older C-mode cells and resulted in a faster weight-gain in mice inoculated with these cells. The 10h C-mode cells still had about 20% of the LPF activity of 10h X-mode cells and this was presumably responsible for the slower weight-gain in mice inoculated with these samples.

Lacey (1960) followed modulation by monitoring changes in surface agglutinogens during growth of X-mode cells in C-medium. He determined the slide-agglutination titres of rabbit X- and C-antisera against C-mode cells of various ages. His results showed that the titre of specific X-antiserum against X-cells inoculated into C-medium decreased from 5120 initially to about 2560 after 12h of growth. The titre

further decreased to 1280 by 18h and dropped to below 5 by 24h. He also showed that the titre of unabsorbed C-mode antiserum against X-mode cells grown on C-medium stayed constant at 20 for 12h then increased slightly to about 60 by 18h then increased rapidly to 1280 by 24h and thereafter stayed constant. He did not give any data to show possible changes in the agglutination titres of X- or C-antisera against X-mode cells grown in X-medium. However, his results suggested that the loss of some surface agglutinogens associated with X-mode cells occurred within 24h of growth in C-medium.

It was shown in the present investigation that while the titres of specific and unabsorbed X-antisera against X-cells stayed relatively constant at 4096 throughout growth, the titres of these antisera against C-mode cells decreased from 2048 and 4096 to 64 and 512 respectively by 24h and thereafter remained relatively unchanged. Furthermore, the titres of the specific and unabsorbed C-antisera against C-mode cells increased from 4 and 64 to 256 and 4096 respectively by 24h and stayed relatively constant while the titres of these antisera against X-mode cells stayed relatively constant at 4 and 32 respectively throughout growth. The results provided some evidence that the surface X-specific agglutinogens were lost by 24h during modulation. This observation agrees with the earlier finding by Lacey (1960). However, one discrepancy was that Lacey showed that the titre of specific anti-X serum against C-mode cells dropped to less than 5 after 24h but in the present project the titre of the specific

anti-X serum against C-mode cells decreased only to 64 by 24h. This apparent difference in titres may be accounted for by the fact that the anti-X serum may not have been completely absorbed by the C-mode cells and thus there was still some C-mode specificity in the serum.

Investigation of the level of the HA activity in C-mode cultures during modulation revealed that activity was not detectable in any samples of C-mode cultures or cell suspensions obtained after 10h during modulation. On the other hand, appreciable levels of activity were detected in all X-mode cultures and cell suspensions. It is possible that if C-mode samples were obtained earlier than 10h during modulation, some activity would have been detected. However, very similar results were obtained by Lacey (1960) when he examined the HA activity of C-mode cells against sheep red blood cells. He was unable to detect any activity in C-mode cells obtained 2h after inoculation of X-mode cells onto C-medium. He therefore concluded that under pro-C-mode conditions, the HA of X-mode cells disappeared long before the X-agglutinogens were lost. Since this investigation showed that by 10h during modulation, C-mode cells still had 10%-20% of the other pathophysiological activities present in 10h X-mode cells and no HA activity, it appeared that the HA activity was more labile and was lost faster than the other pathophysiological activities during modulation in liquid medium.

Although initial experiments showed the loss of the various activities during modulation, there was no clear evidence to show whether loss of activities was due to denaturation or degradation in situ of the cell components responsible or if the loss from the cells was due to liberation of the components into the growth medium. It was known that these components were liberated into the surrounding medium during normal X-mode growth. However, later investigations revealed that the HSF, LPF and HA activities were also lost in C-mode whole cultures and culture supernatants. This loss followed a similar pattern to that observed for the cell suspensions. Thus it was concluded that the loss of activities from C-mode cells during modulation could not be due solely to the liberation of activities into the extracellular medium but was probably due to denaturation or degradation of the cell components in situ.

Wardlaw et al., (1976) correlated the loss of the 28k and 30k envelope polypeptide components with the loss of the PA and HSF activities in C-mode cells. The present study also showed that the 28k and 30k polypeptides were lost in parallel with the immunological and pathophysiological activities. To determine the precise relationship between the bands and activities and to determine the possible mechanism of modulation, investigations were carried out to ascertain whether the 28k or 30k polypeptides were transiently synthesized in C-mode cells during modulation or if synthesis of the polypeptides was switched off

immediately upon inoculation of X-mode cells into C-medium. X-mode cells were grown in X- and C-media with added ^{14}C glutamate and the incorporation of ^{14}C glutamate into the 28k and 30k polypeptides and other cell components, after 8h growth, was monitored. B. pertussis prefers glutamate to other amino-acids as a source of carbon and nitrogen (Rowatt, 1957a). Thus the labelled glutamate in the medium would be incorporated into any polypeptides synthesized in the new growth medium. When cell components were fractionated by SDS-PAGE, the ^{14}C uptake in the region of the various polypeptides would be in proportion to the amount of new protein synthesized. If there was no synthesis of a particular protein in the new medium then there would be no detectable labelled glutamate in that particular region of the gel. Although the results obtained were not conclusive, there was some evidence that polypeptide components with molecular weights of 28k, 30k and approximately 100k were not synthesized within 8h of inoculation of X-mode cells into C-medium. Furthermore, the presence of 28k and 30k polypeptides has been correlated with certain of the activities. The relationship of the 100k polypeptide bands, however, to any of the activities is not known. It has been reported that two high molecular weight (120k and 90k) polypeptides present in Phase I cell envelopes were absent in Phase IV cells (Muse, Findley, Allen and Collier, 1978) but no indication of their relationship to any of the activities was given. However recent fractionation studies (Sato, 1978) have shown that LPF has a molecular weight of about 110k, so it is possible that the 100k polypeptide may

be related to the component responsible for the LPF activity. In the uptake experiment, a protein component of the X-mode cells, with a molecular weight of approximately 25k showed little or no uptake of labelled glutamate after 8h growth. However it is possible that this was a late synthesized protein since it is known that different envelope proteins are synthesized at different stages of growth (Schnaitman, 1974).

Further investigations correlating the increase in viable cell numbers and the rate of loss of activities in C-mode cells suggested that the loss of activities was not due solely to dilution of cell components amongst the progeny cells. This further suggested that the immunological and pathophysiological factors in the original inoculum were presumably degraded or denatured during modulation. One interesting observation in this project was that some of the activities were not completely lost during one subculture. By 72h, after inoculation of X-mode cells into C-medium and when the culture had reached stationary phase, the C-mode cells still had about 2% of the level of HSF activity present in the X-mode cells. It would be necessary to carry out repeated subcultures in C-medium to see if activities were lost completely. However, it is also possible that these residual levels of activities were not due to the presence of the protein components designated as LPF or HSF but to the endotoxin content of the C-mode cells. Endotoxin has been reported to have some HSF activity (Malkiel and Hargis, 1964; Kurokawa and Homma, 1975) and LPF activity (Kurokawa et al., 1978).

Overall results in the present study showed that within 10h of inoculation of X-mode cells into C-medium, the resulting cells had only 2%-20% of the various activities present in their X-mode counterparts and could therefore be described as C-mode. The change from X-mode to this C-mode state appeared to be a rapid process requiring only 1-2 cell divisions. Lacey (1960) reported that the transition from X-mode to C-mode occurred between 21-36h during modulation and required 7-12 cell divisions. There could be several possible reasons for the discrepancy in the number of cell divisions required for modulation in these studies. Firstly, different criteria were used to assess the mode of the organism in the two studies. Lacey's work was concerned primarily with the loss of virulence and surface agglutinogens. In the present study the various biological activities were the focus of investigation. Secondly, different strains of B. pertussis were used. Thirdly, Lacey's work was done with solid medium whereas the present study was done using liquid medium. Some reports have shown that the levels of different activities in B. pertussis could be influenced greatly by the growth medium. For example, Munoz (1971) reported that cells grown on solid medium had more HSF activity than cells grown in liquid medium. Thus, the time loss of activities, i.e. the time required for X-mode cells to modulate, could depend on the type of medium used and the initial level of activities present in the cells.

There was some evidence in this study to suggest that during modulation, the various activities were lost at different rates. The PA and the HA activities appeared to be lost

faster than the HSF and LPF activities. At 10h during modulation, the best estimated potency values showed that the C-mode cells had only 2% of the mouse-protective activity and about 12% of the HSF activity of the X-mode cells. This showed a six-fold difference in the levels of these activities by 10h. Results from individual experiments showed a similar pattern of a faster loss of the mouse-protective activity than of the HSF activity. Wardlaw et al., (1976) also reported that the mouse-protective activity declined more sharply during antigenic modulation than does the HSF activity. Pusztai and Joo (1967) also noted the same effect with three B. pertussis strains grown in high nicotinic-acid medium. However, these workers stated that the changes produced by high nicotinic acid were not quite the same as in antigenic modulation. Dolby (1968) produced some evidence that PA and HSF do not necessarily occur in a fixed ratio of activities.

Because the rate of growth of the organism was slow, samples were not taken earlier than 10h. Furthermore, because of the low precision of the intracerebral mouse-protection test, it might not have been possible to detect accurately small differences in the levels of protective activity within the first few hours of modulation.

Similarly, the HSF assay method used in this study was based on the rate and number of deaths of mice as an estimation of the level of activity. However, a very recent paper (Ishida, Iwasa, Asakawa and Kurokawa, 1978) confirmed that this HSF assay method was too insensitive to detect relatively low HSF content with any accuracy. Instead they proposed an assay method which was based

on body temperature reduction in the mouse as a response. They showed that the body temperature of mice sensitized with pertussis vaccine began to decrease immediately after histamine challenge, reaching the lowest level in 20-40 min, and then began to recover slowly. They added that the temperature of the mice 30 min after histamine challenge was a measure of the HSF response and concluded that the lowest level of HSF content detectable by the temperature measurement was 20 to 30 times less than that detectable by the method used in this study.

If in future work the sensitivity of the assay methods used were improved, the time sequence of loss of each of the activities could be determined more precisely. In the present work, each mouse-protection test in which four samples were assayed involved the use of 180 mice. By increasing the number of mice used by two-fold or three-fold, more precise and reliable results might be obtained. Also the use of the HSF assay method based on mouse body temperature reduction could help detect small changes in the level of HSF activity.

Though the inability to detect HA activity in 10h C-mode samples suggested a faster rate of loss than the HSF activity, the precision and sensitivity of the assay method used is questionable. Though Lacey (1960) was unable to detect any HA activity in C-mode cells grown on solid medium, after 2h of modulation, it might be possible that if C-mode cells grown in liquid medium were assayed earlier than 10h during modulation, some HA activity could be detected. However, some workers, like Keogh and North (1948) have shown that the PA also has HA activity. Also Sato (1978) in his fractionation studies

obtained a fraction which possessed high HA and mouse-protective activities. The faster rate of loss of these two activities in this study tend to agree with the findings of these earlier workers and thus may be a further evidence for the relationship between the PA and the HA.

The most recent fractionation studies suggest that the HSF and the LPF are the same component of the cell (Lehrer et al., 1974; Morse and Morse, 1976). In this study, 10h C-mode cells contained only 20% of the LPF activity but 11% of the HSF activity. Thus a two-fold difference in the levels of the activities was observed. This tends to suggest that the activities were lost at different rates. However, a number of other factors like HA (Arai and Sato, 1976) and endotoxin (Ishida et al., 1978) have been reported to induce leukocytosis in mice. Endotoxin also has HS activity as already stated. Thus because different cell components may exhibit different combinations of the unusual pathophysiological properties of B. pertussis, it is clearly very difficult to draw definite conclusions about the relationship of the various components by monitoring the loss of the activities. It would therefore be necessary to devise a way to assay for LPF and HSF activities due solely to the protein components known as LPF and HSF respectively. For example, the contribution made by endotoxin to these activities could be determined by finding the activity of boiled vaccine since the HSF and LPF but not endotoxin would be destroyed. These specific assay methods are necessary to determine if the two-fold difference in the levels of HSF and LPF activities observed in this study was significant.

Arai and Sato (1976) and Irons and MacLennan (1978) reported the presence of two distinct haemagglutinins, LPF-HA and F-HA, in Phase I B. pertussis. Arai and Sato (1976) stated that the LPF-HA had very low haemagglutinating activity and high leukocytosis-promoting activity while the F-HA, which was associated with the fimbriae, had very high haemagglutinating activity and no leukocytosis promoting activity. Sato (1978) showed that the F-HA also had protective activity. In the present study, haemagglutinating activity was undetectable in C-mode cultures even when HSF and LPF activities could still be detected, i.e. the haemagglutinating activity of the component LPF-HA was undetected. It thus appeared that the low haemagglutinating activity which could be measured in X-mode cells was due to the F-HA component which may be identical with the PA (Sato, 1978) and the fimbriae (Arai and Sato, 1976; Morse and Morse, 1976).

There was also some evidence in this study to suggest that the HLT was lost at a slower rate than the HSF. By 48h during modulation, C-mode cells had 22% of the level of toxicity and 2% of the HSF activity present in X-mode cells. Cells harvested earlier than 48h were not assayed for their levels of toxicity. It is possible that a more reliable result would have been obtained by following the time course of the HLT loss in cells as for the HSF. Livey et al., (1978) showed that the HLT activity was lost at a slower rate than the HSF activity. In four different experiments, after 65h of modulation, C-mode cells had between 3.7%-9.3% of the level of toxicity and between 0.4% and 1.9% of the HSF activity present in X-mode cells of the same age. In the present study, the rate of loss of the HLT and HSF in C-mode whole cultures did not differ significantly.

However, the distribution of toxicity between cells and culture supernatant during growth was not established.

During the early stages of growth in X-medium it was observed, in this study, that the HSF, LPF and HA activities were associated with the cells but by the log phase of growth, the bulk of these activities were contained in the culture supernatant. This suggested that during growth, these components were synthesized by the cells and, at least a proportion of these components, were liberated into the culture medium. This observation was in accord with the reports of Munoz (1971) and Sato et al., (1974) who stated that in theyoungercells HSF was cell-associated but released into the supernatant fluid during the log phase of growth. Fisher (1950) and Masry (1952) also showed that the HA was released into the extracellular medium during growth. Similar results for LPF were reported by Morse and Bray (1969) and Sato et al., (1974) who showed that in Phase I strains, the factor responsible for the activity was associated initially with the cells but the bulk of it was released subsequently into the culture medium as growth progressed.

Despite the liberation of the active factors into the culture supernatant, it was observed in this study that cells obtained at various intervals during growth in X-medium had maintained a constant level of the various activities.

In the course of investigations into the distribution of activities between cells and culture supernatants, some evidence obtained in this study indicated that storage by freezing and thawing of samples prior to detoxification

by heating had some effects on the levels of the various activities present in the samples. When cell pellets, harvested at various intervals during growth from a fixed volume of culture and resuspended in saline to original volume, were treated as described above, the levels of HSF and LPF activities decreased progressively despite a steady increase in cell number during growth. However, in later experiments where all suspensions obtained in the same manner as above were heated immediately after harvest and preserved by the addition of thiomersal instead of freezing, it was found that the level of HSF activity in the cell suspensions increased in parallel with cell numbers. Therefore freezing and thawing prior to detoxification of samples by heating appeared to be responsible for the earlier observation in which levels of activities decreased in cell suspensions despite increase in cell numbers. It may be that freezing and thawing liberated autolytic enzymes which degraded the envelope components before they in turn were destroyed by the detoxification process. The levels of these enzymes could have increased as the culture aged giving a greater reduction of activities in older samples, despite the increase in cell numbers.

During growth of X-mode cells in X-medium, it was observed in this investigation that the levels of the HSF, HLT and LPF activities in whole culture samples increased as growth progressed, reached a peak at about the end of log phase and then started to decline during the stationary phase. This decline in the levels of activities

after synthesis had ceased might have been due to the liberation of degradative enzymes or to an increase in the pH of the medium, causing denaturation. Masry (1952) showed that the HA titres of B. pertussis grown in liquid medium increased after the 3rd or 4th day until a maximum was reached on the 8th day. This was then followed by a rapid decline in titre. He stated that the decline in titres was due to the medium becoming more alkaline. Sato et al., (1974) reported a similar decrease in HSF and LPF activities in Phase I strains after 5 days incubation in liquid medium but offered no explanation for this decrease.

On correlation of the levels of HSF activity of X-mode cultures with increase in viable cell numbers during growth, it was observed in the present project that the increase in the total levels of activity was greater than could be accounted for by a doubling effect resulting from an increase in viable cell numbers. However, Lane (1968) showed that at anytime during growth of B. pertussis in liquid medium, the percentage of viable cells compared to the total number of cells in the culture was less than 15%. Thus in this study, it was possible that the dead cells in the culture presumably still had some HSF activity. Also, during growth, HSF was released from the cells into the supernatant. These facts would explain the observed greater increase in HSF activity compared to the increase in viable cell numbers.

Because of the toxic side effects associated with the present B. pertussis vaccines, a number of fractionation studies have been carried out in order to separate the component of the cell actually responsible for protecting the child against B. pertussis infection from that causing toxicity. In recent years much progress has been made in the isolation of various components, like HSF (Lehrer et al., 1974) LPF (Morse and Morse, 1976) and HA (Arai and Sato, 1976). However, the isolation of the PA still remains elusive. At the beginning of this study, it was hoped that the process of modulation could be utilized in the separation of these components. The initial speculation was that cells with high levels of PA and low levels of HSF, LPF and HLT might be produced by this process. However, evidence obtained in this study indicated that the PA activity was lost at a faster rate than the other activities during X to C modulation. The difference in the rate of loss of the various activities may presumably be due to the fact that different proteins have different stabilities and therefore are denatured at different rates. Unfortunately, the PA appeared to be more labile than the HSF and other potentially toxic factors. If HSF had been more labile than the PA, production of a less toxic vaccine would have been a possibility. However because the rates of loss of the various activities were similar, it might not be possible to separate the various activities by modulation. Thus the present investigation revealed that the approach to the production of a safer vaccine by utilizing the process of

X to C modulation might not be feasible.

However, since Lacey (1960) reported that the C to X modulation does not retrace the path of X to C change, it might still be possible to explore the utilization of C to X-modulation for the production of cells with high PA activity and less toxic activities. In this work only a superficial examination of C to X modulation was made. It was observed that C-mode cells with very low levels of HSF, LPF and HLT activities, when grown in X-medium for 48h, regained these activities almost to the levels present in normal X-mode cells. However, no attempts were made to estimate the levels of the various activities at different intervals during this reverse process of modulation and thus there was no indication of the rate of gain of these activities. A more detailed study of C to X modulation is necessary for future work as it is possible that during the C to X change, the PA activity might be regained at a faster rate than the other activities. If this should be, then the utilization of the reverse process of modulation for the production of a safer vaccine might still be possible.

A number of other factors have been reported to induce modulation. These factors include growing X-mode B. pertussis cells at low temperature, in the presence of certain salts, fatty acids, tellurite or old blood (Lacey, 1960). In the present study only the effect of magnesium sulphate on modulation was investigated. Other workers (Holt and Spasojevic, 1968; Parton and Wardlaw, 1975; Wardlaw et al., 1976 and Livey et al., 1978) also studied the effects of salts on

modulation. Lacey (1960) also looked at the effect of low temperature on modulation. He found that B. pertussis cells at 25^oC grew in the C-mode no matter what salt was included in the medium and at 35^oC they grew like the wild type, with the whole population modulating according to the salt content of the medium. He gave no details of the pattern of changes in the levels of the various activities during modulation induced by low temperature or by other factors. It would be of interest to examine if the same sequence of events occurred in modulation induced by other environmental conditions as in modulation induced by magnesium sulphate. It is possible that the sequence of changes in modulation induced by different factors might vary.

SECTION 3 : POSSIBLE MECHANISM OF MODULATION

Although the sequence of changes in antigenic modulation induced by the different environmental conditions is not known, Lacey (1960) emphasized that there was no evidence to show that any of these factors acted by a different mechanism or gave rise to different types of C-mode cells. Wardlaw et al., (1976) showed that growth of X-mode cells in the presence of high nicotinic acid, high magnesium sulphate, sodium sulphate and magnesium succinate produced C-mode cells as shown by loss of HSF, PA and 28k and 30k polypeptides, though with varying degrees of loss of activities. The fact that a wide

variety of apparently unrelated factors induced modulation poses the question : Why does B. pertussis undergo modulation ? Although this question has not been investigated experimentally, it would seem likely that modulation is a process of adaptation to a new environment. Modulation appears to involve the switching off of the synthesis of components of the cell which are not essential under all conditions of growth. These components include what could be described as the virulence factors, for example, the HSF, HLT and LPF. C-mode cells appeared to grow quite normally and at a rate comparable with that of X-mode cells. Therefore, the C-mode cells are not apparently handicapped in any way, at least in vitro. Thus modulation could be a process of conserving energy under certain conditions. Lacey (1960) reported that C-mode antibodies have been found in several adults with persistent cough and infection, indicating that possibly modulation does occur in vivo. It is difficult to see how loss of the "virulence" factors" could help the organism in vivo but a drastic change in surface antigens, in response to some external stimulus, might help the organism to resist host defence mechanisms. This stimulus might be provided if the organism became dislodged from the upper respiratory tract and transferred to another site in the host. The different environmental conditions could induce modulation, and the organism would conserve resources and resist defence mechanisms until it could regain a suitable site in the host or in a different host and then revert to the original virulent form. However, at present the

significance of modulation in the life cycle of B. pertussis is still not known.

The process of antigenic modulation is not unique to B. pertussis. In a review of non-genetic variation of surface antigens of micro-organisms, Lacey (1961) reported that changes in growth conditions resulted in changes in structural components of other micro-organisms. For example, growth at 26°C led to lack of capsule formation by virulent strains of Pasteurella pestis; also growth of Bacillus subtilis in the presence of manganese increased the levels of certain envelope polypeptides of the organism. Some other bacteria and protozoa, for example, Borrelia recurrentis, Trypanosoma rhodesiense and Trypanosoma gambiense are also known to undergo different types of modulation in infected hosts.

Attempts were made in this study to determine the possible mechanism involved in modulation of B. pertussis. Since most of the properties lost during modulation were associated with cell envelope proteins, one possibility was that pro-C-mode conditions favoured higher proteolytic activity in the cells, which led to degradation of certain envelope proteins in the C-mode cells. However, no significant difference in the proteolytic activities of the X- and C-mode cultures, intact cells and X-preserved cells, was found. Thus loss of activities in modulation was not apparently as a result of enhanced proteolytic degradation of cell components.

As a further attempt to understand the mechanism of modulation, the role of sodium chloride and magnesium sulphate in modulation were examined. Wardlaw et al., (1976) investigated the effect of different concentrations of magnesium sulphate on modulation and showed that the change from X-mode to C-mode occurred when the magnesium sulphate concentration in the medium was raised from 4mM to 20mM. They showed that a medium containing less than 4mM of magnesium sulphate was pro-X-mode. Alphen and Lugtenberg (1971) reported the influence of osmolarity of the growth medium on the outer membrane proteins of E. coli. They stated that the addition of high concentrations of NaCl, KCl or sucrose to the growth medium caused a drastic change in the ratio of two outer membrane proteins of E. coli K12. In the present study the replacement of sodium chloride in the medium with magnesium sulphate would decrease the osmolarity of the medium. Thus it was initially thought that this change in osmolarity might be causing changes in the outer membrane proteins of B. pertussis cells similar to those reported for E. coli.

In order to investigate this possibility, X-mode cells were grown in medium without added sodium chloride or magnesium sulphate. Cells harvested after 48h showed between a two-fold to four-fold decrease (per ou. ml) in the level of HSF activity compared to the level of activity in normal X-mode cells. The growth of cells in C-medium resulted in a ten-fold decrease in the level of HSF activity

compared to the level of activity in X-mode cells. Thus though an increase in the level of magnesium sulphate in the medium induced modulation it does appear that it is not only the magnesium sulphate that has a role in the modulation process since lack of sodium chloride in the medium also resulted in a decrease in HSF activity. Moreover, the observed changes in HSF levels cannot be explained simply by the changes in osmolarity in the different media. Lacey (1960) reported that growth medium without added magnesium sulphate or sodium chloride was slightly pro-X-mode but that this residual pro-X-mode property of the medium varied with the batch of blood added to the medium. Thus the difference between his findings and the observations in the present study may be due to differences in the ionic composition of the basal media used in these studies.

Recently, Parton and Durham (1978) reported the loss of adenylate cyclase activity in C-mode cells. Since the role of cyclic AMP in the regulation of the metabolism of other organisms has been well established (Rickenberg, 1974), the role of cyclic AMP as a possible clue to the underlying regulatory mechanism in modulation was examined. Results revealed that during X to C modulation, the original cyclic AMP content of the culture derived from the inoculum was not detectable after 10h. However, it was observed that during C to X modulation cyclic AMP and the pathophysiological activities were regained.

Hewlett, Underhill, Vargo, Wolff and Manclark (1978) isolated a component from rabbit blood which when added to the growth medium increased the adenylate cyclase activity of the cells by several folds. This activator has since been found in other mammalian tissues and commercial bovine liver catalase is a very rich source. In the present study, attempts were made to determine the relationship between the levels of cyclic AMP in the culture and the levels of activities by using this activator to enhance cyclic AMP levels and possibly to enhance levels of the activities. Even in the presence of an activator, there was no cyclic AMP detected in C-mode cultures. Therefore presumably there was not even unactivated adenylate cyclase activity in the C-mode state, however, it is also possible that the activator could not gain entry into the C-mode cells. The presence of catalase enhanced the cyclic AMP levels in X-mode cells by about two-fold. With B. pertussis strain 18334, the increase in cyclic AMP due to catalase was not accompanied by increased HSF activity. However, with strain D30042I, cells grown in X-medium with added catalase showed a corresponding two-fold difference in the level of HSF activity but this may not be significant.

Different concentrations of cyclic AMP may be required for optimal expression of different operons (Lis and Schleif, 1973) and it may be that only a very low level of cyclic AMP is required to "switch on" the synthesis of HSF. Thus if the HSF was already "switched on" in the X-mode cells, more cyclic AMP would not have had any additional effect on the level of HSF activity.

Furthermore, the effect of catalase on the C to X modulation was investigated to see if synthesis of the HSF, LPF and HLT were switched on faster than would normally be observed. Cultures grown in the presence of catalase had increased levels of cyclic AMP after 8h and also showed a three-fold increase in HSF activity. After 24h the cultures grown in the presence of catalase had a two-fold increase in cyclic AMP and a corresponding two-fold increase in HSF activity compared to control cultures. This clearly suggested some relationship between the presence of cyclic AMP and the presence of the HSF activity. It also gave an indication that cyclic AMP might have some role in the control mechanism of modulation.

Another line of investigation in this study showed that envelope components, which had been correlated with the PA and HSF activities (Wardlaw et al., 1976), were apparently not synthesized in the C-mode cells. Thus, as stated earlier, it appeared that the process of modulation involved the switching off of the synthesis of some non-essential components of the organism immediately upon transfer of X-mode cells to C-medium. In other organisms like E. coli, it is known that cyclic AMP plays an important role in the regulation of certain non-essential function in the organism. Cyclic AMP is required to promote transcription of certain inducible enzymes, for example, in the "glucose effect" where cyclic AMP represses the synthesis of β -galactosidase and other catabolic enzymes. Also E. coli mutants lacking adenylate cyclase and cyclic AMP have been shown to be

unable to ferment various sugars, to have abnormal patterns of membrane fatty acids and to lack flagella. Thus the construction and function of the envelopes of these mutants were drastically altered (Dills and Dobrogos, 1977).

B. pertussis does not utilize carbohydrate but uses amino-acids as both carbon and nitrogen sources. So, cyclic AMP in B. pertussis is presumably doing something other than controlling the synthesis of carbohydrate-degrading enzymes. Since cyclic AMP and the other activities are lost in the C-mode cells, it is possible that in the X-mode cells the cyclic AMP may be involved in the regulation of the genes responsible for the synthesis of the cellular components responsible for the various activities.

One of the main aims of this study was to determine if the various activities were lost in parallel or independently during modulation. If found to be lost in parallel, this would have supported the idea that all the activities were different manifestations of a single complex macromolecule or that the activities were distributed amongst different components of the cell but that their expression was under a common control mechanism. On the other hand, if the activities were found to have been lost independently then it would have been strong evidence that different components of the cell were responsible for the different activities. However, it has become apparent that this initial speculation was too simplistic. The determination of the relationship of the various activities was more complex than was thought at the beginning of this investigation. For example, the LPF

and HSF activities in this study appeared to be lost at different rates despite the fact that a single protein component of the cell is now thought to have both activities. Such observation could only be explained by the fact that other components of the cells, with some activity in the tests used, were not lost or were lost at different rates. For example, there may be some synergistic effect whereby the LPS may effect a leukocytosis response without affecting the HSF activity. Thus results obtained from this study alone may not be sufficient evidence to agree or disagree with the unitarian hypothesis.

However, data obtained from this study showed that the loss of the different activities appeared to occur at different rates during modulation. The PA and the HA activities were lost at a faster rate than the HSF and LPF activities and the HLT activity was lost at a slower rate than the HSF activity. From the known properties of adenylate cyclase (Hewlett and Wolff, 1976), this enzyme is probably quite distinct from these other immunologically and pathophysiologically active components of the cell. Taking into account these observations and the evidence from recent fractionation studies by other workers, it could be speculated that at least four separate cellular components are lost during modulation. These are :

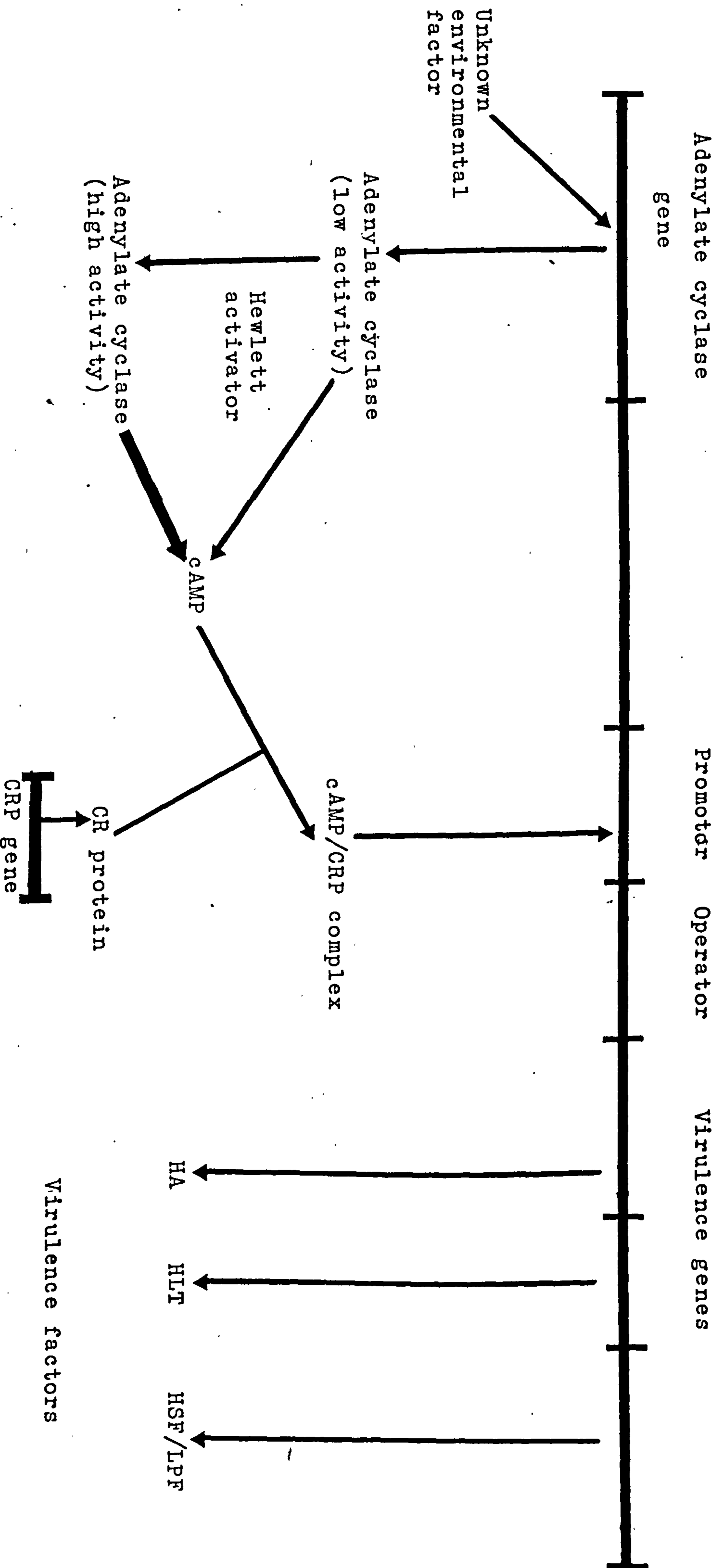
- (1) the PA/HA complex,
- (2) the HSF/LPF complex with a variety of other activities,
- (3) the HLT and
- (4) adenylate cyclase.

The similar pattern of loss of these activities suggests that their expression may depend

on a common control mechanism. It may be that cyclic AMP regulates the expression of the genes which are responsible for the synthesis of the cell components responsible for the various activities of B. pertussis. Different concentrations of cyclic AMP may be required for switching on/off of the different genes, therefore when the production of cyclic AMP in the cell is switched off/on, other activities are also switched off/on at slightly different times. The exact mechanism involved in this regulation is not known but it could be speculated as follows (Figure 35).

In the X-mode, there is an unknown environmental factor which stimulates transcription of the adenylate cyclase gene. Alternatively, conditions giving C-mode cells, actively inhibit transcription of the gene. In the presence of adenylate cyclase, ATP in the cell is converted to cyclic AMP and from there on, the cyclic AMP acts in a way very similar to that in E. coli as described by Pastan and Perlman (1972). Briefly, the cyclic AMP combines with a cyclic AMP receptor protein (CRP). This combination produces an allosteric change in the CRP. The cyclic AMP-CRP complex then binds to the DNA at the promotor region and produces a change in the DNA so that RNA polymerase can bind to the DNA. The RNA polymerase then initiates the transcription of the virulence genes, resulting in the production of the virulence proteins notably HA, HSF, LPF and HLT. It is possible that during modulation, some factor which activates the adenylate cyclase gene is either absent or rendered inactive under

Figure 35 : Possible role of cyclic AMP
in antigenic modulation



pro-C-mode conditions. Also it is possible that this factor is an inhibitor which inhibits the adenylate cyclase gene under pro-C-mode conditions. The factor would appear to be quite distinct from the activator described by Hewlett et al., (1978). For example, adenylate cyclase activity can be switched on or off by alteration of ionic composition of the medium although it is possible that the Hewlett activator works in conjunction with other environmental conditions. Before a firm conclusion can be made concerning the role of adenylate cyclase in antigenic modulation, it will be necessary to see if all conditions which induce antigenic modulation also cause loss of adenylate cyclase activity. It is of interest to note that high nicotinic acid levels which give a C-mode-like state also cause loss of adenylate cyclase activity. (W. McPheat, personal communication).

As a further study in antigenic modulation, it would be of great value to look at the process of modulation in a continuous culture system. In a batch culture, modulation apparently ceases when growth ceases and therefore the process may not be complete when the nutrients run out and waste products of metabolism accumulate. Furthermore, the presence of large numbers of dead cells and the accumulation of the various activities in the culture supernatant might be complicating the actual picture of modulation. With continuous culture, a number of variables could be manipulated at will. These include growth rate of the culture, the concentration of magnesium sulphate and other nutrients in

the culture and the cells could be maintained in the exponential growth phase for days or even weeks. The continuous culture system would provide a continuous supply of cells that were both uniform and in an optimal physiological state. By continual sampling, it would be possible, under this system, to monitor effectively the time taken to achieve a steady state i.e. true X-mode or true C-mode, or stop at intermediate stages when the cells could have an interesting and useful range of properties.

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APPENDICES

Appendix 1Preparation of media1% Casamino acids solution

10g casamino acids (Difco technical)

0.1g $MgCl_2 \cdot 6H_2O$

0.016g $CaCl_2$

5g NaCl

Dissolve in 1 litre of distilled water. Adjust the pH to 7.0 with 1N NaOH. Autoclave for 15 min.

Bordet-Gengou plates

200ml Bordet-Gengou agar base (Gibco Bio-Cult Diagnostics, Paisley, Scotland) melted and cooled at $45^{\circ}C$.

2ml glycerol (autoclaved)

40ml fresh citrated horse blood (Gibco Europe, Scotland) warmed to $37^{\circ}C$

Add glycerol to the agar and add the blood aseptically. Mix gently by rolling and pour into 90mm diameter, triple vent plastic petri dishes. Remove surface bubbles by light flaming. These plates were stored in the refrigerator and used within two weeks of preparation.

Appendix 1 (continued)Modified Hornibrook medium (Parton and Wardlaw 1975).

1.	Casamino acids (Difco technical)	100g
2.	Nicotinamide (B.D.H., Poole, England) 0.1% (w/v) solution	10ml
3.	CaCl ₂ (anhydrous)	0.02g
4.	NaCl	50g
5.	MgCl ₂ .6H ₂ O, 1% (w/v) solution	25ml
6.	KCl	2g
7.	K ₂ HPO ₄ , 10% (w/v) solution	25ml
8.	Soluble starch (B.D.H., Poole, England)	10g
9.	distilled water	800ml
10.	glutathione (Sigma Chemicals, St. Louis, U.S.A.) 0.1% (w/v) solution	10ml

Prepare solutions, 2, 5 and 7 fresh each time before use. Dissolve ingredients 1, 3, 4 and 6 in 800ml distilled water and add solutions 2, 5 and 7. Suspend starch in 25ml distilled water. Separately boil up 175ml of distilled water and add starch suspension to it. Mix immediately and add to the bulk medium. Make volume up to 1 litre with distilled water. Bring pH to 7.0 with approximately 0.45g of Na₂CO₃. Dispense in 100ml amount in screw cap bottles and autoclave at 121°C for 15 min. Store at 4°C. This is a 10 times concentrated medium.

Appendix 1 (continued)

When required for use dispense the 100ml concentrate into 900ml of distilled water in 2-litre dimple conical flasks. Autoclave at 121°C for 15 min. Immediately before use, filter sterilize 0.1% (w/v) solution of glutathione (Sigma Chemical, St. Louis, U.S.A.) and add 10ml aseptically to each dimple flask. This is Hornibrook X-medium. Hornibrook C-medium is prepared by replacing the 50g NaCl with 50g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, other ingredients being the same.

Stainer and Scholte medium (Stainer and Scholte, 1971).

1.	L-glutamate (monosodium salt) (B.D.H., Poole, England)	10.72g
2.	L-proline (Sigma Chemicals, St. Louis, U.S.A.)	0.24g
3.	NaCl	2.5g
4.	KH_2PO_4	0.5g
5.	KCl	0.2g
6.	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.1g
7.	CaCl_2	0.02g
8.	Tris (hydroxymethyl-methylamine) (Koch Light Laboratory, Colnbrook, Bucks., England)	6.075g
9.	L-cysteine (B.D.H., Poole, England)	0.04g
10.	$\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$	0.01g

Appendix 1 (continued)

- | | |
|---|--------|
| 11. ascorbic acid (B.D.H., Poole, England) | 0.02g |
| 12. nicotinic acid (B.D.H., Poole, England) | 0.004g |
| 13. glutathione (Sigma Chemical, St. Louis, U.S.A.) | 0.1g |

Dissolve ingredients 1-8 in 800ml distilled water. Adjust pH to 7.6 with 2.5N HCl. Make up volume to 990ml with distilled water. Autoclave at 15 p.s.i. for 15 min.

Immediately before use dissolve ingredients 9-13 in 10ml distilled water, filter sterilize with millipore filter of 0.45 μ pore size (Millipore S.A. Molsheim, France), and add aseptically to bulk medium. This is Stainer and Scholte X-medium. Stainer and Scholte C-medium is prepared by replacing the 2.5g NaCl with 5g MgSO₄·7H₂O, other ingredients being the same.

Appendix 2Preparation of miscellaneous reagents

Reagents for the Folin test for protein (Lowry modification by Herbert, Phipps and Strange, 1971).

Reagent A.

5% (w/v) Na_2CO_3 in distilled water

Reagent B.

1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water

2% (w/v) aqueous solution of sodium potassium tartrate.

Mix equal volumes of the above two solutions and allow the precipitate to settle before using the supernatant.

Reagent C.

To 50ml of reagent A add 2ml of reagent B.
Prepare immediately before use and discard after one day.

Reagent D.

Folin and Ciocalteu¹¹ phenol reagent (B.D.H.,
Poole, England)

Reagent E.

Protein standard - 1mg/ml Bovine serum
albumin (Sigma Chemical Co., St. Louis, U.S.A.).

Appendix 2 (continued)Triton-toluene scintillant

- | | | |
|----|--|-------|
| 1. | Triton X-100 (Sigma Chemical Co.,
St. Louis, U.S.A.) | 1L |
| 2. | Toluene (Koch Light Laboratories,
Colnbrook, Bucks., England) | 2L |
| 3. | PPO(Intertechnique, Uxbridge,
Middlesex, England) | 8g |
| 4. | Dimethyl-Popop (Intertechnique,
Uxbridge, Middlesex, England) | 200mg |

Mix reagents 1 and 2. Add reagents 3 and 4 and allow to dissolve. Leave for at least 10h in the dark before use.

0.1M phosphate buffer. pH 7.0

39ml of 0.2M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

61ml of 0.2M $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$

Mix and make up volume to 200ml with distilled water.

Appendix 3Slab and tube-gel electrophoresis.Stock solutions

1. Acrylamide solution

acrylamide (B.D.H., Poole, England) 30g

NN'-methylenebisacrylamide (B.D.H.,
Poole, England) 0.8g

Add distilled water to 100ml. Filter and store in
brown bottle at 4°C.

2. Tris/HCl buffer. pH 8.8

1M Tris (hydroxymethyl-methylamine) 50ml

1N HCl 8.1ml

Add distilled water to 100ml

3. Tris/HCl buffer. pH 6.8

1M Tris (hydroxymethyl-methylamine) 50ml

1N HCl 45ml

Add distilled water to 100ml

4. Tris/glycine buffer. pH 8.3 (10x concentrated)

glycine 144.13g

Tris 30.28g

Add distilled water to 1000ml

Appendix 3 (continued)

5. Ammonium persulphate
0.8% (w/v) in distilled water
6. Sodium dodecyl sulphate (SDS)
20% (w/v) in distilled water
7. Bromophenol blue (B.D.H. Ltd., Poole,
England).
0.1% (w/v) in distilled water
8. Solubilizing buffer

Tris/HCl (6.8)	2.0ml
SDS	3.2ml
β -mercaptoethanol	1.6ml
glycerol	3.2ml
Bromophenol blue	0.32ml
distilled water	5.68ml

Preparation of gel plates

The gel plates are prepared with glass plates (80mm x 80mm), separated with spacers and sealed at three sides with adhesive tapes. Seal corners by dipping in molten paraffin wax.

Appendix 3 (continued)Preparation of seperating gel solution

acrylamide solution	14.7ml
Tris/HCl (8.8)	15.0ml
20% SDS	0.2ml
N',N',N',N'tetramethyl-ethylenediamine (TEMED)	10 μ l
ammonium persulphate	4.0ml
distilled water	6.1ml

Mix thoroughly and pipette approximately 14ml into the gel plates standing vertically in a rack. Carefully overlay with 5% (v/v) ethanol without disturbing the surface of the gel. This removes the meniscus from the gel and leaves a perfectly flat surface. Allow to polymerise and prepare stacking gel solution.

Preparation of stacking gel solution

acrylamide solution	1.7ml
Tris/HCl (6.8)	1.25ml
20% SDS	0.05ml
TEMED	2.5 μ l
ammonium persulphate	1.0ml
distilled water	6.0ml

Mix thoroughly but without aeration. Pour off overlay solution from seperating gel. Replace the plates in the rack and fill to within 3-4mm of the top with stacking gel solution. Suspend the sample "combs" in the solution and

Appendix 3 (continued)

fill any remaining space with gel solution. Allow to polymerise then remove the "combs" carefully under running buffer (Tris/glycine buffer, pH 8.8, diluted 1 in 10).

Electrophoresis conditions

Dilute the Tris/glycine stock buffer 1 in 10 and add SDS to give a final concentration of 0.1% (w/v). Fill the lower electrode tank with approximately 2.5L of the diluted buffer.

Remove the tape from the base of the gel plates and insert into the upper electrode tank using a liberal amount of grease around the gaskets to prevent leakage. Fill the vessel with running buffer and then layer 25-50 μ l of samples into the sample wells.

Connect electrode unit to power supply and run at a constant current of 15mA/gel for approximately 2 $\frac{1}{2}$ h. When the tracking dye reaches the end of the gel, switch the power off. Carefully remove the gels from the plates and immerse in fix-staining solution for 90 min. Destain by soaking in several changes of destaining solution.

Fixing and staining solution

Coomassie brilliant blue R250	1.25g
50% (v/v) methanol	454ml
glacial acetic acid	46ml

Appendix 3 (continued)Destaining solution

methanol	50ml
glacial acetic acid	75ml
distilled water	875ml

Appendix 4
Statistical Analysis

Appendix 4a

Comparison of LPF responses in
male and female Ham/ICR mice

Log₁₀ leukocyte counts in individual animals after injection
with *B. pertussis* 18334 heated cell suspensions.

Sex	Dose (ou.ml)		
	0.5	2.0	8.0
Male	4.3541	4.3180	4.7520
	4.2175	4.0890	4.3838
	4.1523	4.3483	4.5185
	4.3674	4.3464	4.6821
	3.9764	4.1703	4.6553
	T = 21.0675(T _A)	21.272(T _B)	22.9615(T _C)
$\bar{X} = 4.2135$	4.2544	4.5923	
$\Sigma X^2 = 88.8730$	90.5554	105.5315	
S = 0.1612	0.1178	0.1425	
Female	4.0253	4.0880	4.5944
	3.9794	4.2528	4.2945
	3.8172	4.0943	4.5038
	4.1703	4.0985	4.5145
	4.1460	4.0492	4.5185
	T = 20.138 (T _D)	20.583(T _E)	22.4255(T _F)
$\bar{X} = 4.0276$	4.1165	4.4851	
$\Sigma X^2 = 81.1904$	84.7551	100.6330	
S = 0.1428	0.0800	0.1131	

T = sum of individual leukocyte counts
 \bar{X} = mean leukocyte count
 ΣX^2 = sum of the square of individual counts
S = standard deviation

Appendix 4a (continued)Interaction table

Sex	Dose (ou.ml)			Totals
	0.5	2.0	8.0	
Male	21.0675(T _A)	21.272(T _B)	22.9615(T _C)	65.301(A+B+C)
Female	20.138(T _D)	20.583(T _E)	22.4255(T _F)	63.1465(D+E+F)
Totals	41.2055(A+D)	41.855(B+E)	45.387(C+F)	

Analysis of variance

Source of variation	Degrees of freedom	Corrected sum of squares	variance or mean square	F-ratio	Probability level
Total	29	$\Sigma X^2 - CF$ = 1.5784	1	-	-
Groups	5	$\frac{\Sigma T^2}{5} - CF$ = 1.1740	2	-	-
Sex	1	$\frac{(A+B+C)^2 + (D+E+F)^2}{15} - CF$ = 0.1509	3	8.93	<<1%
Dose	2	$\frac{(A+D)^2 + (B+E)^2 + (C+F)^2}{10} - CF$ = 1.0114	4	29.923	1%
Interaction between sex and dose	2	2 - 3 - 4 = 0.0117	5	0.349	NS
Residual	24	1 - 2 = 0.4044	6	0.0169	-

CF = Correction factor = $(\Sigma X)^2 / N = 549.96$

N = total number of observations = 30

Appendix 4a (continued)

Tabulated F - value for degrees of freedom (2,24)

= 3.40 (P = 0.05)

= 5.61 (P = 0.01)

<< 1% = there is a highly significant
difference (at the 1% probability
level)

NS = No significant difference
(at 5% probability level).

Appendix 4bComparison of the effect of route
of inoculation on LPF responseLog leukocyte counts in individual animals after injection
with *B. pertussis* 18334 heated whole culture

Route	Dose (ml)			
	0	0.025	0.05	0.1
Intravenous	4.2307	4.3178	4.2249	4.3717
	4.1298	4.2693	4.1899	4.5001
	3.9409	3.9911	4.2714	4.4974
	4.1298	4.2144	4.2300	4.3547
	4.0713	4.2197	4.3198	4.4974
	T = 20.5025	21.0122	21.2360	22.2213
	\bar{X} = 4.1005	4.2024	4.2472	4.4443
	ΣX^2 = 84.1150	88.3654	90.2035	98.7793
	S = 0.1061	0.1254	0.0498	0.0742
	Intraperitoneal	4.1330	4.1200	4.1101
4.0447		4.0287	4.0368	4.2235
3.8509		3.9630	4.0786	4.0910
4.0563		4.1134	4.3072	4.2183
4.0079		4.1756	4.2118	4.2022
T = 20.0928		20.4007	20.7445	20.9364
\bar{X} = 4.0186		4.0801	4.1489	4.1873
ΣX^2 = 80.7876		83.2659	86.1149	87.6787
S = 0.1042		0.0839	0.1096	0.0548

Student t-test

Calculated mean value for control groups = 4.06

Calculated standard deviation = 0.1100

For the mean (\bar{X}) of a test group to differ from control, it should lie outside $4.06 \pm 0.11 = 3.95$ and 4.17 \bar{X} values were inspected for each group and scored as

S = significantly or NS = not significantly different

Appendix 4b (continued)

Route Significance of differences in
 \bar{X} values of test groups and control
 at doses tested

	0.025(ml)	0.05(ml)	0.1(ml)
Intravenous	S	S	S
Intraperitoneal	NS	NS	NS

Appendix 4cComparison of the effect of strain and
age of mouse on LPF response

Log₁₀ leukocyte counts in individual animals after injection
with B. pertussis 18334 heated cell suspensions

Strain	Age of mice	
	5 week old	8 week old
Ham/ICR	4.5634	4.7621
	4.8201	4.4986
	4.7250	4.6630
	4.8597	4.7899
	4.8260	4.6611
	T = 23.794(T _A)	23.3345(T _B)
\bar{X} = 4.7588	4.6669	
ΣX^2 = 113.29	108.96	
S = 0.12	0.11	
NIH	4.72	4.67
	4.76	4.58
	4.66	4.63
	4.75	4.77
	4.87	4.67
	T = 23.76(T _C)	23.32(T _D)
\bar{X} = 4.7520	4.6640	
ΣX^2 = 112.93	108.78	
S = 0.08	0.06	

Interaction table

Strain	Age of mice		Totals
	5 week old	8 week old	
Ham/ICR	23.794(T _A)	23.3345(T _B)	47.1285(A+B)
NIH	23.76(T _C)	23.32(T _D)	47.08(C+D)
Totals	47.554(A+C)	46.6545(B+D)	

Appendix 4c (continued)Analysis of variance

Source of variation	Degrees of freedom	Corrected sum of squares	Variance or mean square	F-ratio	Probability level
Total	19	$\Sigma X^2 - CF$ = 0.02	1	-	-
Groups	3	$\frac{\Sigma T^2}{5} - CF$ = 0.0426	2	-	-
Strain	1	$\frac{(A+B)^2 + (C+D)^2}{10} - CF$ = 0	3	0	NS
Age	1	$\frac{(A+C)^2 + (B+D)^2}{10} - CF$ = 0.0425	4	4.336	NS
Interaction between Age and Strain	1	$2 \cdot 3 - 4$ = 0.0001	5	0.0001	0.01
Residual	16	$1 - 2 = 0.1574$	6	0.0098	-

CF = correction factor = $(\Sigma X)^2 / N = 443.76$

N = total number of observations = 20

Tabulated F-values for degrees of freedom (1, 19)

= 4.38 (P = 0.05)

8.18 (P = 0.01)

NS = no significant difference (at 5% probability level).

Appendix 4dStatistical distribution of
leukocyte countBartlett test for homogeneity of variances -
arithmetic leukocyte count

Number of groups of counts = a = 3

Number of degrees of freedom within each
group = f = 19

$$\text{Calculate } C = 1 + \frac{(a+1)}{3af} = \left(1 + \frac{3+1}{3 \times 3 \times 19}\right) = 1.02339$$

Group	Standard deviation(S)	variance (S ²)	Log ₁₀ of variance (Log ₁₀ S ²)
1	14661	214944921	8.3323
2	10093	101868649	8.0080
3	5500	30250000	7.4807

$$\begin{aligned} \text{Log}_{10} \frac{\sum S^2}{a} &= \text{Log}_{10} \frac{\sum S^2}{3} = \text{Log}_{10} \frac{347063570}{3} = \text{Log}_{10} 115687856.7 \\ &= 8.0633 \end{aligned}$$

$$a \text{Log}_{10} \frac{\sum S^2}{a} = 3 \times 8.0633 = 24.1899 = B$$

$$\begin{aligned} \text{Calculate } M/C &= (B-A) \frac{2.3026f}{c} \\ &= \frac{24.1899 - 23.821 \times 2.3026 \times 19}{1.02339} \\ &= 15.77 \end{aligned}$$

Appendix 4d (continued)

Degrees of freedom associated with $M/C = (a-1)$
 $= (3-1) = 2$

Tabulated chi-square values at 2 degrees of freedom
 $= 5.99 (P = 0.05)$
 $9.21 (P = 0.01)$

Calculated M/C value of 15.77 is greater than 9.21,
 thus at 1% probability level, the variances between
 groups are highly heterogeneous.

Bartlett's test for homogeneity of variances - \log_{10} leukocyte counts

Number of groups of counts = $a = 3$

Number of degrees of freedom within each group
 $= f = 19$

Calculate $C = 1 + \frac{(a+1)}{3af} = (1 + \frac{(3+1)}{3 \times 3 \times 19}) = 1.02339$

Group	Standard deviation (S)	Variance (S^2)	\log_{10} of variance ($\log_{10} S^2$)
1	0.114	0.012996	-1.8862
2	0.14	0.0196	-1.7077
3	0.135	0.018225	-1.7393
		$\Sigma S^2 = 0.050821$	$\Sigma \log_{10} S^2 = -5.3333 = A$

Appendix 4d (continued)

$$\begin{aligned} \text{Log}_{10} \Sigma S^2/a &= \text{Log}_{10} \frac{0.050821}{3} = \text{Log}_{10} 0.01694 \\ &= -1.7711 \end{aligned}$$

$$a \log_{10} \Sigma S^2/a = 3 \times -1.7711 = -5.3132 = B$$

$$\begin{aligned} \text{Calculate } M/C &= (B-A) \frac{2.3026f}{c} \\ &= \frac{(-5.3132 - 5.3333) \times 2.3026 \times 19}{1.02339} \\ &= 0.29 \end{aligned}$$

$$\begin{aligned} \text{Degrees of freedom associated with } M/C &= (a-1) \\ &= (3-1) = 2 \end{aligned}$$

$$\begin{aligned} \text{Tabulated chi-square values at 2 degrees of freedom} \\ &= 5.99 (P = 0.05) \\ &9.21 (P = 0.01) \end{aligned}$$

Calculated M/C value of 0.29 is less than 5.99, thus at 5% probability level, the variances between groups had a satisfactory degree of homogeneity.

Appendix 4eEstimation of LPF potency4-point assayTabulation of Log₁₀ individual counts in relation to dose

X-mode (10h cell suspension)		C-mode (10h cell suspension)	
Low dose	High dose	Low dose	High dose
4.4180	4.4378	4.2250	4.3666
4.2090	4.4941	4.1299	4.2057
4.4310	4.6305	4.1395	4.2891
4.4437	4.5416	4.0248	4.2846
4.2063	4.4378	3.9692	4.4041

Results of analysis of variance

Tabulated F - values at 1,24 degrees of freedom

= 4.26 (P = 0.05)

7.82 (P = 0.01)

Calculated F - values

Source of variation	Degrees of freedom	F-ratio values	Probability
Between preparations	1	26.3	<< 1%
Slope	1	19.3	<< 1%
Parallelism	1	0.28	NS(> 5%)
Residual	12	-	-

Though there was a highly significant difference between potency of preparations, there was no significant difference in parallelism. Thus, the application of the 4-point assay was valid.

Appendix 4fEstimation of LPF potency6-point assay

Tabulation of individual Log_{10} leukocyte count in relation to dose

X-mode (10h cell suspensions)			X-mode (48h cell suspensions)		
Low dose	Medium dose	High dose	Low dose	Medium dose	High dose
4.2118	4.4919	4.5870	4.0618	4.2595	5.3495
4.0080	4.2340	4.5084	3.9570	4.4310	4.5271
4.0677	4.5124	4.8472	4.0693	4.3669	4.7361
3.9742	4.2238	4.4630	4.0580	4.2940	4.7072
3.9615	4.3810	4.6216	4.0909	4.5795	4.5546

Results of analysis of variance

Tabulated F-values at 1, 2 degrees of freedom

= 4.26 (P = 0.05)

7.82 (P = 0.01)

Calculated F-values

Source of variation	F-ratio values	Probability
Between preparations	0.0051	NS (> 5%)
Slope	94.9025	<< 1%
Parallelism	0.0884	NS (> 5%)
Quadratics	1.5025	NS (> 5%)
Difference in quadratics	0.1051	NS (> 5%)

Appendix 4f (continued)

There was no significant difference between the potencies and parallelism of both preparations thus the use of the 6-point assay was valid.

Tabulation of individual Log₁₀ leukocyte counts in relation to dose

<u>X-mode (10h cell suspensions)</u>			<u>C-mode (10h cell suspensions)</u>		
Low dose	Medium dose	High dose	Low dose	Medium dose	High dose
4.2118	4.4919	4.5870	3.9524	4.0447	3.9298
4.0080	4.2340	4.5084	4.1911	4.1698	4.0195
4.0677	4.5124	4.8472	4.0618	3.9132	4.0111
3.9742	4.2238	4.4630	3.7683	3.9745	4.0813
3.9615	4.3810	4.6216	4.1967	4.1871	3.9442

Results of analysis of variance

Tabulated F-values at 1, 24 degrees of freedom

$$= 4.26 \text{ (P = 0.05)}$$

$$7.82 \text{ (P = 0.01)}$$

Calculated F-values

Source of variation	F-ratio values	Probability
Between preparations	42.6106	<< 1%
Slope	20.3017	<< 1%
Parallelism	26.4213	<< 1%
Quadratics	0.7254	> 5% (NS)
Difference in quadratics	0.0000	> 5% (NS)

Appendix 4f (continued)

There was a highly significant difference between the potencies and parallelism of the two preparations. The application of the 6-point assay was therefore invalid.

Appendix 4gMETHOD FOR COMBINING THE RESULTS OF
SEVERAL INDEPENDENT ASSAYS

To illustrate the procedure used to combine the results of two or more HSF or MPT assays take the following two sets of results. The blocked-in figures are the values taken for combining.

RUN 1

	DOSE	LOG DOSE	TESTED	RESPONDING
STD1	1.000000	0.0	20	2.
	0.250000	-0.6021	20.	7.
	0.062500	-1.2041	19.	8.
932A.	1.000000	0.0	19.	0.
	0.250000	-0.6021	20.	1.
	0.062500	-1.2041	20.	5.

	REL POT	95 PC LIMITS	ED50	SLOPE	LOG R	WLOGR	LOGED50	WLED50
STD1			0.07	-0.84			-1.185	2.7
932A	6.338	1.761111.544	0.01	-1.55	0.802	1.2	-1.987	0.7

RUN 2

	DOSE	LOG DOSE	TESTED	RESPONDING
STD2	1.000000	0.0	19.	4.
	0.250000	-0.6021	20.	12.
	0.062500	-1.2041	20.	20.
932D	1.000000	0.0	19.	1.
	0.250000	-0.6021	19.	6.
	0.062500	-1.2041	20.	13.

	REL POT	95 PC LIMITS	ED50	SLOPE	LOG R	WLOGR	LOGED50	WLED50
STD2			0.41	-2.29			-0.390	22.8
932D	3.349	1.761 7.218	0.12	-1.58	0.525	10.7	-0.915	21.4

Appendix 4g (continued)Estimation of \bar{R}

The combined estimate of Relative Potency (\bar{R}) is given by :

$$\overline{\log R} = \frac{\Sigma(W \log R \cdot \log R)}{\Sigma W \log R}$$

$$\Sigma W \log R$$

$$= \frac{(1.2)(0.802) + (10.7)(0.525)}{1.2 + 10.7}$$

$$1.2 + 10.7$$

$$= 0.553$$

$$\therefore \bar{R} = 3.57$$

Note how the effect of the much higher weight of Run 2 causes \bar{R} to be much closer to the $R = 3.35$ of Run 2 than to the $R = 6.34$ of Run 1.

Estimation of 95% Confidence Limits

Note that the computer print out for "WLOGR" incorporates the Student term 't'.

$$\text{i.e. "WLOGR"} = 1/(ts)^2$$

Thus there is no t factor in the expression for the limits because it is already present in the weight.

Appendix 4g (continued)

$$\begin{aligned}
 \text{Limits} &= \text{Antilog } \log R \pm \frac{1}{\sqrt{\Sigma W \log R}} \\
 &= \text{Antilog } 0.553 \pm \frac{1}{\sqrt{1.2 + 10.7}} \\
 &= \text{Antilog } 0.553 \pm 0.2904 \\
 &= \text{Antilog } 0.262 \text{ and } 0.843 \\
 &= 1.83 \text{ and } 7.0
 \end{aligned}$$

Appendix 5Mouse protection test data

Sample	Dose per mouse (ou.ml)	Number of survivors/number challenged in four similar experiments			
		1	2	3	4
X-mode cells (10h)	2.5	9/10	—	—	—
	0.5	9/9	8/10	8/10	7/10
	0.1	7/10	4/10	5/10	4/10
	0.02	—	3/10	3/10	3/10
X-mode cells (48h)	2.5	10/10			
	0.5	9/10	9/10	9/10	8/10
	0.1	8/10	6/10	6/10	5/10
	0.02		3/10	2/10	2/10
C-mode cells (10h)	12.5	8/10	6/10	7/10	6/10
	2.5	5/10	3/10	3/10	3/10
	0.5	4/10	2/10	2/10	1/10
C-mode cells (48h)	25.0	6/10	5/7	6/8	5/10
	5.0	3/10	4/10	4/10	3/10
	1.0	2/10	1/10	1/10	0/10
Control					
Dilution 4	10 ⁵ org/ml	2/10	0/10	1/10	0/10
Dilution 5	10 ⁴ org/ml	3/10	1/10	2/10	2/10
Dilution 6	2000org/ml	5/10	3/10	5/10	4/10
Dilution 7	400org/ml	7/10	7/10	7/10	7/10
No. of LD ₅₀ (Dilution 4)	0.03ml	50	110	50	70
Viable count per ml (Dilution 4)		8.5x10 ⁹	4.1x10 ⁹	3.2x10 ⁹	3.5x10 ⁹

Appendix 6Mouse weight-gain test data

Sample	Dose (ou.ml)	Weight change (in grams) per mouse			
		Day 1	Day 2	Day 4	Day 7
X-mode cells (10h)	10	-1.67	-0.88	+1.44	+2.68
X-mode cells (24h)	10	-2.21	-1.04	+0.95	+2.39
X-mode cells (48h)	10	-2.44	-1.31	+0.86	+2.88
X-mode cells (72h)	10	-2.0	-1.15	+0.84	+3.82
C-mode cells (10h)	10	-1.29	-0.43	+2.12	+3.56
C-mode cells (24h)	10	-1.08	-0.28	+2.83	+5.91
C-mode cells (48h)	10	-1.36	-0.41	+2.57	+6.56
C-mode cells (72h)	10	-1.14	-0.28	+2.63	+6.60
Saline	-	+1.8	+2.4	+4.25	+8.65
Uninoculated	-	+2.21	+3.7	+5.14	+9.07

Appendix 7Leukocyte counts obtained duringX→C and C→X modulation

Sample	Mean arithmetic count per mouse	Log ₁₀ count	Mean Log count (\pm Standard deviation)
X→X	36440	4.5616	4.5178 (\pm 0.06)
	27340	4.4368	
	35140	4.5458	
	33640	4.5268	
X→C	13655	4.1353	4.0712 (\pm 0.0563)
	10255	4.0109	
	12555	4.0988	
	10955	4.0396	
C→C	10855	4.0356	3.9858 (\pm 0.13)
	10355	4.0152	
	6218	3.7937	
	12555	4.0988	
C→X	24855	4.3954	4.3463 (\pm 0.08)
	22955	4.3609	
	16855	4.2267	
	25255	4.4023	
Saline (Control)	7157	3.8547	3.6633 (\pm 0.15)
	4873	3.6878	
	3148	3.4980	
	4097	3.6125	