THE ENTEROVIRUSES

A study of their epidemiology and of some fundamental properties of the ECHO virus group.

A thesis submitted to the University of Glasgow for the degree of Doctor of Medicine

by

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An examination of some fundamental properties of the ECHO virus group

INTRODUCTION

INTRODUCTION

The enteroviruses form a group of recently discovered agents which all share the property of preferentially inhabiting the alimentary tract. To date they have been discovered in many mammals but the group which is associated with man and which contains the polioviruses, the Coxsackie viruses and the ECHO (Enteric Cytopathic Human Orphan) viruses has been subjected to the most detailed examination.

In the pages which follow I shall attempt to describe investigations I have performed on three aspects of the human enterovirus group, and for convenience of description I have laid out my thesis in three parts. The first two parts are clinically orientated in that each contains the results which were obtained from surveys of the distribution of enteroviruses in patients who were admitted to hospital.

In part one the results are described from a study of patients suffering from paralytic poliomyelitis and aseptic meningitis who were taken ill between May 1956, and March 1959, and admitted to hospital in West, Central and East Scotland.

A similar type of survey comprises part two, but in this the diseases which were studied were diarrhoea and acute respiratory illnesses. The patients who made up this survey

were all children five years of age or less and all were admitted to Ruchill Hospital, Glasgow, between April 1957, and March 1959.

The third part of this thesis is based on laboratory experiments in which some of the hitherto undescribed properties of certain ECHO viruses were examined in an attempt to achieve a clearer understanding of the nature of the reaction between this type of virus and a susceptible cell during the process of infection. In particular I wanted to ascertain the reasons for differences in plaque forming ability of the four ECHO viruses which were selected for study. I thought that the differences in plaque formation must reflect differences in the rates of reaction between virus and susceptible cell. In these experiments I examined therefore the rates of attachment of the viruses to cells under various conditions and by various methods; the nature of the growth cycle of each virus; the histological changes produced during the growth of each virus in susceptible cells and the reasons for the inability of one of the selected viruses to form plaques under agar.

The information which I gained from these experiments was then utilised to examine the phenomenon of interference between each of the selected viruses.

A number of tests was also performed on a group of ECHO viruses belonging to type 7, which had been isolated from

the patients in the surveys in parts one and two, in an attempt to demonstrate differences between these agents which would allow classification of at least some as "prime strains". Although I was unable to prove this contention the results, which are included in a separate chapter, are suggestive that ECHO 7 prime strains do, in fact, exist.

PART 1

THE EPIDEMIOLOGY OF POLIOMYELITIS AND ASEPTIC MENINGITIS INFECTIONS IN WEST, CENTRAL AND EAST SCOTLAND, 1956-1959.

CHAPTER I

REVIEW OF THE LITERATURE

Although acute paralysis in children was reported as early as 1795 in England, the condition was apparently regarded as ubiquitous and invariably attributable to teething, "foul bowels" or to an unspecified fever. The first suggestion that such paralysis might be due to "a contagion" can be found in the report by Bell (1836) who described a small outbreak of paralysis in children on the island of St. Helena. Another outbreak which affected four children who were living in a small community in England was described by Badham (1834). Colmer also reported a group of children, all of whom were under two years old, who developed acute paralysis in Louisiana, U.S.A., in 1843.

Recognition of the infectious nature of the condition developed very slowly indeed. Thus although further outbreaks of paralysis were described in Northern Europe by Barlow (1878) and Cordier (1888), in Scandinavia by Medin (1890) and in the U.S.A. by Koplik (1909) it was not until 1908 that Wickman, in a detailed study of an epidemic which occurred in Sweden in 1905, laid the foundations of all modern thought about the disease by emphasising its infectious nature, the importance of

carriers and mild cases and its spread by human contact.

In 1909 Landsteiner and Popper produced flaccid paralysis in a monkey by the injection of spinal-cord material from a fatal human case of poliomyelitis and were thus able to demonstrate the infectious nature of the condition. With the finding of a suitable animal host it required but a short time before the infecting agent was shown to be viral in nature.

The changing pattern of poliomyelitis infection.

The epidemics which were described during the latter part of last century in Europe were relatively small and invariably affected the youngest age groups in the population. The pattern of infection in civilised countries seems to have changed gradually during the first half of this century in that the epidemics increased in size and perhaps in frequency. During the 1930's an older age group was involved and the disease ceased to be truly "infantile paralysis". Whereas 50 years ago all cases occurred in children under 5, by 1952 the maximum attack rate in Sweden was reported by Olin to be in the age group 15-25.

The epidemic nature of outbreaks was described much later in tropical areas than elsewhere and usually it was thought that a new strain of virus had been introduced into a

tropical community. In numerous instances the native disease, which we now know must have been endemic, came to notice in the form of an epidemic only at a time when numerous immigrants arrived in a tropical or subtropical area. Most frequently the epidemic was associated with the arrival of troops and a new virus may have been introduced in this way. Outbreaks, associated with the arrival of troops, in which epidemic proportions were reached have been described by Hillman (1936) and Sabin (1947) in the Phillipines, by McAlpine (1945) in India, by Paul, Havens and van Rooyen (1944) in Cairo and by Paul (1949) in Korea. A similar incident was observed personally in Kenya at the time of the influx of tropps occasioned by the Mau Mau uprising in 1954, although in this instance the highest incidence rate was among troops who were presumably encountering a local strain of virus for the first time.

Importance of asymptomatic carriers

A great advance in the understanding of poliomyelitis developed from the realisation that a paralytic attack was a rather rare event and that for every one person so paralysed hundreds might be infected as symptomless carriers. Various attempts have been made to ascertain the precise ratio of paralytic attacks to symptomless infections since Wickman's (1908) original report. Data produced by Paul, Salinger & Trask (1933)

suggested that in the age groups 1 to 4 and 5 to 14 the ratios of paralytic attacks to minor illnesses (which were taken to be abortive poliomyelitis) were 5.2 and 7.6 respectively. This problem had to wait for the introduction of tissue culture techniques before it became capable of accurate solution. From estimations of the amount of poliovirus in sewage in New York City, Melnick (1947) concluded that the ratio of paralytic to inapparent poliomyelitis infections was about 1:1000. Perhaps the most sensitive index of this ratio however was obtained from a study of antibody distribution in a community which was actually involved in a large outbreak of poliomyelitis. Melnick and Ledinko (1951) were able to demonstrate that the ratio of paralytic cases to inapparent alimentary infections during a large outbreak in North Carolina varied from 1:175 for children less than one year old to approximately 1:75 between 1 and 15 years.

Spread of poliovirus within the body

The realisation of the extent to which a carrier state could exist made necessary a number of studies which were designed to elucidate the spread of poliovirus within the body and also the reasons for the existence of the symptomless carrier of a potentially lethal virus. In the spread of poliovirus through the community there is no doubt that the main link in the transmission chain is man himself. There can

also be little doubt that two possible pathways are available for the spread of virus from an infected person to another individual.

Firstly, pharyngeal secretions are frequently contaminated in the early stages of the attack. Lepine (1955) found poliovirus in the pharyngeal secretions of 95% of cases either on the day before or the day of onset and Howe & Bodian (1947) isolated the virus during the first week of infection from the throat of one of three paralytic cases. They similarly isolated virus from one of six juvenile family contacts and two of 28 school contacts of the paralysed children. Horstmann, Melnick & Wenner (1946) and Bodian (1956) showed that the virus disappears rapidly from the pharyngeal area, but its early presence there helps to explain the large number of persons, both within and without the family, who subsequently become excretors of virus in the course of a few days after contact with a case. Virus can be spread from the nasopharyngeal area in exhaled moisture droplets or it can be swallowed with subsequent internal spread through the alimentary tract.

The swallowed virus multiplies within the alimentary tract and very large amounts are excreted in the stool over a considerable period. Doubt exists as to the actual site of multiplication in the bowel. In his studies with chimpanzees Bodian (1956) found multiplication in Peyer's patches and

mesenteric lymph nodes and postulated by analogy that alimentary tract lymphoid tissue might be the site of multiplication in man. Sabin (1956) on the other hand favoured the view that virus multiplied in the epithelial cells lining the bowel rather than actually within the Peyer's patches.

With regard to the actual amount of poliovirus excreted in the stool Wood (1956) estimated from a study of a series of cases of paralytic poliomyelitis in Southern England that approximately 10^4 infectious particles per gramme of stool per day were excreted during the seven days following the onset of paralysis. In following the duration of excretion of virus from paralytic cases Brown (1955) observed titres of virus ranging from 10^1 to 10^7, with an average titre of $10^{2.8}$, whereas a group of cases who showed no evidence of paralysis had an average titre of $10^{2.7}$.

The duration of excretion varies from case to case. Horstmann, Ward and Melnick (1946) found virus in the stools of 70% of 61 patients during the first two weeks, in 50% during the second fortnight, 27% in the third and 13% at the end of the fourth fortnight. In one case they were able to demonstrate virus at the twelfth week after onset. Between the twelfth and the twentyfourth weeks all specimens were negative. They observed no differences in the duration of excretion between paralytic and non-paralytic patients. Schabel, Smith, Fishbein & Casey

(1950) found 67% of their paralysed patients still excreting virus after 2 weeks and Lepine (1939) found poliovirus in the stool of one patient, by monkey inoculation, 16 weeks after onset.

Spread within the body from the primary site of multiplication, be this Peyer's patch or intestinal epithelium, can occur at any time. The classical theory of central spread along nerves was rendered untenable, at least as the major method of spread by Sabin (1944), Horstmann, Melnick, Ward, and Sa Fleitas (1947) and by Bodian (1954 a & b), by the demonstration of a viraemic phase during the early stages of poliomyelitis infection. All modern thought about the pathogenesis of paralysis is based on the presence of a phase of viraemia as are most theories of the prevention of paralysis by artificial stimulation of antibody by poliovaccines.

Possibilities for spread by extra-human agencies

Within a short time of the discovery of the viral aetiology of poliomyelitis a number of experiments had been made by Flexner & Lewis (1910) in which virus-containing material from experimentally infected monkeys was introduced into the brains of calves, goats, pigs, sheep, dogs, cats, fowls, and the horse in an unsuccessful effort to infect these animals. Flexner & Clarke (1911) were however able to

demonstrate the ability of flies to harbour the virus for at least 48 hours.

Subsequent investigations have been largely repetitions of this original work with the added refinements made available by successive technical developments.

The possibility of extra-human transmission was strengthened by the discovery of Kling, Levaditi and Lépine (1929) that poliovirus could remain infective in water for 114 days and again by Rhodes, Clark, Knowles, Shimeda (1950) who isolated infective virus from sewage after 188 days. Toomey, Takacs and Tischer (1943) made a number of unsuccessful attempts to recover poliovirus from fruit, well water, dogs and chickens during an epidemic. Spinal cords from chickens dying of paralysis were inoculated into Eastern cotton rats. The cord suspension killed the rats and the lethal factor was transmissable for 3 passages but poliovirus could not be demonstrated. Droppings from paralysed birds were not tested for the presence of virus. In this connection Frauchiger & Bourgeouis (1938) made a study of the histological lesions associated with paralysis in fowls and concluded that the paralysis of fowls was an entirely different entity from poliomyelitis.

More recently Gear (1952) investigated the association between simultaneously occurring epidemics of fowl paralysis

and human poliomyelitis in South Africa but was unable to demonstrate a link. Sommerville, Monro & Cuthbert (1958) found that a boy who had been bitten on the lip by a paralysed budgerigar was infected with the same strain of poliovirus (type 1) as the bird. This was the first authenticated account of the isolation of poliovirus from a bird but neither Sommerville (1959) nor Dane, Dick & Donaldson (1959) could demonstrate infection experimentally following inoculation of budgerigars by various routes. In a survey of bird sera collected from various sources Dekking (1958) was unable to demonstrate any poliovirus antibody.

The possible spread of virus by flies has also been the subject of numerous enquiries; perhaps the implication has been strengthened by the curiously high seasonal prevalence of poliomyelitis during summer and autumn. Sabin and Ward (1942), Trask and Paul (1943) and Trask, Paul and Melnick (1943) were able to demonstrate the presence of poliovirus in flies caught either in or outside poliomyelitis epidemic areas. In most instances the flies had had an opportunity of feeding on infected faeces. The fly most commonly incriminated was the "blowfly". Melnick and Penner (1947) showed that poliovirus could survive for prolonged periods in experimentally infected "Blow flies". The wholesale destruction of flies by Melnick, Ward, Lindsay and Lyman (1947) and by Paffenbarger and Watt

(1953) failed to affect the course of epidemics of poliomyelitis, although in numerous instances both sets of investigators were able to demonstrate the contamination of food by flies.

The influence of tissue culture techniques on the differentiation of paralytic poliomyelitis from the non-paralytic syndrome (aseptic meningitis)

Shortly after the publication of the original report by Enders, Weller and Robbins (1949) that poliovirus could be grown in explant cultures of human embryo skin, muscle, gut or nervous tissue a series of other observations on the use of tissue culture techniques for the propagation of poliovirus was published. Thus Weller, Robbins and Enders (1949) described the use of explant cultures of human foreskin epithelium obtained postnatally, while Scherer, Butovac and Syverton (1951) described the growth of poliovirus in explant cultures of monkey testicular tissue. Two major advances in tissue culture techniques were the discovery of Scherer, Syverton and Gey (1953) that the continuous line of tissue culture cells derived from a cervical carcinoma and named strain HeLa would support the growth of poliovirus and Youngner's (1954) observation that monolayer tissue cultures prepared from trypsin-dispersed monkey kidney epithelial cells would also grow the virus.

When these two latter tissue culture techniques were

applied on a large scale to the isolation of viruses from patients with paralytic and non-paralytic poliomyelitis in a number of different laboratories it was found that, allowing for individual variations, poliovirus could be isolated from paralytic patients with a frequency of over 90% when properly collected specimens gathered in the early stages of the attack were used. It could be isolated with less regularity from patients diagnosed as suffering from non-paralytic poliomyelitis or aseptic meningitis. During sharp outbreaks or epidemics of poliomyelitis poliovirus was isolated with greater frequency from non-paralytic cases. This suggests that during such epidemics, poliovirus accounts for more aseptic meningitis cases than it does at times and in areas where poliomyelitis incidence rates are low or when there is a dichotomous relationship between the epidemic curves of paralytic poliomyelitis and the non-paralytic disease. An example of this is found in the paper by Godenne and Reordan (1954), who found poliovirus in the stools of 93% of paralytic cases admitted to hospital as compared with 33% of non-paralytic cases. In contrast 19% of non-paralytic cases harboured an orphan (ECHO) virus, whereas none were isolated from the paralytic group.

A slightly different picture was presented by Svedmyr, Melen and Kjéllen (1956) in describing experiences in Scandinavia in 1953 and 1954. In Stockholm, during the

summer of 1953, poliovirus type 1 was isolated from 91% of paralytic children below the age of 16 years and from 82% of children in the same age group with non-paralytic disease. In 1954 however the situation was quite different in that few paralytic cases occurred. One or other of all three types of poliovirus were isolated from only about 50% of these. An occasional poliovirus grew from a non-paralytic patient whereas ECHO type 6 virus was isolated from 25% of non-paralytic cases. No isolations of ECHO type 6 virus were reported from the paralytic group.

Almost all surveys of the distribution of viruses in paralytic and non-paralytic disease were performed in tissue cultures of monkey kidney epithelial cells or in human amnion, human embryo, trachea or lung because it was quickly realised that the HeLa cell was not only slightly less sensitive to the polioviruses but was unable to support the growth of ECHO viruses and had a restricted range of Coxsackie viruses (Evaluation of 1954 field trial of poliomyelitis vaccine, 1955).

The suggestion that viruses other than poliovirus could be associated with non-paralytic syndrome was strengthened by the description of other instances similar to the Stockholm experience in 1954. ECHO type 6 virus was recovered from 68% of the cases in an epidemic of aseptic meningitis in

Western New York in 1955 by Winkelstein, Karzon, Barron & Hayner (1957). In the same outbreak poliovirus was recovered from only 4% of cases. The same type of virus was grown from 100% of children admitted to hospital with aseptic meningitis in Buffalo, in 1955, by Karzon, Barron & Winkelstein (1956). Poliovirus was not encountered in this outbreak at all. Davis and Melnick (1956) isolated viruses from 50% of patients with non-paralytic poliomyelitis in Connecticut in 1955. Of the 69 agents isolated 41% were polioviruses, 23% Coxsackie viruses of a variety of types and 36% were ECHO viruses, predominantly ECHO type 6.

At about the same time aseptic meningitis was occurring with increased frequency in Europe. Here the illness was almost indistinguishable from the American one except for the presence of a transient morbilliform or scarlatiniform rash in a variable proportion of cases in each outbreak. The virus most commonly isolated was ECHO type 9. Epidemics caused by this virus occurred in England in 1956 and were reported by Tyrrell and Snell (1956), Boissard, Stokes, Macrae and MacCallum (1957), Garnett, Burlingham and van Zwanenberg (1957), McLean and Melnick (1957) in Holland and Belgium by Quersin-Thiry, Nihoul and Dekking (1957) and Nihoul, Quersin-Thiry and Weynants (1957) and in Scotland by Jamieson, Kerr and Sommerville (1958). Each outbreak was also distinguished

by the low frequency of poliovirus isolations. Details of the Scottish outbreak described by Jamieson, Kerr & Sommerville are contained in this thesis in the chapter on poliomyelitis and aseptic meningitis.

By 1957 the disease caused by ECHO type 9 virus had spread to the U.S.A. and to Scandinavia, with only a small number of cases occurring in Britain (Tyrrell 1959). The Scandinavian outbreak was described by Berglund, Bottiger, Johnsson & Westermark (1958) while a description of American experience was published by Sabin, Krumbiegel and Wigand (1958). Since 1957 no large scale epidemics of aseptic meningitis have been reported.

Recently, however, a number of investigators have shown that Coxsackie viruses may also be associated with the production of aseptic meningitis. This type usually differs in that Coxsackie viruses cause only sporadic cases - or at most comparatively minor outbreaks. Clinically the cases are indistinguishable from the ECHO virus produced disease.

A two year study of the syndrome in Connecticut by Davis and Melnick (1958) revealed the extent to which different members of the enterovirus group may be responsible for the same clinical syndrome. During this study period the age, sex, and seasonal distribution of the cases, as well as the clinical syndrome itself simulated that of non-paralytic

poliomyelitis. Polioviruses, Coxsackie virus types A9, B2, B3 and B4 and ECHO virus types 6 and 14 were frequently isolated. Invariably a specific antibody response occurred to the virus which was recovered. Vaccination against poliomyelitis decreased the incidence of poliomyelitis in vaccinated persons but had no effect upon the incidence of the other enteroviruses. Similar studies have been reported by Habel, Silverberg and Shelokov (1957), Kibrick, Melendez and Enders (1957) and by von Zeipel and Svedmyr (1957).

In every outbreak of aseptic meningitis or non-paralytic poliomyelitis caused by an ECHO or Coxsackie virus the disease has been mostly mild, self-limiting and of short duration. In the larger epidemics caused by ECHO viruses one virus has been isolated from the majority of cases and under these circumstances poliovirus has been conspicuous only by its absence. In these instances the term non-paralytic poliomyelitis does not adequately describe the disease and the alternative title aseptic meningitis appears to be a better one. The W.H.O. Expert Committee on Poliomyelitis recommends the adoption of the term aseptic meningitis in its special report (1958). The report stresses that the concept of non-paralytic poliomyelitis is entirely correct but that the term has been used indiscriminately in the past and should now probably be superseded by the description "aseptic meningitis syndrome" (due to ...; - the virus responsible).

Viruses other than poliovirus associated with acute paralysis.

Another aspect of the virology of paralytic illness which has come to light largely as the result of the intensive investigative efforts associated with the development and proof of the effectiveness of the poliomyelitis vaccines is the possibility that viruses other than poliovirus may be associated with acute paralytic illness. The arthropod-borne encephalitis viruses and rabies have been accepted for some time as possible causative agents of paralytic infection which can usually be differentiated clinically by the occurrence of obvious encephalitis preceding the onset of paralysis. Fortunately these agents are not encountered in Britain. More recently occasional cases of paralytic illness have been reported from which the only virus isolated was an enterovirus other than poliovirus. In most instances serological tests have not permitted the conclusion that concurrent infection with poliovirus or an arthropod-borne virus had not occurred.

An unidentified agent, subsequently shown by Habel and Loomis (1957) to be Coxsackie A7 virus, was isolated by Chumakov (1956) from a case of paralytic illness indistinguishable clinically from acute poliomyelitis. Chumakov named his unidentified agent Poliovirus type 4.

The prototype strain of Coxsackie B5 virus was isolated from two brothers aged 3 and 6 by Steigman (1957). One of the children developed a paralytic illness during a large-scale epidemic

of poliomyelitis caused by the type 1 virus. Both children developed rising antibody titres to Coxsackie B5 virus but not to poliovirus type 1. Sabin and Steigman (1949) reported acute poliomyelitis lesions in a paralysed monkey inoculated with human stool material. This condition was shown subsequently to be caused by Coxsackie B2 virus. A virus which was identified as ECHO type 2 was isolated by Steigman, Kokko and Silverberg (1953) from the C.N.S. of a fatal case of clinically and histologically typical bulbo-respiratory poliomyelitis. ECHO type 6 was associated with cases of paralytic illness by Kibrick, Melendez and Enders (1957) and by Karzon, Barron, Winkelstein and Cohen (1956), while ECHO type 9 was incriminated in some instances by Sabin, Krumbiegel and Wigand (1958). In a series of 6 cases reported by Hammon, Yohn, Ludwig, Pavia, Sather and McCloskey (1958) Coxsackie B3, B4, A9, ECHO 4 and ECHO 16 viruses were all isolated from patients with paralytic illness. Antibody rises were demonstrated in this series to each agent except ECHO 16 and Coxsackie A9, and in no instance was a significant increase in poliovirus antibody titre observed.

Effects of inactivated poliovirus vaccines on behaviour of poliovirus in the community.

While this review is not primarily concerned with the development or use of the inactivated poliovirus vaccines, their widespread use and the consequent decrease in the

incidence of paralytic attacks have produced a number of fresh problems directly related to the epidemiology of poliomyelitis.

Perhaps the most important question was whether the use of inactivated vaccine might not interfere with the natural process of immunity following natural alimentary infections or indeed might even interfere with the development of natural alimentary infections. If this interference occurred it would reveal itself as a gradual age increase in paralytic case incidence which would progress until the disease affected young adults predominantly. In an attempt to answer this question Sabin (1957) and Paul, Horstmann, Melnick, Niederman and Deutch (1957) compared the intestinal infection rate in non-immunized persons with that in persons immunized by the inactivated poliovirus vaccine. Intestinal infection occurred equally in both groups. A similar type of study was undertaken by Gelfand, Fox and Leblanc (1957) and by Davis, Lipson, Carver, Melnick and Robbins (1958) using as their index a comparison of the faecal excretion rates in immunized natural contacts of healthy carriers and of clinical cases of poliomyelitis. No significant difference in virus excretion rates were found. In each set of children neither the amount of poliovirus in the faeces nor the duration of excretion was affected. The evidence suggests that a natural alimentary infection within twelve months of vaccination with

inactivated vaccine results in a boost to immunity. Whether this condition applies also at a later stage after the vaccination-induced antibody titre has fallen or even disappeared remains to be discovered.

In general three principal effects have been noted from the increasingly widespread use of inactivated poliomyelitis vaccines. Firstly, there can be little doubt that the number of paralytic cases has been considerably reduced in vaccinated subjects. In the second place it would appear that the rate of natural infection and the incidence of the alimentary carrier states has not been altered in any way by vaccination. This desirable state of affairs means that the opportunity is continually present for an individual to become infected while protected from paralysis by artificially induced antibody, with a consequent increase in the antibody level. The third effect, which has followed the decrease in numbers of paralytic attacks, has been the greater amount of attention given to the problems of the aetiology of aseptic meningitis, the incidence, but not the severity of which has apparently increased in proportion to the number of cases of poliomyelitis.

It is of considerable importance, therefore, that epidemiological surveys should continue in populations of young persons so that the spread of the various types of poliovirus in the community can be followed in relation to the potential

number of unvaccinated individuals and also in relation to the age incidence of paralytic cases in different population groups. In addition it is important that assessment be made of the frequency of paralytic attacks due to viruses other than poliovirus. In aseptic meningitis of both epidemic and sporadic types greater attempts must be made to identify the aetiological agents so that the epidemiology and eventually the control of the condition can be envisaged.

In the following chapters and with these ends in view Scottish experience of poliomyelitis and of aseptic meningitis during the last three years is described from the standpoint of the laboratory worker.

CHAPTER II

THE PLAN OF THE INVESTIGATION AND THE POPULATION WHICH WAS STUDIED.

In 1956 the Medical Research Council organised a "field trial" of the inactivated poliomyelitis vaccine in Britain. A number of laboratory studies were necessary in parallel with the trial, in order that the natural spread of poliovirus and other agents through the community could be followed during the trial and also to facilitate the interpretation of the results obtained with the vaccine, particularly in terms of alimentary infections which were undetectable otherwise. The virus laboratory at Ruchill Hospital was one of the nominated laboratories willing to participate in the epidemiological investigations. The area from which clinical material was channelled through the laboratory comprised the areas administered by the Western and Eastern Regional Hospital Boards. The area is approximately the part of Scotland south of a line drawn from Fort William to Montrose and west of a line drawn from Dundee to Dumfries.

Every hospital within this area which was likely to admit cases of either paralytic poliomyelitis or of aseptic meningitis was asked to cooperate in the investigation. Every person admitted to any such hospital within the area of the

study was to be included if the diagnosis on admission was either paralytic poliomyelitis or aseptic meningitis.

A specimen of stool and of serum was collected from each case as soon as possible after admission to hospital, and transmitted to the laboratory. After an interval of 10 to 14 days a second sample of stool and serum were collected and sent for examination. This system worked well until December 1957 when the investigation seemed to have served its purpose in that the Medical Research Council no longer required information about the epidemiology of naturally transmitted infections. Because the organisation for collection of specimens was functioning so very well however, the same hospitals were invited privately to continue the study for a further period so that continuing observations might be obtained. The required cooperation was readily continued and it is thus possible to report the results obtained from the full three-year investigation.

Throughout the investigation each specimen of faeces was homogenised to approximately 10% (V/V) and extracted by centrifugation as will be described in the section on materials and methods. The bacteria-free faecal extract was separated into 2 or more aliquots which were then frozen at either -20°C or at -40°C. In the early stages of the investigations one aliquot of the stool extract was examined for the presence of virus by inoculation into cultures of HeLa and one was often also examined

in human kidney cells.

This part of the investigation which was carried out principally by a team of science graduates ended in December 1957 with the ending of the M.R.C. investigation. The results have been analysed and published (MacGregor, Larminie, McLaughlin, Sommerville & Grist, 1958., and Grist, Larminie, MacGregor, McIntosh, McLaughlin & Sommerville, 1958.)

With increasing experience of tissue culture techniques, however, it became apparent that a combination of HeLa and human kidney cells would not support the growth of as wide a range or as great a number of viruses as would monkey kidney cells by themselves. From January 1958 onwards the first specimens were accordingly examined only in cultures of monkey kidney cells, and in order to bring the remainder of the investigation into line the remaining aliquots of first stool specimens which had been collected between May 1956 and December 1957 and which had been stored frozen in the interval were re-examined in monkey kidney cell cultures. This was possible in almost every case, but the numbers involved are slightly different from those which have been published already because in some instances the aliquots of stool extract had all been used.

In effect then, the investigations to be described here represent the results of a 3 year survey of the distribution of enteroviruses in the stools of patients suffering from paralytic

poliomyelitis or aseptic meningitis. The results have been obtained throughout from the examination of specimens of stool in monkey kidney cell cultures.

The population which was studied.

Although the population which made up this survey was drawn from a very large geographical area the greatest numbers of cases came from zones with the greatest population density. Most of the cases of either disease came in fact from Glasgow and its surrounding areas. Many came from the East coast area around Dundee.

The number of cases of either disease occurring in the two parts of the survey are shown in tabular form in Table 1. The survey includes results from 283 cases of paralytic poliomyelitis and 445 cases of aseptic meningitis. In total and in the individual parts of the survey aseptic meningitis was more common than was paralytic poliomyelitis. A small increase in the numbers of cases of aseptic meningitis occurred between the first and second parts but this was not so marked as the increase in numbers of cases of paralytic disease in the second, shorter part of the investigation.

The number of cases occurring at each age group is shown in the fourth column and it will be seen that when either disease is considered the proportion of cases in each group is

TABLE 1

Total number of cases of paralytic poliomyelitis and aseptic meningitis occurring in the study area between May 1956 and March 1959

Disease	Period of study	Age	Number in group	Total for period	Total cases
Paralytic poliomyelitis	May 1956 - December 1957	<3 3-5 6-8 >9	33 (36%) 25 (27%) 12 (14%) 22 (24%)	92	283
	January 1958 - March 1959	<3 3-5 6-8 >9	60 (32%) 40 (21%) 26 (14%) 65 (34%)	191	
Aseptic meningitis	May 1956 - December 1957	<3 3-5 6-9 >9	20 (13%) 38 (19%) 55 (22.5%) 81 (40%)	200	445
	January 1958 - March 1959	<3 3-5 6-8 >9	42 (17%) 57 (20%) 47 (19%) 89 (36%)	245	

remarkably constant between the two parts of the investigation. One noticeable feature is the greater proportion of paralytic cases which occurred in the under 3 age group and this contrasts sharply with aseptic meningitis where the smallest proportion of cases occurred in this group. In the 9-years and older group the incidence of paralytic disease is again high but the incidence of aseptic meningitis is greatest. Inevitably in a survey of this nature where the selection of cases is left to large numbers of individuals who apply different personal criteria for the diagnosis of either disease a number of cases which were initially thought to be paralytic poliomyelitis or aseptic meningitis prove to be instances of unrelated disease. Because these were thought on admission to hospital to be cases for inclusion in the survey specimens of blood and stool were collected and examined, and the results are available for comparison with those obtained from the survey itself. Classification of a large number of other diseases into categories for the purposes of description is rather difficult because of the heterogeneity of this population but basically these cases can be divided into either

1. "other paralytic" which included cases of encephalitis, Bell's palsy or other isolated lower motor neurone paralyses, and
2. "other non-paralytic" which was a larger group and could be sub-divided into

   (a) respiratory infections

(b) specific bacterial infections (e.g. meningococcal or tuberculous meningitis)
(c) miscellaneous infections (which included a large number of cases of pyrexia of unknown origin.)

No cases classifiable as "other paralytic" were examined during the first part of the survey and 35 were encountered during the second. This group consisted of 28 cases of encephalitis, 6 of Bell's palsy and one bilateral external rectus palsy. In the "other non-paralytic" group the following numbers of cases were encountered. (Table 2).

TABLE 2

Numbers of cases of different diseases comprising "other non-paralytic" group.

Period of study	Disease	Number	Total
May 1956 - December 1957	Respiratory infection	23	78
	Specific bacterial infection	16	
	Miscellaneous	39	
January 1958 - March 1959	Respiratory infection	16	75
	Specific bacterial infection	17	
	Miscellaneous	42	

These cases were treated in the same way as cases of either paralytic poliomyelitis or aseptic meningitis in that stools were homogenised and examined in monkey kidney cells after

freeing from bacteria by centrifugation.

CHAPTER III

MATERIALS AND TECHNICAL METHODS.

The descriptions which follow are included at this point for the sake of convenience in description. It should be noted however that the same materials and methods were used in fact throughout the entire work without alteration. Reference has been made to this chapter at other points in the text where methods are mentioned in an effort to avoid duplication.

MATERIALS

MEDIA

Hank's solution

This was made according to the Enders B modification of Hank's original formula.

Solution A (stock)

NaCl	40.0 gm.
KCl	2.0 gm.
$MgSO_4$ $7H_2O$	0.5 gm.
$MgCl_2$ $6H_2O$	0.5 gm.
$CaCl_2$	0.7 gm.

These were dissolved in 250 ml. de-ionised water in the order shown and autoclaved at 10 lbs. for 15 minutes.

Solution B (stock)

Na_2HPO_4 $12H_2O$	0.76 gm.
KH_2PO_4	0.3 gm.
Dextrose	5.60 gm.

These were dissolved in 200 ml. de-ionized water to which 50 ml. of 0.2% phenol red solution had been added and autoclaved at 10 lbs. for 15 minutes.

For use 50 ml. of Solution A was mixed with 50 ml. of Solution B, made up to 1 litre and 5 gm. lactalbumen hydrolysate was added. The mixture was bottled usually in 95 ml. quantities and autoclaved at 10 lbs. for 15 minutes.

Earle's solution

To make this medium the following chemicals were weighed and dissolved in the order shown.

NaCl	67.0 gm.
KCl	4.0 gm.
$MgSO_4$ $7H_2O$	2.0 gm.
NaH_2PO_4 $2H_2O$	1.4 gm.
Dextrose	10.0 gm.
$CaCl_2$	2.0 gm.
0.2% phenol red solution	50 ml.
De-ionised water	950 ml.

This medium was bottled in 10 ml. quantities and autoclaved at 10 lbs. for 15 minutes.

Human serum

This was obtained from the West of Scotland Blood Transfusion service and stored frozen at -20°C. Before use it was thawed, filtered through a bacteria-retaining Seitz pad,

bottled in 20 ml. amounts and inactivated at 56°C. for 30 minutes.

Calf serum

This was obtained as clotted blood from the abbattoir. The separated serum was treated in the same manner as the human serum.

Sodium bicarbonate solution

This was made by dissolving 4.4 gm. sodium bicarbonate in 95 ml. de-ionised water and adding 5 ml. of 0.2% phenol red solution.

CO_2 from a gasifying block of solid carbon dioxide was passed through the bulk solution until the phenol red colour became orange. The solution was then bottled in 5 ml. amounts and sterilised by autoclaving at 10 lbs. for 15 minutes.

Phosphate buffered saline

This solution was made according to the formula of Dulbecco (1954) but the calcium and magnesium ions were omitted.

The following chemicals were weighed and dissolved in the order shown.

NaCl	8.0 gm.
KCl	0.2 gm.
Na_2HPO_4	1.15 gm.
KH_2PO_4	0.2 gm.
De-ionised water	1000 ml.
Phenol red solution 0.2%	2 ml.

The solution was bottled in 100 ml. amounts and sterilised by autoclaving at 10 lbs. for 15 minutes.

Trypsin solution

Difco 1:250 trypsin was dissolved at a concentration of 0.25% in Hank's solution with added 0.5% lactalbumen hydrolysate. It was filtered through a bacteria-retaining Seitz pad, bottled aseptically in 100 ml. amounts and stored frozen at -20°C.

Antibiotic mixture

This contained in 1 ml. the following concentrations of antibiotic.

Penicillin	100 units
Streptomycin	100 microgrammes
Mycostatin	50 units

Growth medium for monkey kidney cells (GM)

This consisted of the following:-

Hank's solution	83 ml.
Human serum	10 ml.
Calf serum	5 ml.
4.4% sodium bicarbonate	1 ml.
Antibiotic mixture	1 ml.

Maintenance medium for monkey kidney cells (MM)

This consisted of the following:-

Earle's saline	10 ml.
De-ionised water with 0.5% lactalbumen hydrolysate	80 ml.
Calf serum	4 ml.
4.4% sodium bicarbonate solution	5 ml.
Antibiotic mixture	1 ml.

This medium was preferred to a modified Hank's solution for the maintenance of monkey kidney cells because the MM as used retained the pH of the cultures for about twice as long and enabled isolation of viruses from stool specimens without the necessity of frequent medium changes and passages when the cells became degenerate due to low pH.

METHODS

Preparation of monkey kidney cells

Tissue cultures were obtained from the kidneys of rhesus monkeys for the greatest part of the work. At one point, however, rhesus monkeys became unobtainable in Britain and for this period cynomolgous (Java) monkeys were used. The substitution was only made after extensive testing to ensure that the cynomolgous kidney cell susceptibility to viruses was identical to the rhesus one.

The procedure adopted for the preparation of monolayer cultures was established at an early stage of the work and used without modification thereafter. It is based on the method described by Bodian (1956).

The kidneys were removed from the animal under Nembutal anaesthesia and after decapsulation the cortices were separated from the medullae with sharp pointed scissors in a sterile petri-dish. The cortices were chopped to fragments of not more than

2 mm. and the "mince" was washed repeatedly in phosphate buffered saline (PBS) containing 1% of the standard antibiotics mixture already described. The washings removed almost all the blood. The "mince" was then suspended in 100 ml. of 0.25% trypsin solution (Difco 1:250 trypsin) at 37°C in a sterile 500 ml. blood transfusion bottle and with a plastic covered sterile magnet added, the bottle was placed on a magnetic stirrer in an incubator at 37°C.

After 1 hour's stirring the trypsin solution was removed completely by pipette and discarded. 100 ml. of fresh trypsin solution at 37°C were added to the fragments which were then agitated by the stirrer for about 4 hours at 37°C. By this time most of the kidney had disintegrated. The turbid supernatant was removed and 100 ml. of fresh trypsin pre-warmed to 37°C. was added. The bottle was replaced on the magnetic stirrer for a further period of two hours by which time the kidneys had invariably disintegrated completely.

The turbid supernatant fluids were pooled, centrifuged at 1000 r.p.m. for 10 minutes, and the cell sediment collected after decantation. Approximately 50 ml. growth medium was added to the cells which were evenly re-suspended by gentle agitation. The count was performed on a 1:20 dilution of this in a Spencer "Bright-line" chamber and the dilution of the cells adjusted thereafter so that 1 ml. of medium contained approx-

imately 150,000 cells if tubes were required and 300,000 cells if bottles or petri dishes were to be inoculated.

For the inoculation of test tubes about 30 sterile 12 x 100 mm. tubes at a time were laid at a slight slope in a suitable size of tin so that the rims overhung the edge. After flaming, 1 ml. of cell suspension was added to each with an automatic syringe of the Cornwall type.

If bottles or petri dishes were required the "double strength" suspension was used. Approximately 8 ml. of this were added to a 4 or 6 oz. medical flat bottle or a 9 cm. petri dish. 5ml. was required for a 2 oz. medical flat bottle or a 6 cm. petri dish.

Petri dishes of cells were placed in a large 14 lb. biscuit tin the lid of which had been drilled to allow 2 pieces of glass tubing to pass through. The tin with its charge of petri dishes was placed in a humidified incubator at 37°C. and one of the tubes through the lid was connected to a supply of 5% CO_2 in air obtained in cylinders from the British Oxygen Company. The CO_2:air mixture was bubbled through a wash bottle of water before entering the biscuit tin. This modification of a gassed incubator worked perfectly during the entire series of experiments. All cells, whether in tubes, bottles or petri-dishes, were ready for use, having formed confluent monolayers, in 7 days. Before use the GM was discarded and replaced with the same amount

of MM.

The preparation of stool extracts

The stool was homogenised to a 10% (V/V) suspension in Hank's solution as soon as possible after its receipt in the Virus Laboratory. It was shaken in a closed universal container until even suspension resulted and then centrifuged at 3,000 r.p.m. in a M.S.E. angle minor centrifuge for 30 minutes. This was adequate for the removal of bacteria in all but a very rare instance. The supernatant fluid was removed carefully and divided into two or three aliquots of 2 ml. each. These were labelled and stored at -40°C. until required for testing.

The examination of stool cultures

The stool extract was inoculated in 1 ml. amounts into each of two tubes containing 1 ml. MM. After one hour's incubation the 2 ml. fluid was removed from each tube and replaced with 1 ml. of MM. The tubes were examined microscopically each day for 14 days and if no cytopathic changes were observed within this time the specimen was considered to be negative. The 14 day incubation period could frequently be managed without recourse to a change of medium or passage. If a toxic substance was present in the stool the toxic fluid from the primary culture was passaged after pooling by inoculating

0.2 ml. into each of two further tubes.

If a cytopathic effect was observed during the 14 day incubation period the material was passaged twice in 0.1 ml. amount into each of two further tubes. If degeneration was observed in these the degenerate cells plus the supernatant fluid from the third passage were pooled, titrated in log steps in two tubes per dilution, and checked for neutralization by mixing 100 ID_{50} (50% infectious doses) in 0.2 ml. with an equal amount of a suitable dilution (usually 1:8) of high titre antiserum prepared against each ECHO virus from 1 to 20, Coxsackie virus B1-5 and A9 and poliovirus types 1-3. The "mixtures" were incubated for one hour at room temperature and inoculated into each of two tubes per "mixture". The tubes were incubated for 3-4 days before reading and neutralization was usually found with one of the viruses. The isolated agent was identified in this way.

CHAPTER IV

RESULTS OBTAINED.

The types of virus which were isolated from cases of various ages and with various diagnoses are shown in tables 3, 4, and 5. It was possible to confirm the diagnosis of paralytic poliomyelitis by isolation of the virus from 66% of cases in the first part of the survey and from 65% in the second. In both instances the greatest proportion of isolations was made in the less-than 3 age group.

Among cases with aseptic meningitis poliovirus was isolated from a total of 10% in the first part and 30% in the second with the greatest proportion of viruses (14 and 43% respectively) isolated in the 6-8 age group in both parts of the survey.

In the "other paralytic" group which consisted of 35 cases examined during the second part of the survey a poliovirus was isolated from only 3.5% whereas in the 153 cases comprising the "other non-paralytic" group a poliovirus was isolated from 1.3% and 7% respectively in the first and second parts of the survey. 3 of the latter were isolated from the stools of patients admitted to hospital with respiratory illness and the remaining three came from persons admitted with pyrexia of unknown origin.

TABLE 3

Type of virus isolated from 283 patients admitted to hospital between May 1956 and March 1959 suffering from paralytic poliomyelitis.

	Age (Yrs.)	Number in group	Poliovirus				ECHO	Coxsackie	Other
			1	2	3	All types			
May 1956 - December 1957	<3	33	11	10	5	26 (78%)	0	0	0
	3-5	25	5	3	4	12 (50%)	0	0	0
	6-8	12	2	1	5	8 (66%)	0	0	0
	>9	22	6	1	8	15 (70%)	0	0	0
	Total	92	24	15	22	61 (66%)	0	0	0
January 1958 - March 1959	<3	60	49	0	0	49 (81%)	0	0	0
	3-5	40	28	1	0	29 (72%)	0	0	0
	6-8	26	14	0	0	14 (54%)	0	0	0
	>9	65	32	0	0	32 (50%)	0	0	0
	Total	191	123	1	0	124 (65%)	0	0	0

TABLE 4

Types of virus isolated from 445 patients admitted to hospital between May 1956 and March 1959 suffering from aseptic meningitis.

	Age (Yrs.)	Number in group.	Poliovirus				ECHO	Coxsackie	Other
			1	2	3	All types			
May 1956 - December 1957	<3	26	2	2	0	4 (8%)	3	0	0
	3-5	38	1	0	2	3 (8%)	1	2	0
	6-8	55	4	2	2	8 (14%)	2	2	0
	>9	81	2	0	4	6 (7%)	3	4	0
	Total	200	9	4	8	21 (10.5%)	9	8	0
January 1958 - March 1959	<3	42	14	0	0	14 (33%)	0	1	0
	3-5	57	20	1	0	21 (37%)	2	0	0
	6-8	47	20	0	0	20 (43%)	1	1	0
	>9	89	18	0	0	18 (20%)	0	0	0
	Total	245	72	1	0	73 (30%)	3	2	0

TABLE 5

Types of virus isolated from 35 and 153 patients admitted to hospital between May 1956 and March 1959 suffering from "other paralytic" and "other non-paralytic" syndromes.

Clinical category	Period of observation	Diagnosis	No. in group	Poliovirus				ECHO	Coxsackie	Other
				1	2	3	All types			
"Other paralytic"	January 1958 - March 1959	Encephalitis	28	0	1	0	1 (2.5%)	0	0	0
		Bell's palsy	6	0	0	0	0	2	0	0
		Other	1	0	0	0	0	0	0	0
	Total		35	0	1	0	1 (3%)	2	0	0
"Other non-paralytic"	May 1956 - December 1959	Respiratory	23	1	0	0	1 (4%)	0	1	0
		Specific bacterial	16	0	0	0	0	0	0	0
		Other	39	0	0	0	0	0	0	0
	Total		78	1	0	0	1 (1.3%)	0	1	0
	January 1958 - March 1959	Respiratory	16	2	0	0	2 (12.5%)	0	0	0
		Specific bacterial	17	0	0	0	0	2	0	0
		Other	42	3	0	0	3 (7%)	0	2	0
	Total		75	5	0	0	5 (7%)	2	2	0

In monkey kidney cells no viruses belonging to the ECHO, Coxsackie or other groups were isolated from the 283 cases of paralytic poliomyelitis, whereas a total of 12 ECHO viruses and 10 Coxsackie viruses were isolated from the patients with aseptic meningitis during the entire survey. The presence of these agents in the stool of 5% of 445 cases of aseptic meningitis together with polioviruses in a further 21% is in sharp contrast with the corresponding figure of 0% and 65.5% for paralytic illness.

The ECHO and Coxsackie viruses which were isolated are shown arranged according to their types in table 6. In aseptic meningitis it will be seen that the commonest viruses were ECHO types 7 and 9. As will be shown in the chapters on juvenile diarrhoea ECHO type 7 was also encountered frequently in this condition. ECHO type 9 was met in one outbreak of aseptic meningitis mostly in the Dundee area and this outbreak will be described shortly in detail because although only 5 viruses were isolated from the 24 cases which were examined from the one area it was possible to demonstrate a significant antibody increase in almost all. Apart from this specific outbreak which occurred in the autumn of 1956 no other series of cases within the survey could be consistently labelled as to cause from the virus isolation study.

The two Coxsackie A9 viruses shown in table 6 under

TABLE 6

Types of ECHO and Coxsackie viruses which were isolated from the stools of patients suffering from aseptic meningitis and "other non-paralytic" illnesses between 1956 and 1959.

Clinical category	ECHO virus types					Coxsackie virus types				
	7	9	11	13	18	A9	B1	B3	B4	B5
Aseptic meningitis	4	6	1	0	1	3	1	3	1	2
"Other non-paralytic"	1	0	0	1	0	2	0	0	0	1

the heading of "other non-paralytic" were both from cases of respiratory illness in February 1958. In the same month a number of Coxsackie A9 viruses were isolated in tissue culture from completely unrelated illnesses and there appeared to be a small outbreak of infection about this time. These cases were encountered only in the Glasgow area. The two ECHO viruses shown in table 5 under the heading of "other paralytic" were an ECHO 7 and an ECHO 18. Curiously both were isolated from children with Bell's palsy. The number of cases of each type and the viruses which were isolated are shown diagrammatically on a monthly basis in figure 1. The date of onset has been used throughout in placing cases within this diagram. The distribution of types of poliovirus varied considerably in the early stages of the survey until 1958 when type 1 was the only virus encountered in the paralytic and aseptic meningitis groups. The distribution of each type of poliovirus between these two groups of cases is shown in table 7.

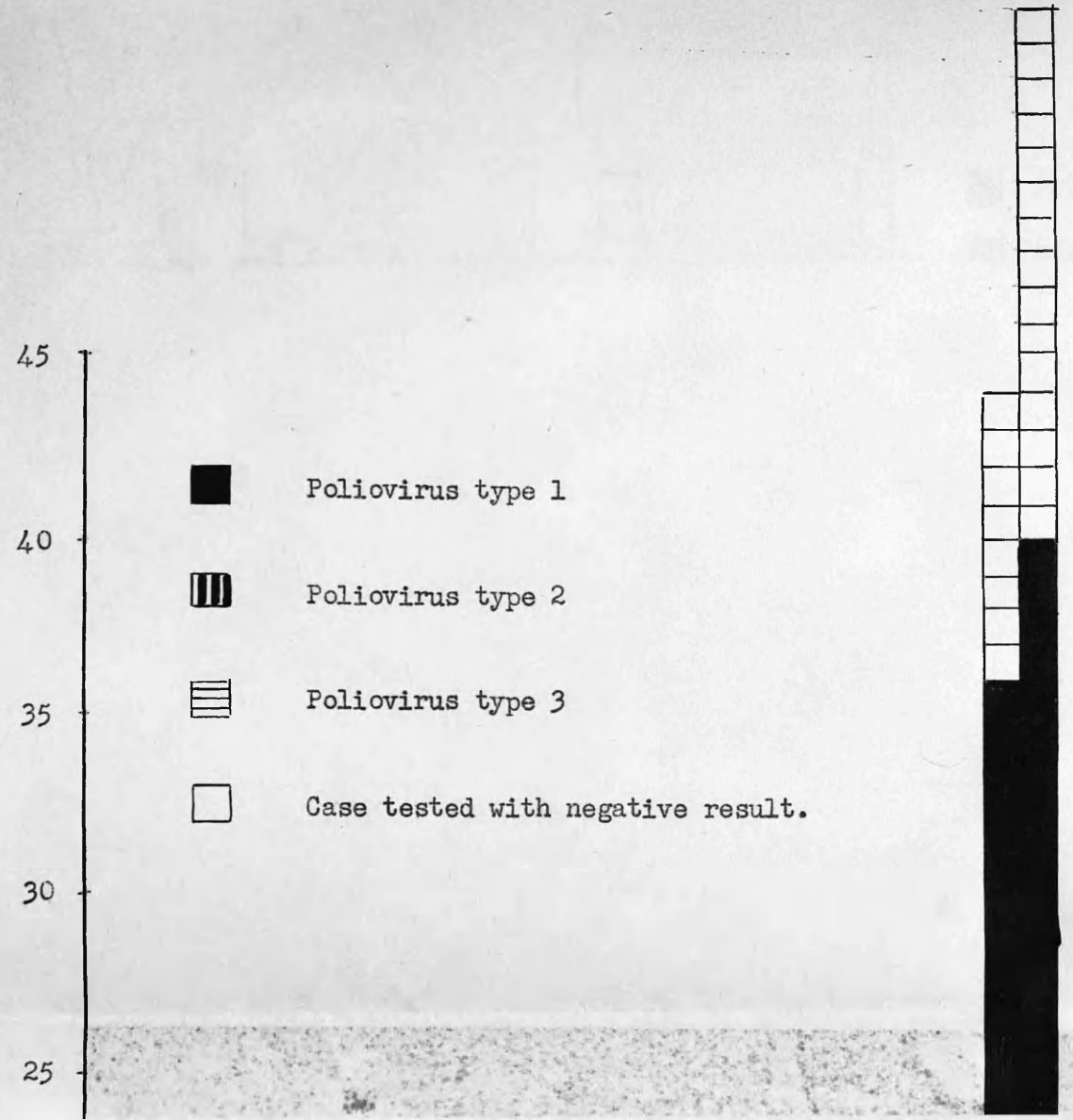
TABLE 7

Distribution of polioviruses by type and year in cases of paralytic poliomyelitis and aseptic meningitis.

Type of virus	1956	1957	1958-59
Poliovirus type 1	4	29	197
Poliovirus type 2	16	2	0
Poliovirus type 3	16	14	0

Inspection of figure 1 shows that both types of disease reached a maximum between August and November in 1956, and that a dissociation occurred in 1957, when paralytic cases were maximally prevalent between August and September and aseptic meningitis was most prevalent between May and August. In 1958 both reached a maximum between June and September.

Figure 1 also shows the localised nature of the outbreak of aseptic meningitis in the Autumn of 1956 which was associated with ECHO virus type 9.


45
40
35
30
25
Poliovirus type 1
Poliovirus type 2
Poliovirus type 3
Case tested with negative result.

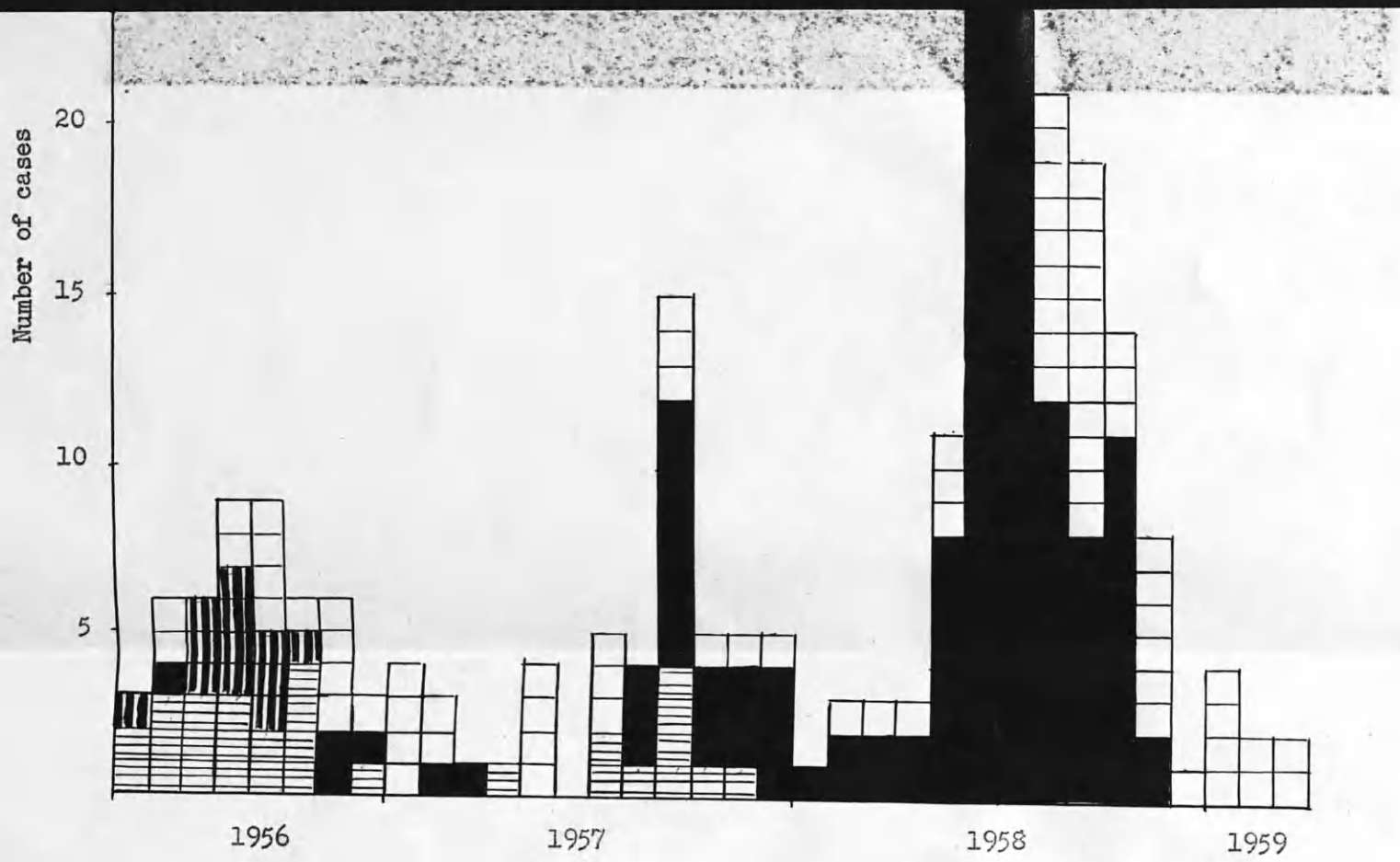


Figure 1

Number of cases of paralytic poliomyelitis studied between May 1956 and March 1959 showing the distribution of viruses isolated from each group.

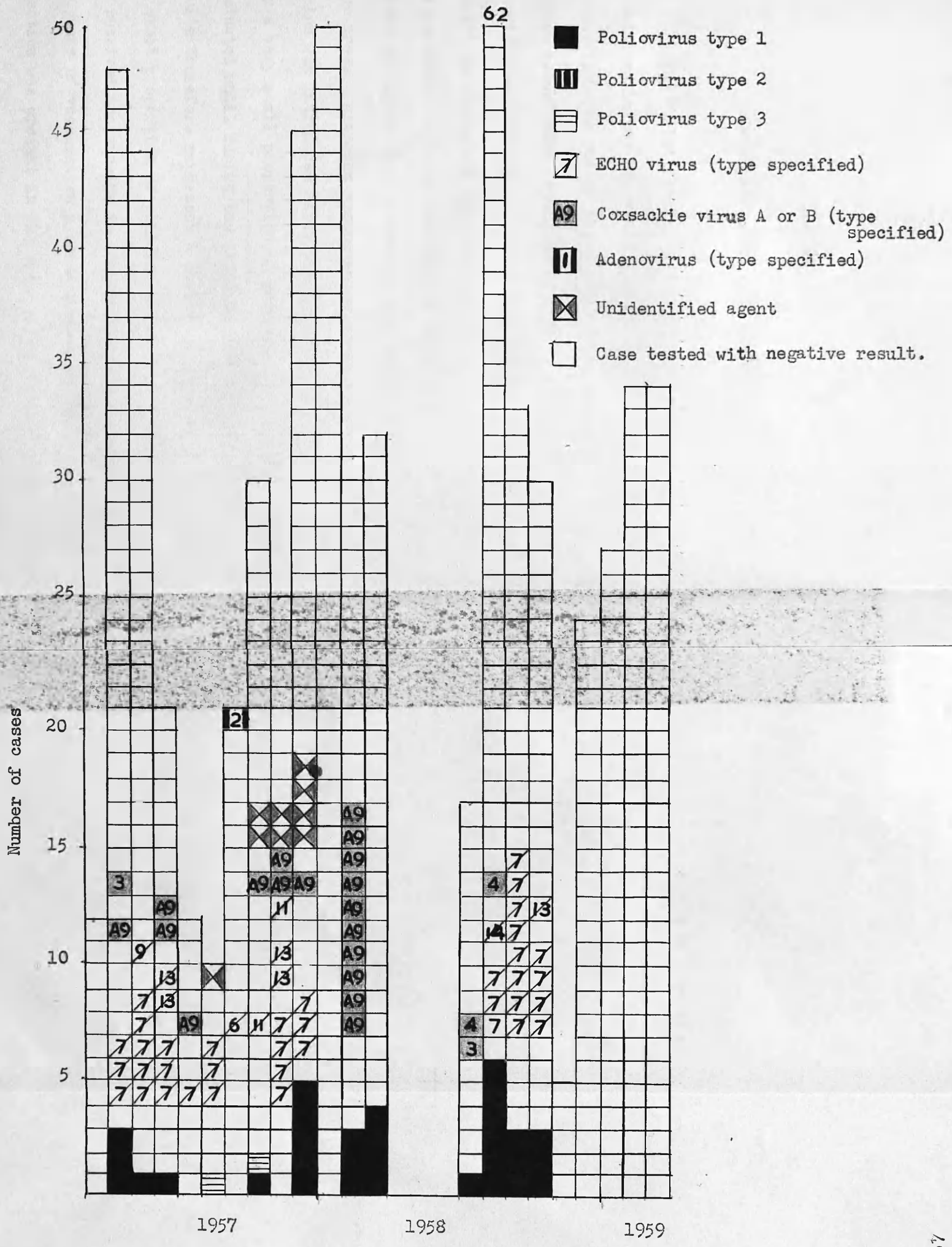


Figure 1 continued.

Number of cases of aseptic meningitis studied between May 1956 and March 1959 showing the distribution of viruses isolated from each group.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The variability of the type of poliovirus which predominates in any one year in cases of paralytic poliomyelitis is borne out by the results just reported. As it has been established beyond doubt that immunization with poliovirus vaccine has no effect upon either the ability to contract an alimentary infection or its duration the variability probably reflects the locally predominant type as determined by the antibody status of the child-population in any given area. Although immunization with inactivated polio vaccine was introduced after the beginning of this survey only a very small proportion of possible children had been vaccinated until during the third year of the study and the results therefore represent a series of non-epidemic years. The exact proportion of children at various age-levels who had received the vaccine is unknown but even in 1958 after two years of vaccination a considerable amount of paralytic infection was present in the area as judged by the proportionately very large numbers of polioviruses which were isolated.

It is of interest that in 1956, when the locally isolated strains were predominantly types 2 and 3, type 1 composed 91% of strains isolated at the Virus Reference Laboratory

at Colindale (Stokes and Macrae, 1957). Type 2 virus, which was the most commonly encountered in 1956 disappeared in 1957 and was isolated from the present survey twice only in 1958. At the same time the incidence of type 1 varied from 11% of strains in 1956 to 64% in 1957 and 99.5% in 1958.

The figures reported here bear out the truly seasonal distribution of the majority of poliomyelitis infections which are confined to the summer and early autumn months. A pointer to the type of virus present in the population which will probably cause the majority of cases in the height of the season can, however, be obtained from a study of the cases of poliomyelitis occurring in the early months of the year. During the 3 years of this investigation a few sporadic cases always were found between January and May and the viruses isolated from these invariably determined the types for the summer months and gave advance warning. During the investigations which make up the epidemiological part of my thesis I found also that a study of such unrelated ailments as diarrhoea, croup or acute bronchopneumonia in children during the early months of the year would equally easily produce the predominant type of poliovirus for the following summer.

The results reported here also bear out the many differences between paralytic poliomyelitis and aseptic meningitis. Although the time-incidence in 1956 and 1958 was approximately

similar, a wave of aseptic meningitis occurred in 1957 before paralytic disease occurred in the population to any appreciable extent. The differences in age incidence between the syndromes are significant also. Paralytic disease afflicted 55% of the 5 years or less age group whereas aseptic meningitis affected only 36% of this group. A comparison of the incidence in the under-3 age group reveals a more striking difference in incidence, when paralytic disease affected 34% and aseptic meningitis 15%.

The other main difference lies in the total absence of isolations of agents other than poliovirus from the paralytic group at all ages as compared with ECHO and Coxsackie viruses from 5% of stools of aseptic meningitis patients. Although this figure is low a number of explanations for it are possible. In a survey of this kind, when specimens are being collected at various hospitals, under conditions which cannot be standardised, for subsequent transmission to the examining laboratory by post, a number of viruses other than poliovirus may be lost through relative instability at room temperature. (Poliovirus is known to be stable for long periods but the stability of ECHO and Coxsackie viruses is unknown.) Also this type of survey involving large numbers of cases is only possible if a single stool specimen from each patient is used as the sample. Poliovirus is excreted for two to three or more weeks from cases of

paralytic illness but no figures are available for the duration or constancy of the excretion of agents other than poliovirus and it may be that the one-stool-per-patient sample will prove completely inadequate for the isolation of optimal numbers of agents other than poliovirus. Finally the viruses causing the bulk of aseptic meningitis in any one outbreak may not yet have been isolated at all because presently used tissue culture systems are inadequate for their growth. A number of these factors were operating in the only definite and quite localised outbreak of aseptic meningitis which occurred in the present survey. A brief description of this will show the difficulties and emphasise the importance of obtaining adequate and enthusiastic clinical cooperation.

The details of this outbreak have already been published by Jamieson, Kerr & Sommerville (1958). Between July and November 1956 32 patients were admitted to King's Cross Hospital, Dundee, suffering from aseptic meningitis. Although this was the total number admitted to hospital reports from general practitioners in the area indicated that very many more, but milder, cases were occurring in the area and being treated at home. Most of the patients admitted to hospital came from the Dundee area but a few were admitted from outlying villages in the area. The incubation period of the illness was estimated from family studies to be about 6 days.

Equal numbers of males and females were affected mostly between the ages of 5 and 20. The onset was sudden, often with a combination of pyrexia, headache, vomiting and meningeal irritation. Mild muscular pain was present in 9 cases and 3 had a rash - morbilliform in 1, and scarlatiniform in 2.

The illness, which was pyrexial, lasted 7-8 days and usually ended with an abrupt but short lived rise in temperature. Clinically the impression was formed that this was a mild illness.

Cell counts on the cerebrospinal fluid revealed an increase of up to 1,500 per c.mm. In 8 cases polymorphs predominated initially but after the fourth day in all there was a lymphocytosis. The protein content was raised and the sugar level was usually within the normal range.

The clinicians were able to select 24 cases which appeared to them to be typical of this type of aseptic meningitis and from each case a stool sample and a serum specimen were collected on admission to hospital. A second serum specimen was collected in the convalescent phase of the illness. These were sent by post to Glasgow.

The stool specimen, extracted as already described, was examined in tissue cultures of HeLa and monkey kidney cells.

Nothing was isolated in HeLa cells but from the monkey

kidney cultures 5 viruses were grown, all of which belonged to ECHO type 9. This represented an isolation rate of 21%, but left 19 clinically identical cases unaccounted for.

Under these circumstances it seemed reasonable to examine the paired sera for antibody to ECHO type 9 virus and these tests were performed in duplicate in monkey kidney cell cultures using a constant virus dosage of 50 ID_{50}, (50% infectious doses) as calculated from a standard infectivity titration in test tubes, of a strain of ECHO 9 which had been isolated from one of the patients. The virus dose was mixed with an equal amount of each serial dilution of the patient's serum and one hour at room temperature was allowed for the reaction before inoculation into monkey kidney cultures. The tests were read when the virus controls showed complete degeneration, usually about the fourth day. The reciprocal of the highest serum dilution producing neutralization is recorded as the end point of the titrations.

The results of the serological tests and also of the virus isolations are recorded in table 8. A fourfold or greater increase in antibody titre to ECHO 9 virus was observed in 20 instances. In 2 a twofold rise occurred and no significant antibody was found in the remaining two cases. From 4 patients who showed a fourfold rise ECHO type 9 virus was isolated, but the fifth isolation was from a patient in whom no increase in an

TABLE 8

Results of virus isolation and serum-virus neutralization tests on acute and convalescent phase sera from 24 cases of aseptic meningitis tested with ECHO virus type 9.

Case	Age (yr.)	Day of disease acute/ convalesc-ent	ECHO-9 antibody titre acute/ convalescent	Monkey-kidney tissue-culture of stool.
1	3	5/15	4/16	Negative
2	7	6/15	<2/16	Negative
3	8	6/17	<16/128	ECHO 9
4	4	12/20	<16/64	ECHO 9
5	5	5/17	<16/32	Negative
6	1	6/18	<16/64	Negative
7	2	4/13	8/16	Negative
8	10	7/19	16/64	Negative
9	7	4/13	<16/128	Negative
10	6	9/18	<16/128	Negative
11	13	7/19	<16/64	Negative
12	6	4/16	16/32	Negative
13	3	4/16	4/16	Negative
14	5	6/17	<16/64	Negative
15	4	6/17	<16/128	Negative
16	17	9/20	16/128	Negative
17	11	5/23	8/8	Negative
18	34	2/21	16/64	ECHO 9
19	18	7/18	<16/32	Negative
20	15	10/21	16/>128	Negative
21	8	9/21	<2/16	ECHO 9
22	6	11/23	4/16	Negative
23	2	11/26	<16/32	Negative
24	0.3	9/27	4/4	ECHO 9

initially very low level of antibody was found by the 27th day of the illness.

The small series of cases demonstrates very well the difficulty of isolating the aetiological virus from the stool of sufficient numbers to be certain of the diagnosis in any outbreak. The factors against virus isolation here were the delay in transmitting specimens to the laboratory occasioned purely by the distance and the necessity of sending by post.

The difficulty in this instance lies in the 24 hour minimum postal delay coupled with the relative instability of the virus concerned, particularly when kept at room temperature. Also, the study was concerned only with one stool specimen and theoretically at least this is a sub-optimal sampling procedure.

On the other hand this small series illustrates well how useful serological tests can be when applied to a disease population which has been carefully selected. The results are biassed in favour of the satisfactory serological findings because of the careful selection of cases by the clinicians but this type of study is only possible during an epidemic or an outbreak in which a highly infectious organism is actively spreading through a non-immune population.

The greatest difficulty encountered in the series of cases with aseptic meningitis in the main survey at times when no major outbreaks of obviously infectious nature were occurring,

and in the absence of virus isolation from the stool, lay in deciding how best to examine the sera which had been collected. In the absence of an outbreak the choice of a suitable virus for neutralization tests is very difficult from the many possibles when no lead has been found from virus isolation studies. The quantity of serum available was not adequate to allow testing with more than two or three viruses and so a compromise was selected in which the sera would be examined for antibody to the most prevalent viruses in the series as judged by isolation results.

In this event two viruses were selected:- ECHO type 9 and Coxsackie B3. ECHO 9 was chosen because of its obvious association with cases in 1956 and Coxsackie B3 because it occurred most commonly in the aseptic meningitis cases but least commonly in the group labelled "other non-paralytic". Coxsackie A9 was excluded because it had been encountered not only in the "other non-paralytic" group but also frequently in the diarrhoeal children to be described in Part 2.

The serological testing was done after the aseptic meningitis survey had been completed and the sera which were chosen were the group which had been most recently collected and which were therefore "cleanest" (least likely to be bacterially contaminated or decomposed in other ways). The group of sera chosen were collected during 1958. Large numbers were found to

be in insufficient quantity for neutralization tests with two viruses and were accordingly omitted. After selection in this way 84 paired sera were available for testing.

The sera were tested by mixing 0.1 ml. volumes of each serial dilution of each serum, in pairs, with an equal quantity of a suitable dilution of either ECHO type 9 or Coxsackie B3 viruses. The dilution was adjusted to contain between 50 and 100 ID_{50} in 0.1 ml. After one hour's incubation at room temperature the mixtures were inoculated into monkey kidney cell cultures. Results were read after 3-4 days of incubation at 37°C, and these are summarised in table 9.

In the absence of isolation of either virus from the patients whose sera were tested a surprising number were found to be positive in each instance. No serum was found which was positive for both viruses in terms of rising titres but 5 were discovered in which a rising titre for one agent was associated with a high but static titre in both sera of the pair for the other agent. If twofold rising titres which are not generally regarded as being significant are omitted from the consideration and fourfold rises, high titres in both sera or falling titres are included in the positive series, then each virus can be incriminated in about 28% of cases of aseptic meningitis in this selected series for 1958. If on the other hand the criterion of positivity is restricted to fourfold rises about 10% of the

TABLE 9

Results obtained from neutralization tests with ECHO type 9 and Coxsackie B3 viruses on 84 paired serum specimens from patients with aseptic meningitis.

Virus	Age (Yrs.)	Total tested	Negative	Rise x 2	Rise x 4	High in both sera	Fall
ECHO 9	<3	16	12	0	0	3	1
	3-5	21	14	2	0	3	2
	6-8	19	13	0	2	3	1
	>9	28	18	1	5	2	2
	Total	84	57	3	7	11	6
Coxsackie B3	<3	15	11	0	2	2	0
	3-5	22	12	1	0	6	3
	6-8	18	12	2	0	4	0
	>9	29	19	2	0	6	2
	Total	84	54	5	2	18	5

series can be accounted for by the neutralization tests. Probably the ideal would be to accept some figure between the two as the most accurate estimate available.

It seems therefore, even in the absence of positive evidence from each case by virus isolation studies, that it is possible to account for at least a proportion of cases of aseptic meningitis retrospectively by judicious selection of the viruses for neutralization tests. No doubt in the present series if serum had been available for further testing with additional viruses the proportion which could be accounted for in terms of viral aetiology would have been increased, perhaps even considerably.

PART 2

THE ASSOCIATION BETWEEN ENTEROVIRUSES AND DIARRHOEA IN YOUNG PERSONS.

CHAPTER VI

REVIEW OF THE LITERATURE

A number of observations have appeared recently in the literature on the possibility of an association between certain enteroviruses and diarrhoea in young persons. These have arisen largely from the great number of surveys which have been undertaken in many parts of the world, particularly in the U.S.A., of the distribution of enteroviruses in various disease states and in poliomyelitis and its allied syndromes in particular. The only members of the enterovirus group for which a relationship with diarrhoea has been postulated are certain ECHO viruses. There has been no suggestion that polioviruses or Coxsackie viruses per se may cause diarrhoea.

The first ECHO virus to be associated with diarrhoea was ECHO type 10. Although the prototype virus was isolated from a normal person by Sabin and Ramos-Alvarez, this virus was reported by Sabin (1957) as being associated with a rather vague clinical picture of steatorrhoeic enteritis in humans, particularly young persons, and also with a coryza-like syndrome without diarrhoea in chimpanzees. The precise clinical pattern of symptomatology in humans remains to be determined in the light of increasing experience.

ECHO type 18 has been reported by Eichenwald, Ababio,

Arky and Hartman (1958) as the causal agent of two related outbreaks of diarrhoea among infants. The virus could be isolated readily and antibody response could be demonstrated. A similar finding was also reported by Ramos-Alvarez & Sabin (1958). Clinically the disease was not severe; diarrhoea persisted for from one to five days with a mean duration of three days. The disease was treated by a reduction in the caloric content of feedings and this measure was supplemented by parenteral administration of water and electrolytes as required. After the diarrhoea had subsided the infants were gradually returned to their normal dietary formula without recurrence of the diarrhoea.

ECHO types 19, 22, 23 & 24 as prototype strains were isolated by Sabin & Ramos-Alvarez from persons with diarrhoea but to date no details of the conditions in the original or subsequent patients who were infected with these agents have been released.

A number of investigations have been reported in which surveys have been undertaken of the distribution of enteroviruses in the stools of normal healthy children. In particular these have been conducted by Ramos-Alvarez & Sabin (1956), Sabin (1956) and by Gelfand, Fox & Leblanc (1957). In each survey a group of normal healthy children was examined with reference to the possible presence in the stools of any type of enterovirus.

These agents were found with great regularity and in an astonishing complexity of types.

Ramos-Alvarez & Sabin (1956) compared the enteroviral flora of children, all four years of age or less, in Mexico City and in Veracruz. The former at 7,000 feet is warm and dry, whereas the latter, at sea level, is hot and intensely humid. They found the incidence of polioviruses to be greatest in both cities in the 1-2 year age group, whereas the "other viruses" were commonest in the first year of life in Mexico City and between the second and third years in Veracruz. No particular significance is attached to these results. Gelfand, Fox & LeBlanc (1957) also divided the agents isolated into polioviruses and "other agents" and in a two-year survey of 150 normal healthy children were able to demonstrate a marked seasonal incidence (greatest in summer) reaching a combined total of 29.5% in July, and an association with the socio-economic status of the child which allowed the lower class children to harbour the greatest number of viruses.

After the investigations which are about to be described here were commenced Ramos-Alvarez & Sabin (1958) published an account of a survey of the distribution of enteroviruses in children suffering from summer diarrhoea. They compared a test group of 153 children with summer diarrhoea with a matched control group of 100 children without diarrhoea. Faecal samples

were collected with rectal swabs and the number of enteroviruses recovered was 2.5 times greater from the diarrhoeal children. ECHO viruses were recovered six times more frequently from the children with diarrhoea and a statistically significant role was assigned to these viruses. The authors suggest that summer diarrhoea of very young children is not an entity but rather a consequence of transitory infections with a large variety of bacterial and viral pathogens.

That is the extent of the literature on the association between enteroviruses and diarrhoea, but small though it is there are enough pointers to indicate that a study of the enteroviral flora of children admitted to hospital with diarrhoea should yield an unending supply of enteroviruses. The survey to be described was accordingly undertaken with this end in view.

CHAPTER VII

THE PLAN OF THE INVESTIGATION, 1957-1959 AND THE POPULATION WHICH WAS STUDIED.

The study was commenced on 1st April 1957 and continued until 31st March 1959. A break occurred in the regular sequential testing of stools from newly admitted children during the months of April, May & June 1958. During these three months very few children suffering from diarrhoea were being admitted to hospital and the opportunity was taken to discontinue the survey temporarily in order to allow the examination of a very large back-log of material which had accumulated. The survey was re-commenced on 1st July 1958 and continued for the following nine months.

A single stool specimen was collected on admission from each of 674 children admitted with diarrhoea to one or other of two wards of Ruchill Hospital, Glasgow. This number is made up of 338 children in the first and 236 in the second part of the survey. The clinical diagnosis on admission was either "gastro-enteritis" or "clinical dysentery". In age the children ranged from 1 week to 5 years, and of those with the clinical diagnosis of "gastro-enteritis" the majority were less than 6 months old. In this younger age group, as will be described in detail in the next chapter, many children were found

to be suffering from the effects of some minor feeding disorder. The majority of these were afebrile and recovered within one or two days and I think it is questionable whether they should be included in an investigation of the viral causes of diarrhoea. It was not possible however, to screen out these cases on admission because the general rule had to be made that a stool specimen was collected at the first opportunity after admission to each ward. The cases were therefore sampled and examined in tissue culture before the accurate clinical diagnosis was available.

The stool specimen was collected either from the 'nappy' or bed pan by scraping up a sample with a wooden spatula into a waxed carton with a screw-on lid, and transmitted to the laboratory as quickly as possible. Each stool was homogenised to a 10% suspension (V/V) and extracted as already described.

The collection of specimens worked well in practice and was organised by the ward staff to suit their own convenience. One ward admitted "clinical dysentery" and the other "gastro-enteritis". In October 1958, however, the ward which was admitting "clinical dysentery" was closed and another which belonged to a different medical unit was substituted for it. This new ward also admitted "clinical dysentery" from the same type of social background and the change-over took place quite smoothly. It is a curious fact, however, that no viruses were isolated

after the changeover took place.

Controls

Control material for this type of investigation is very difficult to obtain. Under ideal conditions the controls should be selected from a healthy population and should be matched for age, sex, socio-economic status and time of sampling. With the resources at my disposal such a control series was unobtainable and I had to utilise such clinical material as was to hand. By selecting controls from children admitted to Ruchill Hospital it was possible to obtain stools from young persons of the same socio-economic background, and as far as possible the control children were of similar age and sex to those admitted with diarrhoea. The controls for the first year's survey were all admitted to the hospital suffering from an acute respiratory infection. These were all admitted to one ward and the same general instructions were given about the collection of specimens as soon as possible after admission to hospital. If the stool was not forthcoming within 3 days of admission the child was removed from the series to minimise the risk of ward cross-infection by enteroviruses. Within the laboratory the stools received identical treatment to those obtained from the diarrhoeal group of children.

During the second part of the survey children admitted

with respiratory illness were again used as controls but in this instance the cases were all admitted to one ward as clinical "croup". The procedure for collection and subsequent processing of the stool in the laboratory were unchanged.

One very great difficulty encountered in this type of control series lies in the fact that children are only admitted to this hospital with respiratory illness during the winter months. This applied to both parts of the survey; for neither was there a control group during the summer and early autumn months. In consequence the control groups are much smaller than the survey groups admitted with diarrhoea. During the first part of the survey the control group totalled 115 (c.f. 338 in the diarrhoea group) and in the second the number was only 45 (c.f. 236).

CHAPTER VIII

RESULTS

The distribution of cases in both the diarrhoea and respiratory groups is shown on a monthly basis in figure 2. During the first year admissions to hospital on account of diarrhoea were fewest between June and September, and in November in the second part of the survey. The first admissions in each section for respiratory illness were in September and October respectively.

1957-58

In the first year's survey from 338 diarrhoeal cases a total of 75 viruses was isolated, representing an isolation-rate of 22%; whereas 17 viruses were isolated from the 115 cases in the respiratory control group for the corresponding period. In the seven months during which respiratory control cases were being admitted, 46 viruses were isolated from 223 cases of diarrhoea. The percentage isolation-rate for the seven months is thus 21, compared with 14 for the control group.

The distribution of virus types within both groups as a whole and also the isolations during the seven months from September 1957, are compared in table 10. The percentage

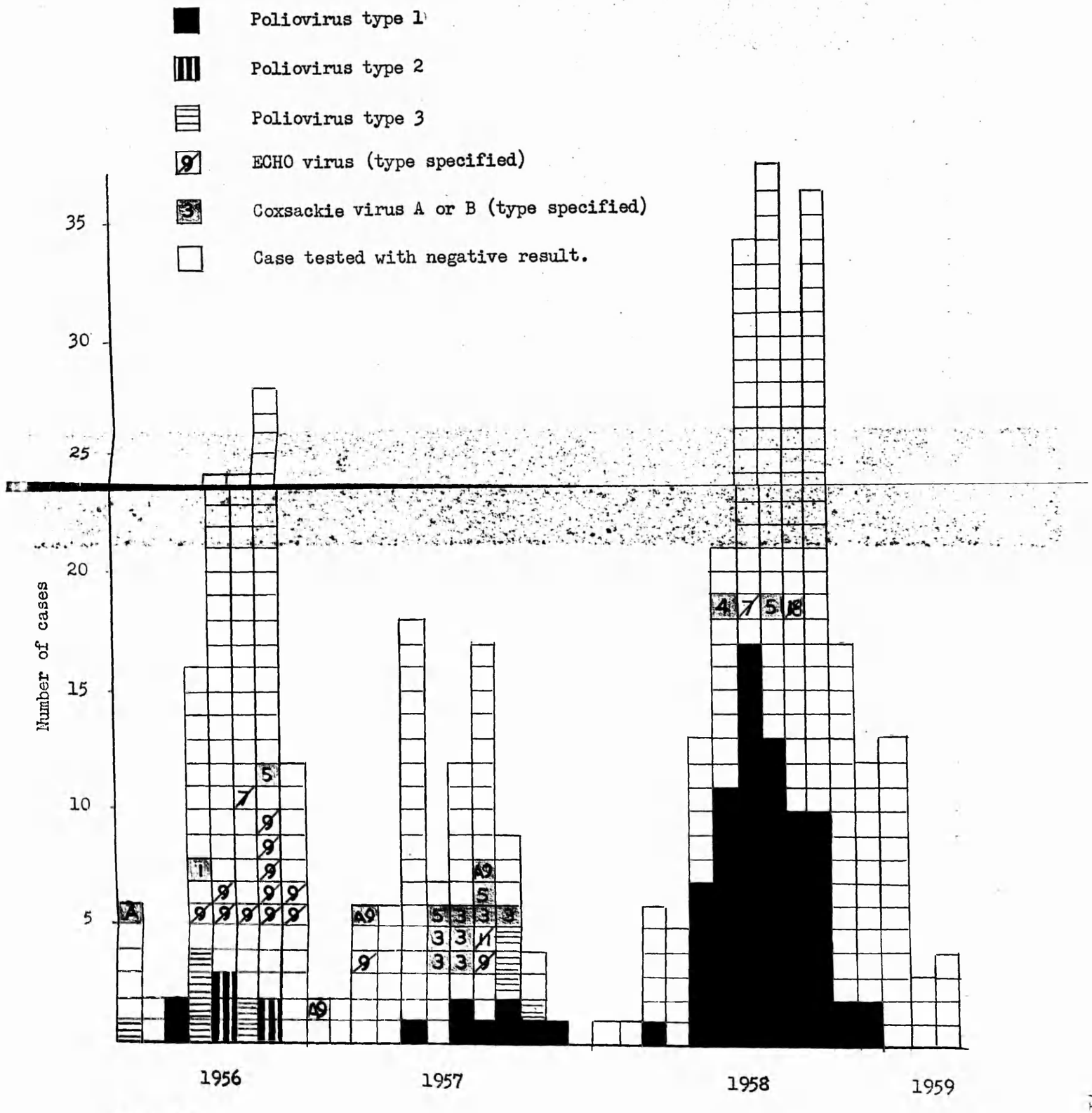


Figure 2

The number of children admitted to hospital with diarrhoea between April 1957 and March 1959 showing the distribution of viruses isolated from each group.

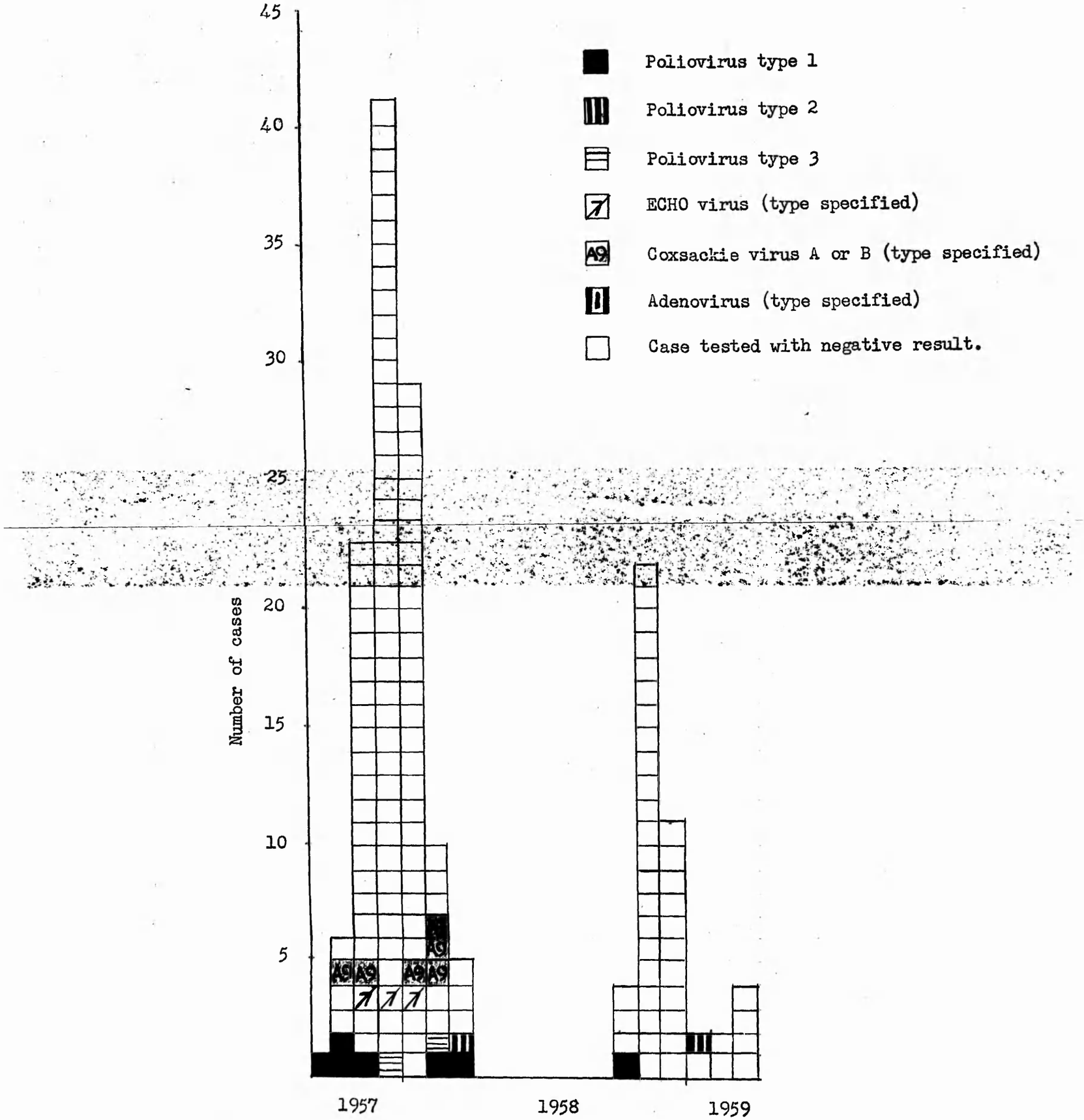


Figure 2 continued.

The number of children admitted to hospital with respiratory illness between April 1957 and March 1959 showing the distribution of viruses isolated from each group.

TABLE 10

Isolation-rates and distribution of virus types isolated from stools of diarrhoeal and respiratory (control) children during twelve months from April 1957, and during seven months from September 1957.

	Diarrhoea		Respiratory control (7 months)	Total series
	12 months	7 months		
Total cases	338	223	115	453
Total isolations	75 (22%)	46 (21%)	17 (14%)	92 (20.3%)
ECHO virus	29 (8.5%)	12 (5.4%)	3 (2.5%)	32 (7%)
Poliovirus	20 (5.9%)	14 (6.3%)	8 (7%)	28 (6.2%)
Coxsackie virus	16 (4.7%)	11 (4.9%)	6 (5%)	22 (4.9%)
Other agents	10* (2.9%)	9 (4%)	-	10* (2.2%)

* includes 1 adenovirus type 2.

isolation-rates for poliovirus and Coxsackie viruses are similar in diarrhoea and control groups. The rate for ECHO viruses from diarrhoeal children varied between rather more than three times and twice the rate for the controls, according to the period under consideration.

1958-59

During the second part of the survey a total of 34 viruses was isolated from 236 cases of diarrhoea, representing an isolation rate of 14.3%. In the six months during which the respiratory control group was available the total isolation rate from the diarrhoeal children was 8 viruses from 154 cases (5.3%) as compared with 1 virus from 45 controls (2.2%).

The distribution of virus types for the nine and six month periods are shown in table 11. Again the percentage isolation rates for poliovirus and Coxsackie viruses are similar in both groups. As no ECHO viruses were isolated from the control group the rate for the diarrhoeal children varied between three and seven times this, depending upon the period under consideration.

No significant differences were observed in the total isolation-rates of each type of virus at three different age levels within the entire diarrhoea group (Table 12), although

TABLE 11

Isolation-rates and distribution of virus types isolated from stools of diarrhoeal and respiratory (control) children during nine months from July 1958, and six months from October 1958.

	Diarrhoea		Respiratory control (6 months)	Total series.
	9 months	6 months		
Total cases	236	154	45	281
Total isolations	34 (14.3%)	8 (5.3%)	1 (2.2%)	35 (12.5%)
ECHO virus	18 (7.6%)	5 (3.3%)	-	18 (6.4%)
Poliovirus	13 (5.5%)	3 (2%)	1 (2.2%)	14 (5%)
Coxsackie virus	3 (1.2%)	-	-	3 (1%)
Other agents	-	-	-	-

TABLE 12

Frequency of isolation of viruses from stools of 674 children admitted to hospital with diarrhoea at different age levels.

	Age (yr).	No. in group	ECHO virus	Poliovirus	Coxsackie virus	Other agent	Totals
1957-58	<1	195	15 (7.5%)	12 (6%)	7 (3.5%)	8* (4%)	42 (21.5%)
	1-2	99	11 (11%)	5 (5%)	7 (7%)	2 (2%)	25 (26%)
	3-5	44	3 (7%)	3 (7%)	2 (4.5%)	-	8 (18.5%)
	Total	338	29 (8.5%)	20 (5.9%)	16 (4.7%)	10* (7.9%)	75 (22%)
1958-59	<1	113	7 (5.3%)	6 (5.3%)	2 (1.8%)	-	15 (14%)
	1-2	89	9 (10%)	5 (5.5%)	-	-	14 (15.5%)
	3-5	34	2 (5.7%)	2 (5.7%)	1 (2.8%)	-	5 (14%)
	Total	236	18 (7.6%)	13 (5.5%)	3 (1.2%)	-	34 (14.3%)
Combined total		674	47 (7%)	33 (5%)	19 (2.8%)	10 (1.5%)	109 (16.2%)

* includes one adenovirus type 2.

a greater number of ECHO and Coxsackie viruses was isolated in the 1-2 year age group in the first year and in the second period the same age group yielded the greatest number of ECHO viruses. In the first year the majority of unidentified agents were found in children of less than 1 year.

When the total isolation rates at 3 different age levels were considered for the control group, the greatest number of ECHO viruses was again encountered in the 1-2 year age group during the first period but a striking absence of viruses in all age groups was observed during the second period (table 13).

The distribution of types within each virus group is shown in table 14 for both diarrhoea and control groups. The predominance of type 1 in the polioviruses reflects the predominant type causing paralytic illness in West Scotland during the entire survey period. An outbreak of infection caused by Coxsackie A9 in February, 1958, in the Glasgow area was reflected by the increase in numbers of isolations in both diarrhoea and control groups. Throughout the survey type 7 was the most frequently encountered ECHO virus.

Whereas a virus was isolated from the stool of 16% of diarrhoeal children during the entire survey, a bacterium was found in 30% of 201 instances. The distribution of bacterial types encountered is shown in table 15. The highest isolation

TABLE 13

Frequency of isolation of viruses from stools of 160 children admitted to hospital with acute respiratory illness (control group) at different age levels.

	Age (yrs).	No. in group	ECHO virus	Poliovirus	Coxsackie virus	Other agent	Totals
1957-58	<1	66	1 (1.5%)	5 (7.5%)	4 (6%)	-	10 (15%)
	1-2	33	2 (6%)	3 (9%)	2 (6%)	-	7 (21%)
	3-5	16	-	-	-	-	-
	Totals	115	3 (2.6%)	8 (7%)	6 (5.2%)	-	17 (14%)
1958-59	<1	12	-	-	-	-	-
	1-2	21	-	-	-	-	-
	3-5	12	-	1 (8%)	-	-	1 (8%)
	Totals	45	-	1 (<1%)	-	-	1 (<1%)
Combined total		160	3 (2%)	9 (5.6%)	6 (4%)	-	18 (11.5%)

TABLE 14

Numbers of isolations from diarrhoea and respiratory (control) groups classified by virus type.

Virus type	1957-58		1958-59	
	Diarrhoea	Control	Diarrhoea	Control
ECHO 6	1	-	-	-
ECHO 7	21	3	16	-
ECHO 9	1	-	-	-
ECHO 11	2	-	-	-
ECHO 13	4	-	1	-
ECHO 14	-	-	1	-
Coxsackie B3	1	-	1	-
Coxsackie B4	-	-	2	-
Coxsackie A9	15	5	-	-
Poliovirus 1	18	6	13	1
Poliovirus 2	-	1	-	-
Poliovirus 3	2	2	-	-
Unidentified agents	10	-	-	-

TABLE 15

Frequency of isolation of a pathogenic bacterium from the stools of 674 children admitted to hospital with diarrhoea at different age levels.

	Age (yr.)	No. in group	Sh. sonnei	Sh.flexneir	Pathogenic Esch.coli	Other	Total
1957-58	<1	195	21 (11%)	5 (2.5%)	15 (7.5%)	3* (1.5%)	44 (22.5%)
	1-2	99	45 (46.5%)	7 (7%)	-	-	52 (52%)
	3-5	44	25 (57%)	2 (4.5%)	-	1ǂ (2%)	28 (63.5%)
	Total	338	91 (27%)	14 (4%)	15 (4.5%)	4 (<1%)	124 (36.5%)
1958-59	<1	113	13 (11.5%)	3 (2.5%)	15 (13%)	1*- 1ǂ (1.7%)	33 (28%)
	1-2	89	24 (27%)	5 (5.5%)	-	-	29 (32%)
	3-5	34	14 (40%)	1 (2.8%)	-	-	15 (44%)
	Total	236	51 (21%)	9 (3.8%)	15 (6.5%)	2 (<1%)	77 (32%)
Combined total		674	142 (21%)	23 (4%)	30 (4.4%)	6 (<1%)	201 (30%)

* A staphylococcus was isolated in each instance.
ǂ This organism was a member of the salmonella group.

rate of pathogenic Escherichia coli occurred, as expected, in the under 1 age-group, while in both seasons the proportion of Shigella sonnei increased to a maximum in the 3-5 year group. The overall incidence of each type of bacterium remained reasonably constant during both seasons.

A specific intestinal pathogenic bacterium and a virus were isolated from the same stool specimen in 30 instances (4.6%) in the diarrhoea group. No systematic bacterial examinations were performed on stools from the control group without diarrhoea and therefore the corresponding rate of double isolations cannot be calculated. The types of combination found in the diarrhoea group are shown in table 16. By far the commonest finding was the combination of Sh.sonnei with Coxsackie A9, ECHO 7 or poliovirus type 1.

During the first year of the survey a total of 9 unidentified agents was isolated from the faeces of patients in the diarrhoea group. These were not neutralized by antiserum prepared against the 3 polioviruses, Coxsackie types B1-5 and A9, ECHO types 1-14, and adenovirus types 1-8. Each agent was examined for pathogenicity in suckling and adult mice. One was found to paralyse sucklings but no effects were produced in adults by any of these agents. Before attempts could be completed to identify these viruses by further neutralization tests using antiserum to higher numbered ECHO viruses the entire group

TABLE 16

Virus and bacterial types found in the same stool specimen from 674 children admitted to hospital with diarrhoea.

Virus type	Bacterial type			
	Sh.sonnei	Sh.flexneri	Pathogenic Esch.coli.	Other
ECHO 7	7	1	1	-
ECHO 9	1	-	-	-
ECHO 13	1	-	-	-
Coxsackie A9	9	1	-	1
Poliovirus 1	4	-	1	1
Other	-	1	1	-

was accidentally destroyed. No unidentifiable agents were recovered from the stools of children in either the second part of the diarrhoea survey or in the entire control group.

CHAPTER IX

DISCUSSION AND CONCLUSIONS

The proof that a causal relationship exists between one enterovirus and the disease it may have produced depends largely on two factors. Firstly the agent must be isolated from cases of the disease with sufficient frequency to demonstrate a significant association; secondly there must be a demonstrable significant increase in antibody titre during the illness to the agent isolated. In the case of diarrhoea evidence is being accumulated which strongly suggests that a causal association may exist with certain enteroviruses.

Eichenwald, Ababio, Arky and Hartman (1958), both by demonstrating rising antibody titres and by virus isolation, were able to state categorically that ECHO virus type 18 was the causal agent of two related outbreaks of diarrhoea among young children in hospital. Ramos-Alvarez & Sabin (1958) were also able to show a rise in antibody titre to the agent isolated from the stool in 24 young persons with diarrhoea. The agents in their group included several strains of ECHO virus, Coxsackie B2, B4, B5 and A9, and poliovirus type 1.

During the first year of the present investigation no attempt was made to undertake antibody studies on the children included in the diarrhoea group. The numbers of agents which

were isolated during the first year made it desirable however, to attempt such an antibody survey in the ensuing 9 months. The greatest difficulty was found in attempting to organise such a study for several reasons. Firstly, most of the children were admitted to hospital for a very short time, and almost always for less than one week. As the onset of the diarrhoea was frequently explosive in nature the child was usually admitted to hospital within 48 hours of onset. This meant that dismissal had taken place before antibody might reasonably be expected to have developed, and as the nature of the illness was so comparatively slight, it was not possible to organise a follow up attendance as an outpatient. Secondly, the majority of patients yielded negative virological results from their stools in tissue culture and, although an attempt was made in every instance to examine the stool specimen immediately it was received, it was not always possible to say whether a positive or negative result had been obtained in tissue culture within the limits of the patient's stay in hospital. Equally it was out of the question from the point of view of ward and laboratory staff to bleed each and every child admitted on two occasions in the hope that a virus might be isolated subsequently from the stool. Thirdly and lastly, very many of the children were under one year old and without some selection procedure such as a positive stool culture in tissue culture it would have been pointless to attempt

jugular or fontanelle puncture as a routine on every young child to obtain blood specimens.

Within these limitations a small antibody survey was attempted; almost invariably the blood came from two sources. In a small number of instances tissue culture had produced a virus within the duration of stay in hospital and these children had two venipunctures at each of which a small quantity of blood was withdrawn and allowed to clot. The other type of case examined serologically was the one which had a more severe illness which necessitated residence in hospital for more than one week. In the course of the second week these children had a second venipuncture at which a small amount of blood was withdrawn and allowed to clot. The separated sera in each instance were inactivated and frozen pending testing. Due to the size of the child and the technical difficulty involved in obtaining the samples the 1-2 ml. optimum was rarely obtained. In practice it was only feasible in most instances to test for one antibody and because of this shortage of serum the tests were directed at finding antibody to the most commonly encountered enterovirus during the latter part of the diarrhoea survey - ECHO type 7.

Altogether 42 paired sera were collected in this way and the results obtained from testing these for neutralizing antibody to ECHO type 7 virus are shown in table 17. Only four

specimens were obtained from cases from which the homologous virus had been isolated. One case gave a stable, high titre of antibody at 128 in both sera, another showed a four-fold fall in titre over 10 days and the remaining two were negative. Of 38 paired sera collected from "ECHO 7 negative" cases, two showed a four-fold rising titre, and two a high static titre. One of these latter had a titre of >128 in both specimens but poliovirus type 1 grew from the stool. 3/4 paired sera had titres of less than 8. In this very small series half of those persons from whom ECHO type 7 virus was grown had increase in homologous antibody titre in at least one specimen whereas 3% of persons from whom the virus was not isolated also showed an increase in antibody titre commensurate with infection with the virus. The number of cases involved is too small however, to allow any conclusions to be drawn. The greater number of ECHO viruses (and untyped agents) isolated from the diarrhoea group in both parts of the survey is suggestive that a causal association may exist. Despite the inconclusive serological results the suggestion is particularly strong in the case of ECHO 7 which was isolated more times than any other virus. If the two parts of the survey are restricted to only those months in which control studies were being carried out, however, the number of ECHO viruses isolated is not statistically significantly greater than in the controls. Neither is there any significance

TABLE 17

Incidence of ECHO 7 antibody in children with diarrhoea who gave positive or negative stool cultures for ECHO virus type 7.

	Neutralization Test. Antibody titre			
	Nil	Rising x4	High static (>32)	Falling x4
ECHO 7 virus isolated	2	0	1	1
Tissue culture negative	34	2	2*	0

* includes one child from whom poliovirus type 1 was isolated.

in the number of polioviruses isolated. Their presence in the stools of children in hospital probably only reflects the very large amount of latent poliovirus infection in the community.

Whether the possibility of a causal association between viruses and diarrhoea is accepted or not, however, it is of interest to compare the rates of isolation of viruses and bacteria in relation to different age groups as in table 18. The smaller number of viruses isolated, which represents an approximately constant proportion of the population at each age level, contrasts sharply with the increasing proportion of bacteria as the age of the patient increases. Also of interest is the increasing number of total isolations as the age of the patient increases. As already mentioned the lowest age group contains a high proportion of children who are admitted to hospital with a poor social background and a mild feeding disorder. These facts probably contribute towards the apparently lower total numbers of isolations in the youngest children.

TABLE 18

The frequency of isolation of a virus or a pathogenic bacterium from the stools of 674 children at different age levels admitted to hospital with diarrhoea.

Age (yr.)	No. in group	Bacterium	Virus	Total isolations
<1	308	77 (25%)	57 (18.5%)	134 (43.5%)
1-2	188	81 (42.6%)	39 (20.5%)	120 (63%)
3-5	78	43 (55%)	13 (16.6%)	56 (72%)
Totals	674	201 (30%)	109 (16%)	310 (47.5%)

PART 3

AN EXAMINATION OF SOME FUNDAMENTAL PROPERTIES OF THE ECHO VIRUS GROUP.

CHAPTER X

A REVIEW OF EXISTING KNOWLEDGE ABOUT ECHO VIRUSES.

The total available literature on this topic is very small for two reasons. Firstly, the ECHO virus group was initially recognised as recently as 1955 and secondly because the greatest amount of investigative work on the group has undoubtedly gone into the questions of their pathogenicity and assessment of their distribution in healthy and diseased populations at different age levels.

The group contains at least twentyfour viruses most of which are antigenically distinct. The viruses all grow preferentially in freshly trypsinised rhesus or cynomolgous monkey kidney cells or in human amnion cells. They were shown to grow in human carcinoma cells type Maben by Ormsbee and Melnick (1957) and they were adapted to grow in strain HeLa cells by Archetti, Weston & Wenner (1957). I have not observed any evidence of growth by unadapted prototype strains in our own stocks of HeLa cells.

The size of a number of the ECHO viruses has been estimated by ultrafiltration experiments by Benyesh, Pollard, Opton, Black, Bellamy and Melnick (1958). With the exception of ECHO type 10 all the other viruses examined behaved similarly and appeared to have a particle diameter of approximately 24 mμ.

The size of the ECHO type 1 virus particle was determined by the use of ionizing radiation of various types by Benyesh et al. (1958). They studied the inactivation of two different properties of the virus at 4°C. (infectivity and complement fixing antigen) following bombardment with high energy electrons. Infective virus was found to have a diameter of 26 mμ by calculating the amount of energy necessary to bring the infective component to the 37% survival point. By the same method the size of the complement fixing antigen was estimated to be 13 mμ. The same authors examined the size of the complement fixing antigens of several ECHO viruses and found that they had a size range varying between 7 and 13 mμ, which is the size of a moderately large protein molecule.

Archetti et al. (1957) demonstrated the presence of a complement fixing antigen in HeLa cell grown ECHO virus cultures up to type 13. The higher numbered types had not been isolated at this time. ECHO type 4 which grew very poorly in HeLa cells could not be shown to possess a complement fixing antigen.

A number of ECHO viruses possess the ability to agglutinate suspensions of human group O erythrocytes. Goldfield, Srihongse, and Fox (1957) found agglutinin in cultures of ECHO types 3, 6, 7, 10, 11 and 12. Their observations suggested that the agglutinin is intimately associated with the infectious virus particle because both sedimented at the same rate in the

ultracentrifuge, and are adsorbed together by erythrocytes during the process of agglutination. They claimed that virus and agglutinin were able to elute from the red cells and that the red cells became exhausted in the process. Although the red cells were no longer agglutinable by the same strain of virus a receptor gradient was present in that the red cells could still be agglutinated by certain other ECHO viruses. The group can be subdivided by the optimum temperature at which agglutination occurs. Types 3, 4, 6, and 11 all agglutinate optimally at 25°C. whereas types 7 and 12 give identical agglutinin titres at 25 and 37°C.

Although the prototype ECHO 6 (D'Amori) strain does not agglutinate human group O cells, Lahelle (1958) demonstrated that a number of locally isolated Scandinavian strains from cases of aseptic meningitis did possess agglutinin. He claimed that the agglutinating strains would also elute. My own observations on the haemagglutinin of ECHO type 7 virus are recorded in this part of the thesis. This differs from that of ECHO type 6 in that it can not be eluted by any of the conventional techniques.

The process of infection of susceptible cell cultures by ECHO viruses was described in detail by Ormsbee and Melnick (1957). The initial lesion tends to occur as a discrete focus of degeneration. Scattered degenerate cells quickly appear

around each original focus, and these in turn each become the centre of a small zone of degeneration. Neighbouring zones then coalesce and the culture gradually exhibits the characteristic complete cytopathic effect. I have also published a detailed description of this process dealing in particular with the instance where susceptible cultures are infected with a small number of virus particles. This work is included in my description of the micro-plaque technique for the enumeration of ECHO virus particles (Sommerville, 1959 a) and a description of the technique itself is contained in the appropriate chapter of the thesis.

The actual cytopathic effect produced in the unstained tissue culture tube cannot be distinguished from that produced by poliovirus although in general the stage of complete cytopathogenicity is reached more slowly than with poliovirus. The cytopathic effect of enteroviruses in general is however distinct and characteristic. The stained histological changes produced by the growth of ECHO viruses in susceptible cells have also been described by Melnick (1958) as being indistinguishable from those produced by polioviruses. This is not so, and a description of the changes is contained in the following pages. This information is also being published (Sommerville, 1959 c).

Enteroviruses in general have the property of producing plaques in cultures of monkey kidney cells. With the ECHO

viruses these usually appear more slowly than with polioviruses. By the standard agar method described by Hsiung and Melnick (1957) in this connection ECHO viruses can be segregated into 4 groups by their ability or otherwise to form plaques. The groups depend upon the size and shape of the plaques and the speed with which they grow to a measurable size. Large plaques which appear and grow rapidly and have smooth circular outlines closely resembling those produced by polioviruses are produced by ECHO types 7 (Garnett and Wallace strains), 8 (Bryson) and 12 (Travis). Types 1 (Farouk) and 13 (Hamphill) produce plaques of medium size and irregular boundaries between 4 and 5 days whereas very small plaques of completely irregular shape were produced by types 3 (Berradi), 4 (Pesascek), 6 (Di Meo), 9 (Quigley), 11 (Gregory) and 14 (Tow). After repeated testing types 2 (Cornelis), 3 (Morrisey), 5 (Noyce), 6 (D'Amori) and 10 (Lang) have not produced plaques.

The reasons for the differences in plaque producing potential have been examined in detail and the results obtained form the subject matter of the greater part of this section of my thesis.

CHAPTER XI

THE GROWTH CYCLE OF ECHO VIRUS TYPES 1, 2, 7 AND 11 IN TISSUE CULTURES OF MONKEY KIDNEY CELLS.

(a) Maturation and release of the viruses from monolayer cultures.

I thought that the likeliest explanation for the differences in plaque potential between the four groups of ECHO viruses defined by Hsiung & Melnick (1957) was that the viruses had different rates of interaction with host cells. This might take the form of different rates either of maturation and release or of attachment to the cells composing the monolayers. The first possibility, that the actual rate at which fully infectious virus was formed in infected cells and the speed with which this virus was released would subsequently affect the total number of contiguous cells destroyed and the size of the plaque produced, was the favoured one. A number of experiments designed to observe the length of the growth cycle and the rate of release of the newly formed virus from the cell were performed. In these experiments one member of each plaque-producing group was selected quite arbitrarily for study. The viruses chosen and the data relevant to their plaque producing potential are recorded in table 19.

ECHO type 7 gives large circular plaques which are clearly demarcated by a smooth edge from the surrounding normal

tissue and which are distinguishable only by their slow rate of appearance from the plaques produced by polioviruses. ECHO type 1 produced clearly marked but smaller plaques which have very irregular edges. ECHO type 11 produced tiny plaques with irregular boundaries. These appear much more slowly than the plaques of ECHO type 1. ECHO type 2 does not produce plaques at all.

TABLE 19

Plaque-producing characteristics of the ECHO virus prototype strains selected for study.

Virus type	Designation	Days to plaque appearance	Average diameter of plaque at 10 days after seeding (mm.)
ECHO 1	Farouk	4.5	5.6
ECHO 2	Cornelis	not produced	-
ECHO 7	Wallace	3-4	11.4
ECHO 11	Gregory	7-10	- *

* 1.5 - 2 mm. measured at 13 days.

MATERIALS AND METHODS

Viruses

Each prototype strain selected for study had been

sent by Dr. G.C. Brown, University of Michigan. ECHO 2 was in its 13th monkey kidney passage and the other three had been passed 11 times in monkey kidney. Each had been passed three times at limiting dilution and bulk stock had been built up rapidly thereafter by the inoculation of each virus into and subsequent harvesting of 200 monkey kidney tubes. The pooled material had been frozen and thawed rapidly three times before centrifuging to remove cell debris. Immediately before use in the growth cycle experiments the infectivity titre of each virus was ascertained in monkey kidney cells.

Media

Growth medium (GM), Maintenance medium (MM), phosphate buffered saline (PBS) and trypsin have already been described in Part I.

Preparation of primary monkey kidney culture

This has also been described in Part I. Rhesus monkey kidneys were used throughout these experiments. The cells were grown in 12 oz. "medical flat" bottles with screw caps or in 12 x 100 mm. test tubes with red rubber bungs. One or two changes of GM were required before confluent monolayers were produced in bottles, usually after 7-10 days incubation at 36°C. Test tube cultures grew readily into a monolayer without a change of medium, usually within 7 days.

Preparation of secondary monkey kidney cultures for growth-cycle experiments.

Monolayers in 12 oz. bottles were washed twice with PBS and were then suspended by incubating for up to 30 minutes in 1:5,000 sodium versenate solution at 36°C. The suspended cells were washed by low-speed centrifugation, re-suspended in growth medium and an aliquot was counted in a haemocytometer. A concentration of 3×10^6 cells were added in 8 ml. of GM to each 9 cm. Petri dish and these were incubated in an atmosphere consisting of 95% air and 5% CO_2 for 3 days.

Plan of experiment

Each growth-cycle experiment can be most easily described in four stages.

1. Infection of the monolayer

These were secondary cultures prepared in petri dishes and grown in a humidified air/CO_2 atmosphere. For each virus two growth cycle experiments were run simultaneously. This necessitated the use of 20 Petri dishes per virus. Each monolayer was washed twice with PBS. Two monolayers were then removed with versene and the number of cells present were counted in a haemocytometer to obtain the average number of cells per petri-dish. To each of the remaining 18 dishes virus was added in the form of a 0.5ml. inoculum containing 4 ID_{50} per cell of the monolayer. Each dish was incubated for 1 hour at 37°C. in

the 5% CO_2/air mixture with gentle agitation in two planes every 5 minutes to ensure even dispersion of the inoculum. After one hour's adsorption the inoculum was removed and each plate washed rapidly 5 times with 10 ml. PBS at 4°C. each time. Thereafter 5 ml. GM was added and the plate returned to the 5% CO_2/air atmosphere.

2. Method of harvesting aliquots for infectivity titrations.

Beginning one hour after the virus inoculum had been removed the supernatant fluid from 2 petri dishes was removed completely at hourly intervals for 8 hours, and again at 24 hours. Fluids removed 6 hrs. or more from the start of the experiment were centrifuged at 3,000 r.p.m. for 5 min. in an M.S.E. Minor angle centrifuge to remove any cells which might have become detached from the glass as a result of infection. Each aliquot was then frozen at -40°C.

The monolayer then had 5 ml. GM added and the cells were completely scraped off by a rubber-tipped glass rod. Where cells had been found in the supernatant fluid the packed cell pellet remaining after centrifugation of the supernatant fluid was returned to the cell fraction which was then also frozen at -40°C.

3. Method for disruption of cells to obtain "cell associated" virus.

Each cell fraction was thawed and centrifuged to deposit the cells. The packed cells were then separated from

the fluid which was retained. The cell fraction was ground in a Ten Broeck grinder for 5 minutes before being returned to the separated fluid for infectivity titration. Experiments in the early phase of this work revealed that after one cycle of freezing and thawing followed by 5 minutes of grinding complete disruption of the cells occurred.

4. Virus titrations.

The amount of virus present in each duplicate sample collected hourly and consisting of either supernatant fluid or disintegrated cells was then estimated by infectivity titration in monkey kidney cells. This method was chosen deliberately in an effort to standardise technique in view particularly of the inability of ECHO type 2 virus to produce plaques. Serial dilutions of each fraction were titrated using 5 tubes per dilution and an inoculum of 0.1 ml. per tube. The tubes were read microscopically after 4 days incubation at 36°C. and the results were graded 0 to 4+ (++++) according to the degree of cytopathic effect present. The infectivity titre was then calculated in terms of 50 per cent infectious doses (ID_{50}) by the method of Kaber (1931).

Results

The results which were obtained from 2 identical experiments with each virus are shown graphically in figures 3-6 and average figures obtained for selected characteristics of

the growth cycles of each virus are shown in Table 20.

TABLE 20

Properties of ECHO virus types 1, 2, 7 and 11 derived from growth-cycle experiments in monkey kidney cell monolayers.

Virus type	Cell associated virus			Free virus	
	First increase (Hr.)	Average maturation time (hr.)	Average yield per cell (ID_{50})	First increase (hr.)	Proportion Free:total virus at average maturation time (per cent)
ECHO 2	3	7.3	496	6	1.5
ECHO 11	5	7.2	142	5.5	2.6
ECHO 1	2	3.2	0.34	7	1.2
ECHO 7	4.5	7.05	59.5	5	0.08

Cell associated virus

The most important point which arises in connection with this virus fraction is that, despite the input multiplicity of 4 virus particles to each cell of the culture, only a small proportion of cells were actually infected. This proportion is lowest with ECHO type 2 and greatest with ECHO types 7 and 11 but even with these the total number of cells infected falls well short of 100%. The results which were obtained have therefore to be interpreted in the light of these initial

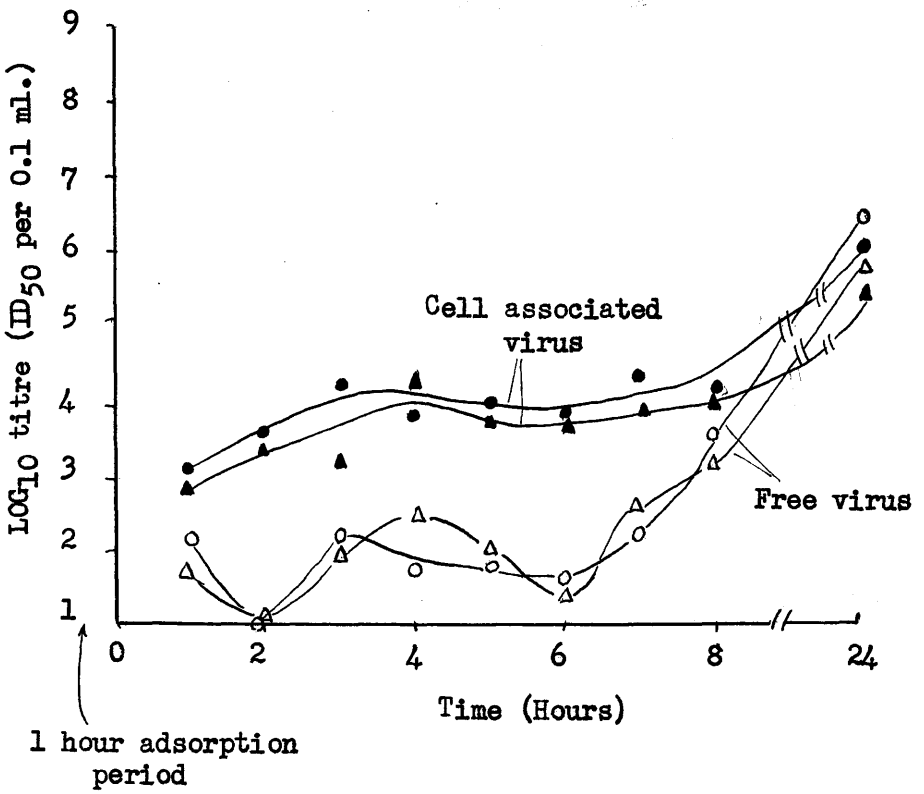


Figure 3

The growth cycle of ECHO virus type 1 in monkey kidney cells. (The results of two identical experiments are shown.)

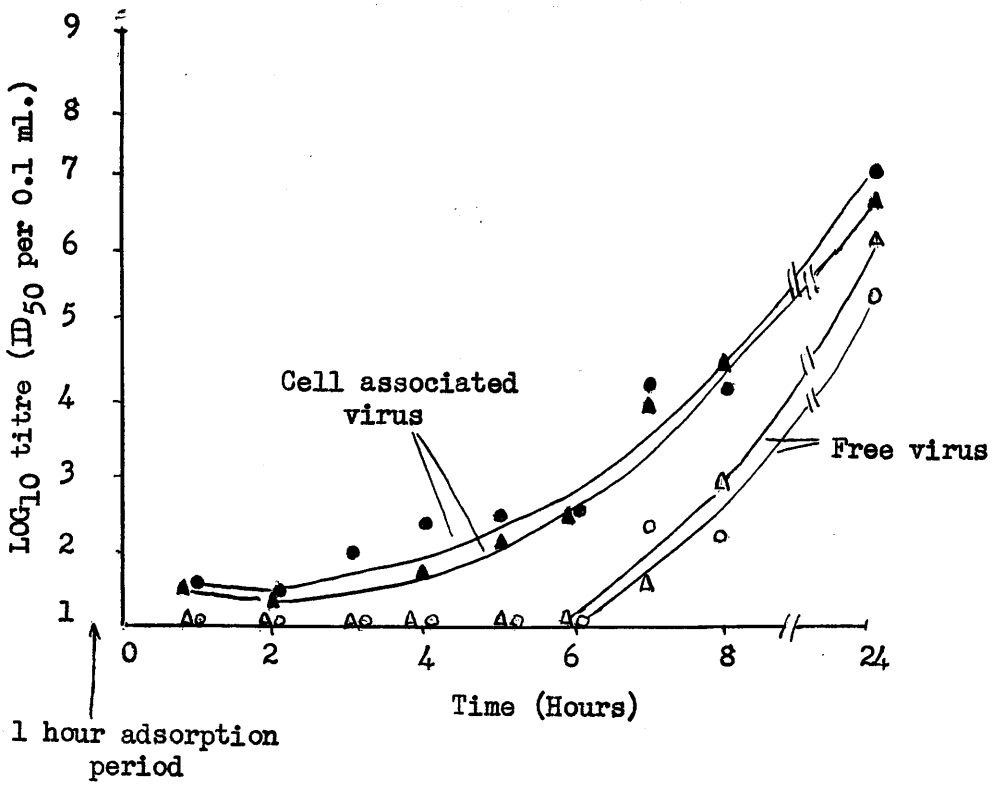


Figure 4

The growth cycle of ECHO virus type 2 in monkey kidney cells. (The results of two identical experiments are shown).

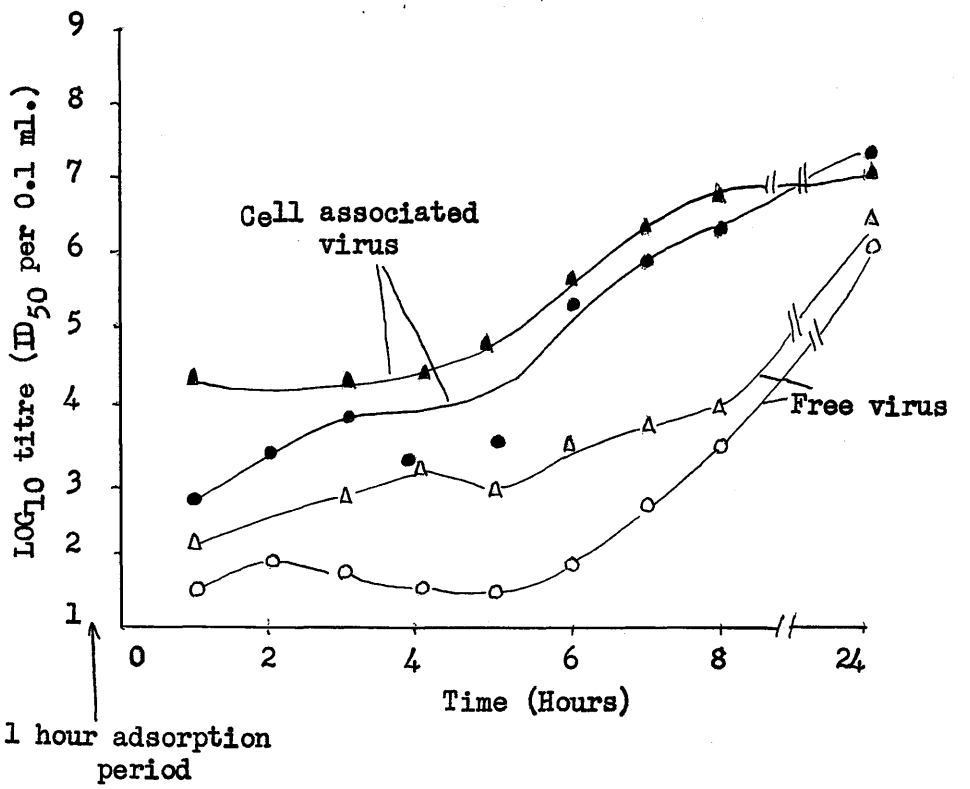


Figure 5

The growth cycle of ECHO virus type 7 in monkey kidney cells. (The results of two identical experiments are shown).

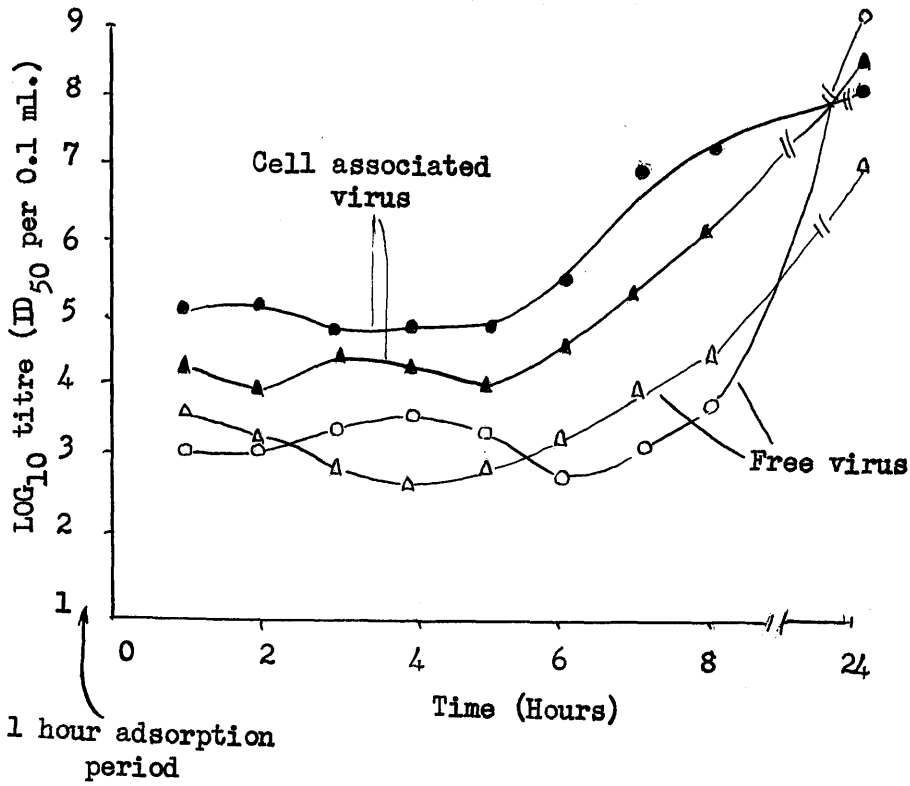


Figure 6

The growth cycle of ECHO virus type 11 in monkey kidney cells. (The results of two identical experiments are shown).

observations.

Although each growth-cycle experiment was continued for 24 hours the end of each cycle was taken to be the point at which the amount of virus contained within the cells reached its maximum. As will be seen from figures 3-6 this point was reached at about 8 hours in every instance and the figures obtained at 24 hours are merely included in the graphs for interest. The calculations in table 20 are based on an 8-hour end-point for each experiment. One parameter which has been used in Table 20 and which requires explanation is the average maturation time. This is a useful measurement in connection with growth cycle experiments and has been defined by Howes and Melnick (1957) as "the time elapsing between inoculation of virus and the appearance of 50 per cent of the total mature (infectious) virus produced by the cells". The figures are obtained graphically but are based on an initial calculation of 50% of the total virus produced by 8 hours. The shorter average maturation time was observed with ECHO type 1, and this is probably consistent with the appearance in figure 3 of an increase in the cell associated virus fraction from the second hour of the experiment. With the other 3 viruses a lag-phase ranging in duration from 3-5 hours was evident before cell-associated virus began to increase in amount. Although an average maturation time of 7.3 hours is recorded in table 20 for ECHO type 2, it will be seen from a

comparison of figure 4 with figures 3, 5 and 6, that by 8 hours the curves obtained for cell-associated virus with ECHO type 2 show least evidence of the levelling which would suggest the end of the growth cycle. It is certainly possible that, in this instance, the growth cycle was not complete at this stage and a more accurate representation of the average maturation time for ECHO 2 virus would be greater than the 7.3 hours recorded. By 8 hours, however, the average yield of virus per cell of the culture was so much greater than for any of the other viruses that it was felt to be justifiable to retain an 8 hour end point for the experiment.

It is of interest also to note that despite the average maturation time of 3.2 hours recorded in table 20 for ECHO type 1, by 8 hours only an average of 0.34 ID_{50} of virus per cell had been produced. This should be contrasted with the greater average maturation times observed for the other 3 viruses and compared with the associated greater average yields of virus per cell.

Free virus

The first increases in free virus occur 5 and 3 hours after cell associated virus production has commenced with types 1 and 2, whereas the delay in appearance of free virus is reduced to 0.5 hour for types 7 and 11.

The proportion of free virus produced by the average maturation time in each instance is very low indeed and this is taken to indicate either a delay in the release of cell-associated virus or rapid re-adsorption of virus already released to neighbouring cells in the monolayers.

Marked fluctuations occur in the curves for free virus in the early stages of both experiments with ECHO 1 (figure 3). One possible explanation of this behaviour might be that the virus enters into a phase of unstable equilibrium in its relationship with the cell surface before penetration of the cell wall occurs. The repeated washing at the end of the hour-long adsorption period may disturb this equilibrium and cause the release into the fluid phase of the culture of numerous particles which then become re-adsorbed to the cells over the next two hours. As a check that the curious re-adsorption cycle might not have been produced accidentally in the first two experiments another experiment was made in which identical conditions of monolayers and amount of infecting virus were produced. After one hour's adsorption the cultures were again washed five times with ice-cold PBS. Samples consisting of supernatant fluid and cells fractions were collected this time at half hourly intervals. The results are recorded in figure 7 and it will be seen that an identical set of fluctuations occurred in this experiment. The two apparent phases of re-adsorption of free

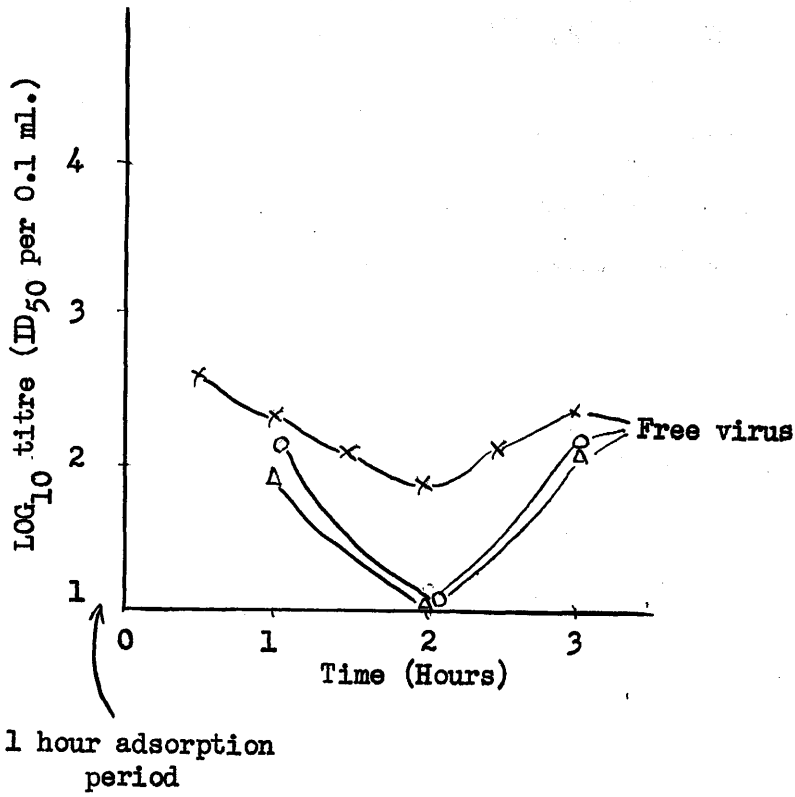


Figure 7

The results obtained from three attempts to examine the behaviour of the "free virus" fraction during the early stages of the growth cycle of ECHO virus type 1.

virus are also of considerable interest in that during this time a build up of cell associated virus was occurring in all three experiments.

Discussion

From a consideration of these results it is apparent that no direct correlation exists between them and the plaque producing properties of the viruses under investigation. If ECHO type 1 be omitted from the consideration, however, an inverse relationship exists between the average yield of virus per cell and the average size of plaque produced by the other 3 viruses. Perhaps the most striking anomaly, however, is seen with ECHO type 2 (table 20) where the average yield of virus per cell was recorded as 496 ID_{50} at 8 hours. Although a relatively small proportion of this amount was present as free virus it would be reasonable to assume that this would be perfectly capable of infecting fresh cells, even under agar, and thereby initiating the "chain reaction" which would lead to the production of a plaque. Even if, as suggested by Howes and Melnick (1957), virus released from cells is retained mechanically in an inter-cellular location within the monolayer itself (thus favouring re-adsorption of newly released virus) it would appear reasonable that ECHO virus type 2 should produce plaques perhaps with even greater speed than any of the other three types tested. In an identical series of experiments but

using poliovirus type 1, Howes and Melnick concluded that between 20 and 40 per cent of the virus produced in the monolayer was released directly into the medium from the free surface of the cells. Another possible but rather unlikely explanation in view of results to be reported in another chapter would be that only a small proportion of cells in the monkey kidney monolayer were competent to receive an infecting dose of ECHO type 2. Rubin (1959) describes a situation in which only about 10 per cent of cells (chicken fibroblasts) in a monolayer are competent to receive an infecting dose of Rous sarcoma virus. Given that no direct correlation exists between the growth cycles of the four ECHO viruses and their plaque producing capacity it is of interest to compare the characteristics observed with those reported by Howes & Melnick (1957) for poliovirus type 1. In their experiments cell-associated virus first began to increase after 3 hours and the growth cycle was virtually complete within about 6 hours. The average maturation time was recorded as 5.4 hours. With poliovirus type 1 increase in free virus commenced shortly after cell-associated virus was found to be increasing but by the time 50 per cent of the mature virus had been produced, free virus accounted for only 0.13 per cent of the total virus present. The average maturation times and the relatively short growth cycles observed with the ECHO viruses might also be compared with the 23 to 26

hour growth cycles for adenovirus types 1-4 in HeLa cells reported by Ginsberg (1958).

As with poliovirus type 1 and adenovirus types 1-4 there appears to be a dissociation between the rate of release of free virus from infected cells and the total amount of virus present within the monolayer when ECHO types 1, 2, 7 and 11 are considered. These results represent one type of virus release and should be compared with the findings of Rubin, Baluda and Hotchin (1955) for Western equine encephalomyelitis virus growing in chicken fibroblasts in cultures, when the release of virus apparently occurs very rapidly indeed.

From the evidence produced it appears that the ECHO viruses selected for study as a group closely resemble poliovirus in their type of growth cycle but that a consideration of their growth cycles cannot be invoked to explain the differences in plaque producing potential.

(b) Rates of attachment of the viruses to cells.

Having found that the amount of virus and the rate of its release from infected cells bore no relation to the size of plaque which the virus would produce, I turned to study the possibility that the relevant factor might be a differing affinity between cell receptors and the viruses concerned.

The hypothesis here was as follows:- Two sets of cells were infected each with a different ECHO virus at the same multiplicity. If one set released 1,000 infectious units of virus A per infected cell and these could attach to fresh cells in the culture with an efficiency of only 0.1% in a given time whereas 100 infectious units of virus B could be released from the other set and attach to fresh cells with an efficiency of 100% in the same time, both growth cycles being of identical length, then plaques of very different size and appearance would result within a few growth cycles under agar.

Several methods were tried in attempts to measure the rates of attachment of the four ECHO viruses and although each produced different numerical answers the rates of attachment formed a gradient which was relatively constant by each method.

The methods will be described individually but may

be summarised as follows.

Measurements of the rate of attachment to monkey kidney cells:-

1. Directly to monolayers.

In this method the amount of virus remaining free in the supernatant fluid was measured at varying times after infection.

2. Directly to monolayers by the micro-plaque method.

This measured the actual amount of virus attaching to a monolayer in a given time.

3. Indirectly to monolayers.

In this method the length of time taken by a virus to produce cytopathic effects was measured following adsorption to a susceptible monolayer for varying times.

Each method and the results obtained by it will now be described separately.

1. Measurement of the rate of attachment to monkey kidney cells in monolayers.

Principle

The direct adsorption of each virus was measured over four hours by following the rate of disappearance of virus which was overlaid susceptible cells in fluid medium.

Preparation of monkey kidney monolayers

The cells were grown for this series of experiments

in 6 oz. "medical flat" bottles with screw tops. After 7-10 days of incubation each bottle contained an average of 3×10^6 cells.

Cells from the same monkey were also grown in 12 x 100 mm. test tubes for titration of the infectivity remaining after each time interval.

Plan of the experiment.

For each experiment two bottles, with an average count of 3×10^6 cells, were emptied of old GM and washed twice in freshly prepared GM. A fresh sterile empty bottle was similarly washed. This bottle served as a control for each experiment and was sampled at the same time and with the same frequency as the "test" bottles. In this way the possible attachment of each virus to the glass in the absence of cells was assessed and the effect of repeated sampling on its contained virus could be studied.

The virus under investigation was diluted appropriately in 30 ml. GM previously warmed to $37^{\circ}C$ so that a multiplicity of one virus particle to 1,000 cells would be present in each bottle. Ten ml. of this suspension was pipetted into each of the "test" bottles and into the control bottle.

Each bottle was then laid on its side so that the monolayer was bathed in virus containing fluid and replaced in the incubator at $37^{\circ}C$. The bottles were agitated gently every 15

minutes to ensure dispersion of the virus, and from each bottle a sample of 0.3 ml. fluid was removed at half hourly intervals. This was frozen at -40°C, thawed and titrated for infectivity titre by making appropriate dilutions. Each dilution was inoculated in 0.1 ml. quantity into each of 5 tubes and whenever possible the infectivity titre was estimated in cells from the same monkey. The infectivity titrations were read after 4 days incubation and scored 0 to 4+ (++++) according to the degree of cytopathic effect present. The infectivity titre was then calculated according to the method of Karber (1931).

Results

Throughout the adsorption experiments several parameters have been utilised to characterise the reaction between virus and susceptible cell. The average adsorption time can be defined as the time elapsing between inoculation of the culture and the uptake of 50% of the inoculated virus. The maximal adsorption time and maximal percentage adsorption are self explanatory. The percentage adsorption at 30 and 60 minutes have also been calculated with reference to the graphs of adsorption rate shown in figures 8-11 for each virus. The characteristics of each virus are recorded in table 21. Also included in this table are the results obtained when the adsorption rate of Poliovirus type 1 (Mahoney) was observed experimentally under the same conditions. The adsorption curves obtained for poliovirus type 1 are shown

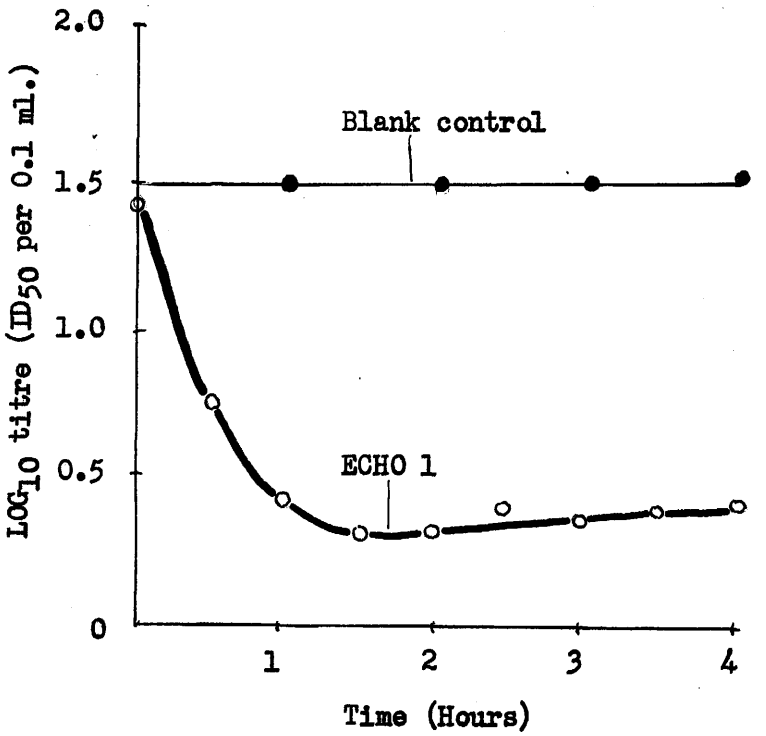


Figure 8

The rate of attachment of ECHO virus type 1 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection.

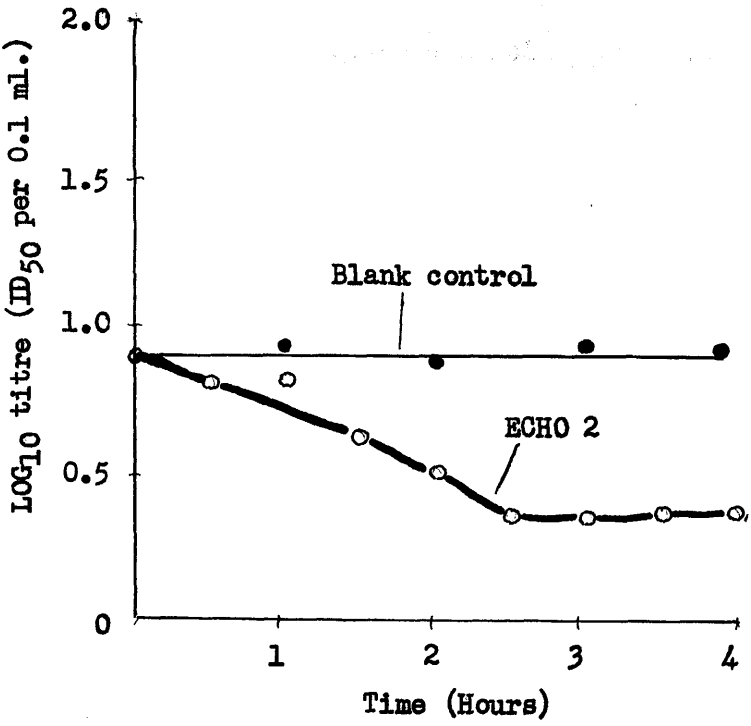


Figure 9

The rate of attachment of ECHO virus type 2 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection.

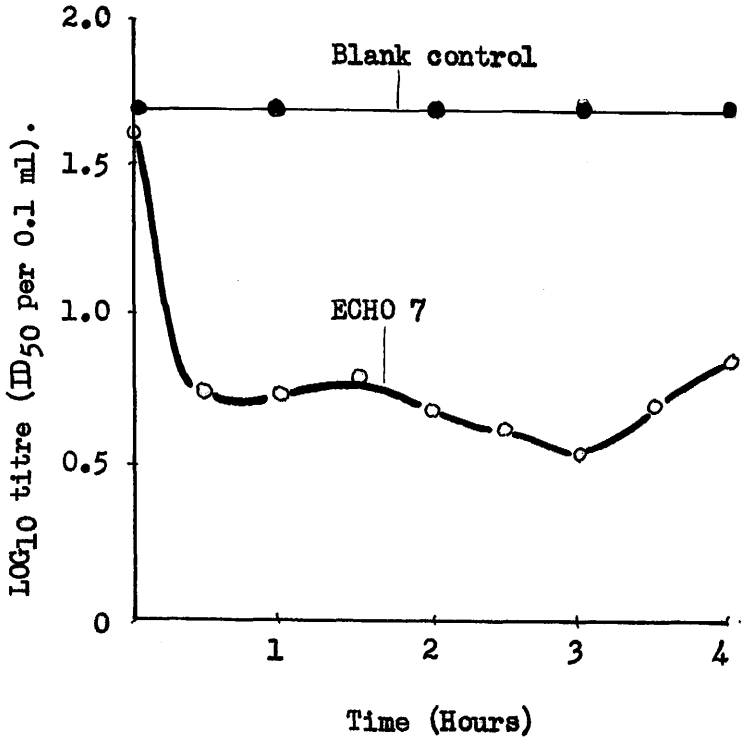


Figure 10

The rate of attachment of ECHO virus type 7 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection.

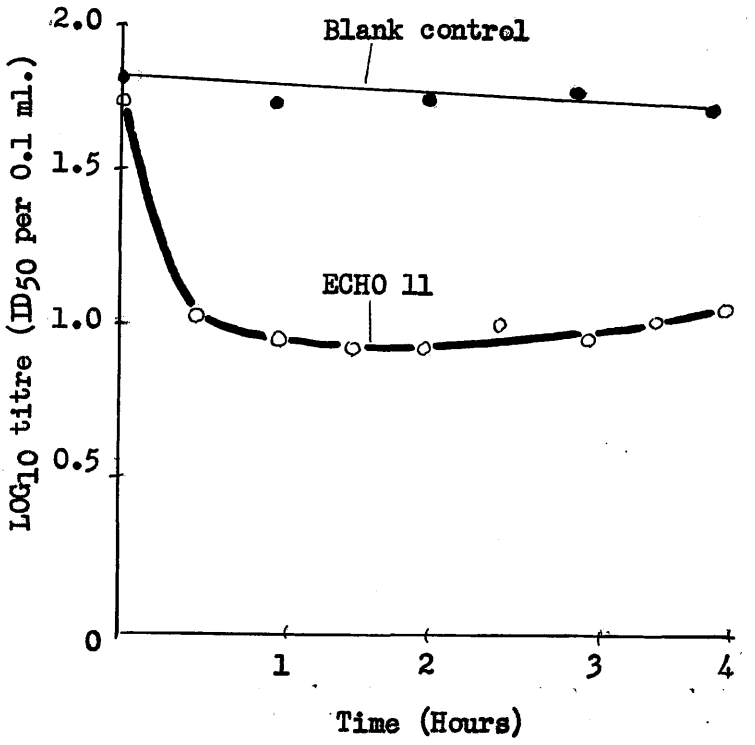


Figure 11

The rate of attachment of ECHO virus type 11 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection.

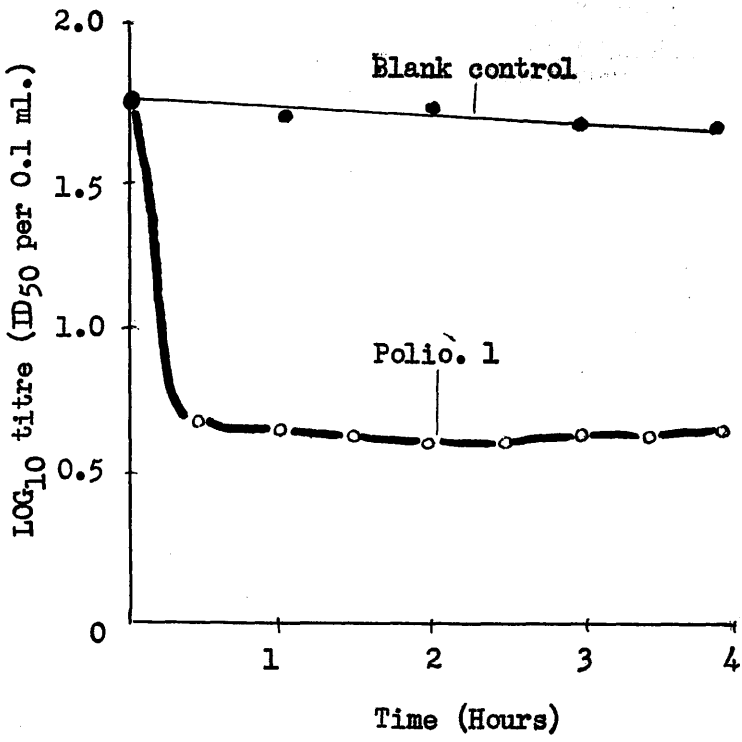


Figure 12

Rate of attachment of poliovirus type 1 (Mahoney) to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection.

in figure 12.

TABLE 21

Properties of ECHO virus types 1, 2, 7 and 11 and Poliovirus type 1 derived from adsorption rates to monkey kidney cells in "bottle" monolayers.

Virus	Average adsorption time (mins).	Adsorption at		Maximum adsorption (hrs.)	Maximum adsorption (percent)
		30 min. (percent)	60 min. (percent)		
ECHO 7	9	81	81	2.5	90
ECHO 11	12	80	84	2.0	85
ECHO 1	12	79	90	2.0	93
ECHO 2	90	19	35	3.0	72
Polio 1	7.5	91	92	1.5	92.5

As can be seen from figures 8-12, when the virus dilution was incubated in a clean sterile bottle and sampled at regular intervals, a remarkably constant result was obtained. Adsorption of virus to the glass can be discounted for practical purposes and under the experimental conditions used, the frequency of sampling did not affect the concentration of virus in any way. Great care was taken to agitate the bottles at regular intervals and in two planes to keep such virus as was unattached

to cells evenly dispersed throughout the medium.

Although the actual figures calculated from the curves for ECHO types 1, 7 and 11 and poliovirus type 1 which are shown in table 21 differ slightly from each other, the adsorption curves for these viruses in figures 8, 10, 11 and 12 are strikingly similar in shape. So far as the shape of the curve is concerned ECHO type 2 is unique. In the four similarly shaped curves there is an initial period of extremely rapid attachment, the duration of which is approximately 30 minutes. The average adsorption time, representing the time at which 50 per cent adsorption has occurred is very short with these viruses and varies from 7.5 minutes for poliovirus type 1 to 12 minutes for ECHO types 1 and 11. Thereafter each curve flattens as the maximum adsorption point is approached. This is reached in from 1.5 hours for poliovirus type 1 to 2.5 hours for ECHO type 7, and the maximum amount of virus adsorbed varied from 85 per cent for ECHO 11 to 92.5 per cent for poliovirus type 1. It will be seen then that these three ECHO viruses behave very similarly to poliovirus type 1 with regard to speed of adsorption to cells.

ECHO type 2 on the other hand has very different properties. The initial period of rapid adsorption with this virus is prolonged to 2.5 hours before the curve shows any evidence of flattening (figure 9). The average adsorption time

is 90 minutes, and maximum adsorption is achieved in 3 hours by which time 72 per cent of virus has become attached to the monolayer.

Discussion

Two points immediately arose from these results. Firstly, from a consideration of the average adsorption times, a possible relationship between plaque characteristics and adsorption rates is evident. This is only a potential relationship, however, because the size and speed of plaque production shows the following descending gradient: ECHO 7, ECHO 1, ECHO 11, (ECHO 2). A similar gradient is apparent in the average adsorption times. Plaques which appear more rapidly than those of ECHO type 7 are produced by poliovirus type 1 and this virus has a correspondingly shorter average adsorption time than ECHO type 7. I think it rather questionable, however, whether a difference in average adsorption times of the magnitude shown between the other ECHO viruses and ECHO type 2 would account for the inability of the latter to produce plaques. Also, if a true relationship were present a difference in the average adsorption times between ECHO 1 and ECHO 11 should have been evident. To elucidate this point a further but similar series of adsorption experiments was undertaken with ECHO types 1, 7 and 11.

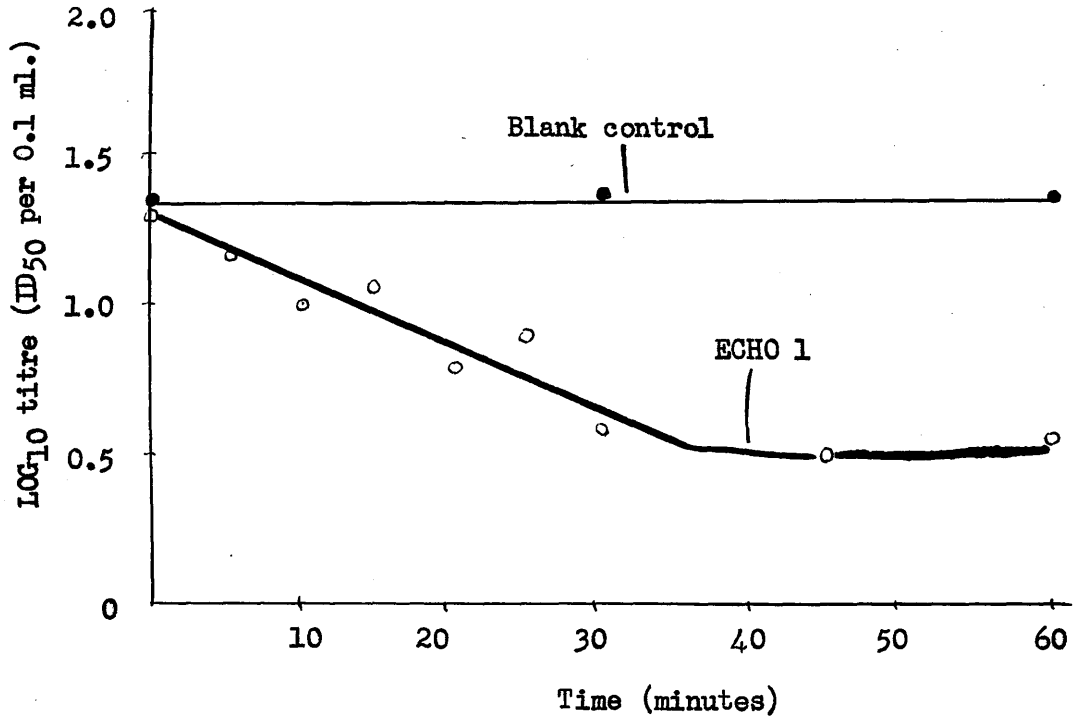


Figure 13

Rate of attachment of ECHO virus type 1 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection. In this experiment samples were taken every five minutes for the first half-hour and the duration of the experiment was limited to one hour.

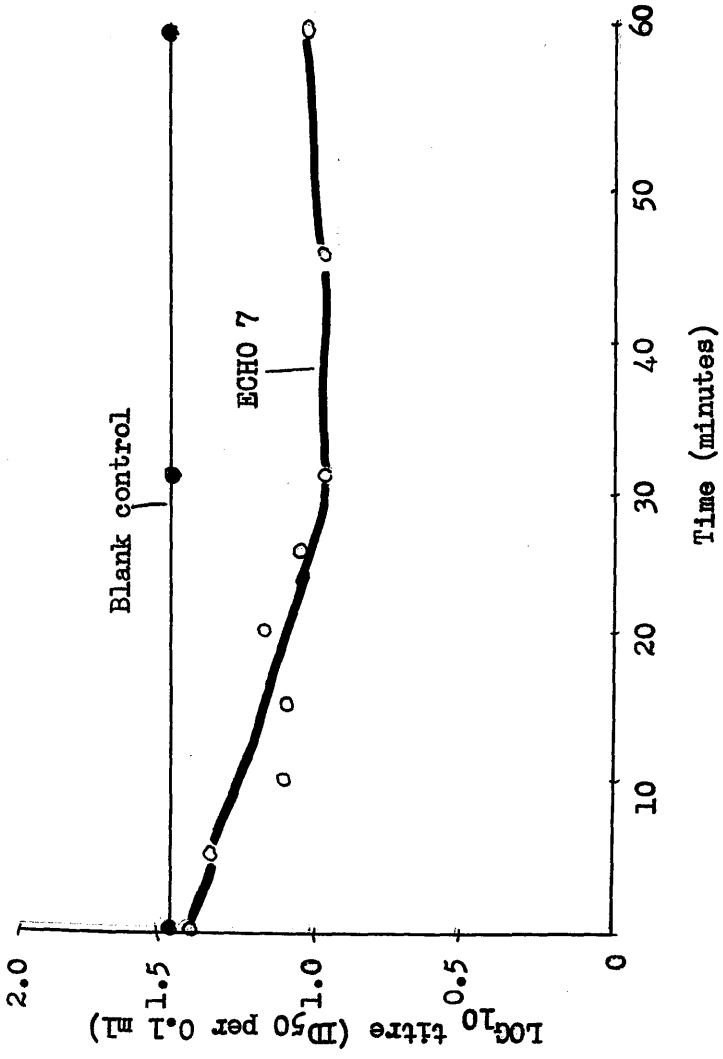


Figure 14

Rate of attachment of ECHO virus type 7 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection. In this experiment samples were taken every five minutes for the first half-hour and the duration of the experiment was limited to one hour.

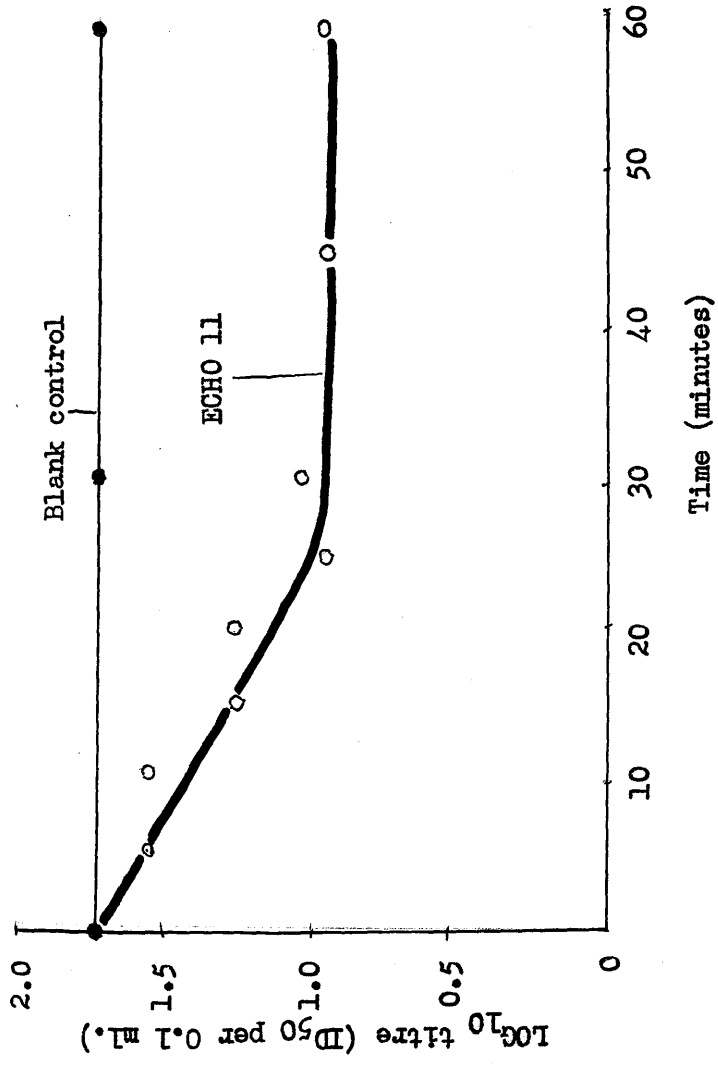


Figure 15

Rate of attachment of ECHO virus type 11 to monolayers of monkey kidney cells measured by the amount of free virus in the supernatant fluid at varying times after infection. In this experiment samples were taken every five minutes for the first half-hour and the duration of the experiment was limited to one hour.

Measurement of the average adsorption time of ECHO types 1, 7 and 11 to monkey kidney cell monolayers.

In this series of experiments the method was identical to that used in the preceding set with the exception that sampling was performed every five minutes during the phase of rapid virus adsorption. In practice this meant that the experiments were only run for one hour. Each sample was then titrated for infectivity in the usual manner using five tubes per dilution in each titration. The average adsorption time of ECHO type 2 was sufficiently different from the other three that a separate estimation was considered unnecessary.

Results

The results obtained are shown graphically in figures 13, 14 and 15, and the calculated average adsorption times are given in table 22.

TABLE 22

Average adsorption times for ECHO virus types 1, 7 and 11 calculated from experiments in which adsorption was observed by frequent sampling over a short period.

Virus type	Average adsorption time	Slope of graph
ECHO 1	12.75 mins.	0.48
ECHO 7	12.5 mins.	0.61
ECHO 11	11 mins.	0.56

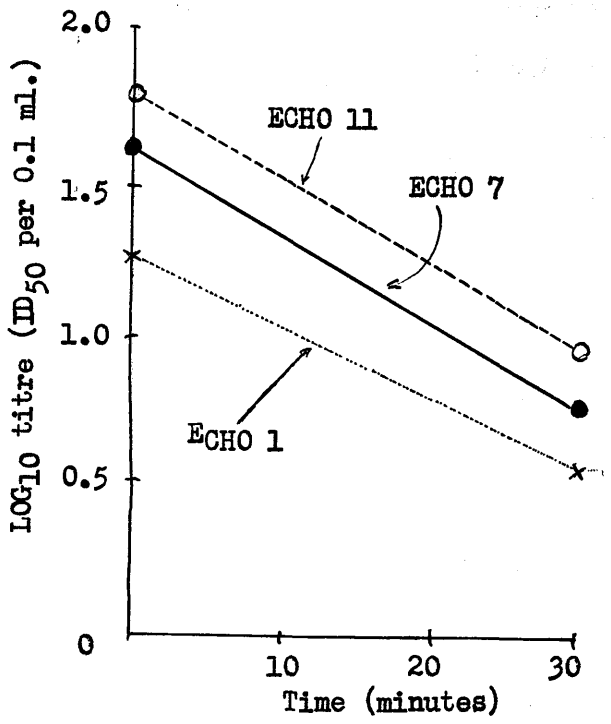


Figure 16

Rates of attachment of ECHO virus types 1, 7 and 11 to monolayers of monkey kidney cells. In this graph the first 30 minutes of adsorption from figures 13, 14 and 15 are superimposed for the sake of comparison of the adsorption "slopes".

In each instance the control bottles show very little variation in the amount of virus between start and finish of each experiment. The results obtained for each virus with regard to average adsorption time do little to substantiate the claim that a relationship exists between this and the size or shape of plaque formed by these viruses, but the times expressed here do not differ significantly from those shown in table 21. In each of these experiments the end of the phase of rapid adsorption can clearly be seen to occur at about 30 minutes (figures 13, 14 and 15). The arithmetical values for the slopes are expressed in table 22 and the slopes themselves, superimposed for comparison upon one graph are shown in figure 16. Again no precise correlation is visible with the known plaque forming characteristics of these viruses.

The second point which arose fairly early on in the adsorption measuring experiments was the difficulty which was persistently encountered in trying to obtain reproducible results for the adsorption rate of ECHO virus type 2. The results quoted in table 21 and the curves shown in figure 9 were obtained only after very many attempts. Despite the most careful prior titration of the virus and the most thoughtful technique for measuring adsorption, aliquots of the specially prepared stock of ECHO type 2 frequently showed either no adsorption to the cells in 3 hours, or adsorption appeared to be

so irregular that non-sensical results were obtained.

Several other types of experiment were performed in which the adsorption rates of these ECHO viruses to susceptible cells were measured partly as a check on the rates already reported and particularly in attempts to overcome this difficulty with ECHO type 2 and to produce reproducible results with this virus.

2. Direct measurement of the uptake of virus to monkey kidney cells by the micro-plaque technique.

Principle

In this type of experiment the speed of attachment of virus to cell was measured directly by counting the increasing number of virus particles which attach to a monolayer of susceptible cells with increasing time. This can readily be achieved by a micro-plaque technique which was developed for the purpose. The details of the technique are to be published but will be described shortly here.

Micro-plaque technique

The technique developed from the observation that cultures of monkey kidney cells infected with small doses of ECHO viruses degenerate in a regular sequential manner. The first signs of degeneration occur in tiny discrete foci which enlarge gradually and coalesce by peripheral spread. After

inoculation of a small number of virus particles the foci first appear at about 36 hours and degeneration is complete within 4-5 days. The little foci remain discrete for up to 24 hours after their first appearance and in a tube culture may be counted quite accurately within this time. It can be proved that the foci originate from one virus particle infecting one susceptible cell and that the foci are in fact micro-plaques.

The method utilises monolayers of monkey kidney cells grown in 12 x 100 mm. test tubes. These have been produced in GM and before use the medium is changed to MM. Micro plaques have been produced with all 20 ECHO strains available in this laboratory, with the exception of ECHO 10 which was not tested.

For the production of micro-plaques the virus is diluted appropriately to contain between 10 and 50 50% infectious doses (ID_{50}) as measured by a standard infectivity titration in an inoculum of 0.1 ml. With most ECHO strains this entails dilution to between 10^{-6} to 10^{-8}. The MM. is discarded from the tubes to be used and replaced with 0.1 ml. of the appropriate virus dilution. The tubes are re-stoppered and returned to the incubator for 1 hour at 37°C. Thereafter the monolayer is washed three times with phosphate buffered saline (pH 7.2) and each tube receives 1 ml. MM as before. The re-stoppered tubes are again slanted, incubated at 37°C and examined

microscopically 36 hours later for the appearance of micro-plaques. These can almost invariably be counted without difficulty. The method used is shown diagrammatically in figure 17 and this should be compared with the standard plaque producing method which is shown in figure 18. The typical appearance of a micro-plaque is shown in figure 19.

When the method was being developed it was noted that the micro-plaques produced by the ECHO viruses differed a little among themselves in structure, in that, for instance, ECHO types 1 and 7 produced larger and more diffuse lesions than ECHO types 2 and 11. This observation accords with the already well known plaque producing properties of these viruses in the familiar method as described by Hsiung and Melnick (1957). The micro-plaque method has the particular merit that it permits the production of micro-plaques with ECHO type 2, and also with types 3, 5 and 6, none of which produce standard plaques.

Proof that the foci which are here called micro-plaques are produced by the interaction of one virus particle with one susceptible cell lies in the observation that a series of dilutions of virus covering 1.2 log steps produces a geometrical increase in the number of micro-plaques upon inoculation of each dilution into several replicate culture tubes. This satisfied the criteria suggested by Dulbecco & Vogt (1954) and, as shown in figure 20, produces a straight line graph.

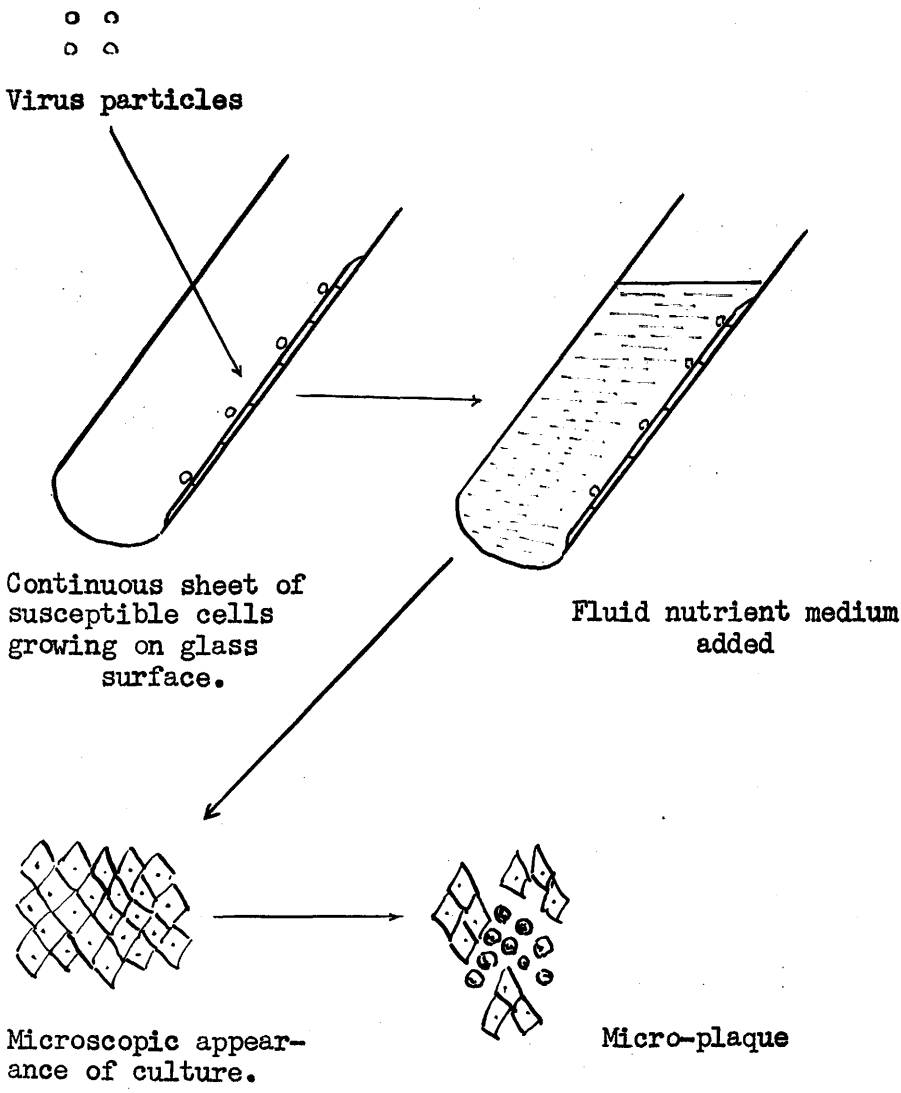


Figure 17

Diagrammatic representation of the method used for the production of micro-plaques.

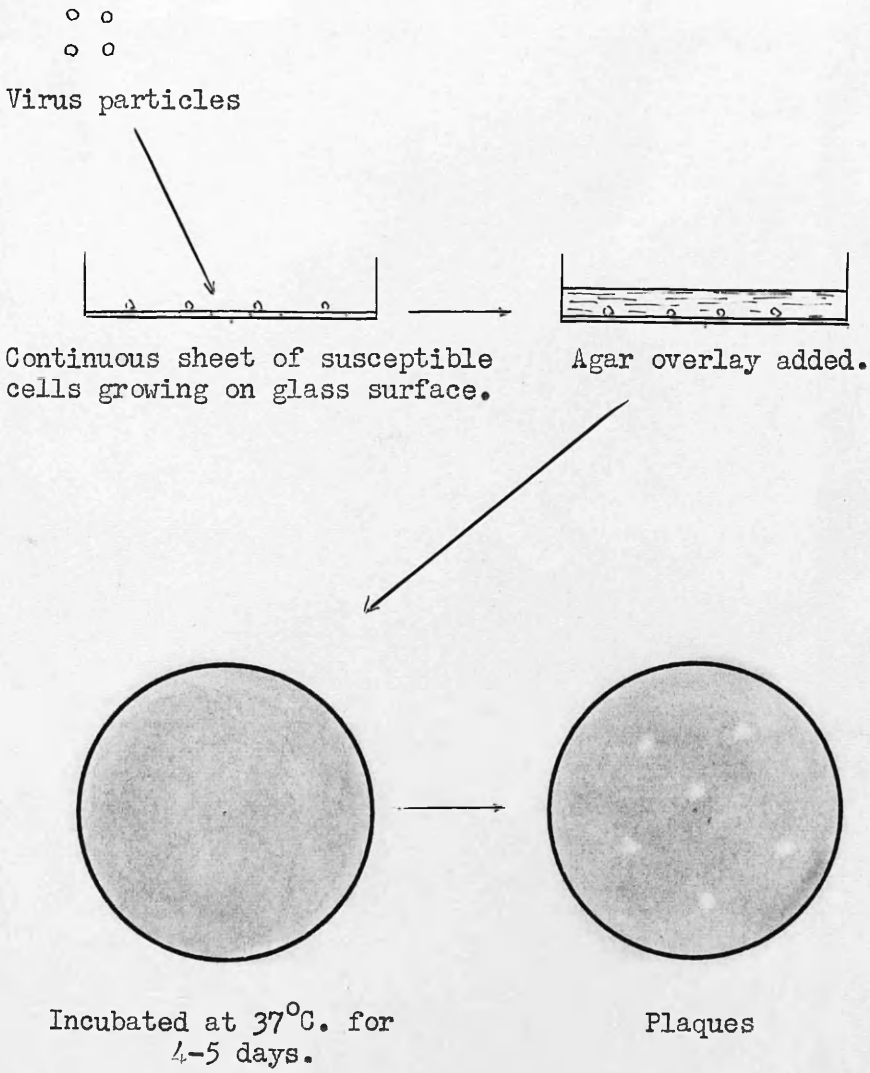


Figure 18

Diagrammatic representation of the method for the production of "standard" plaques described by Hsiung and Melnick (1957).

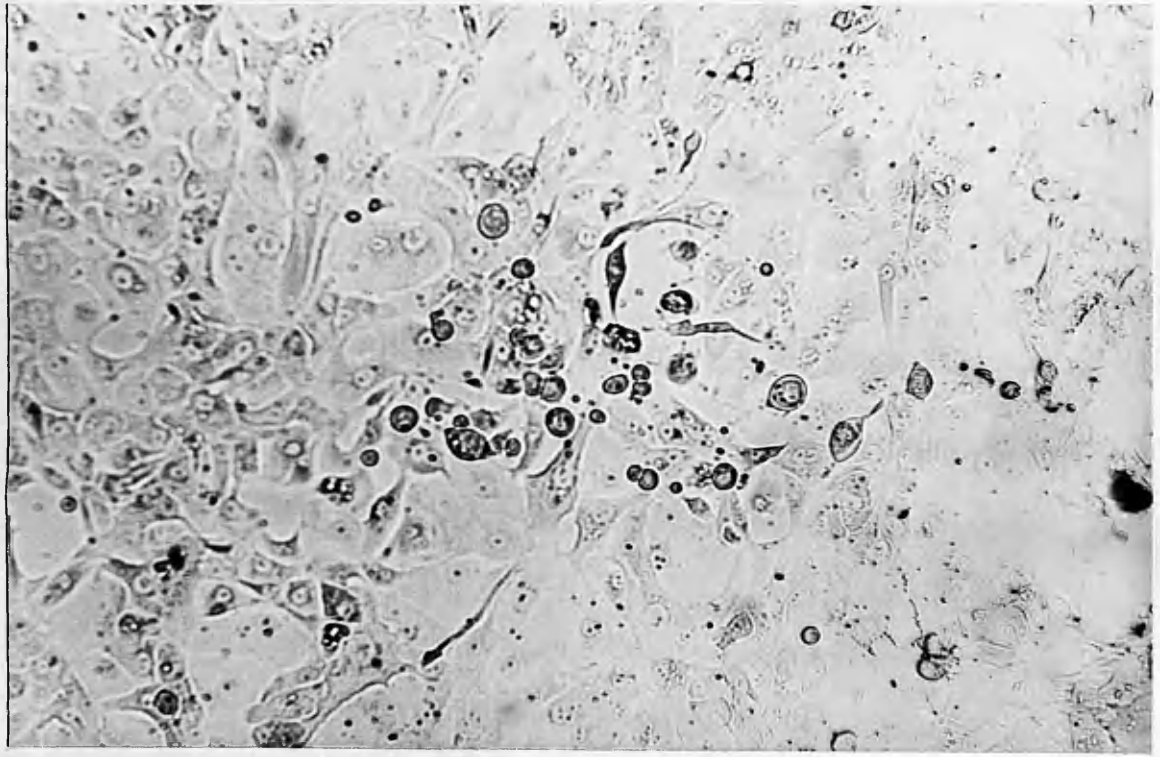


Figure 19

This photograph shows the typical appearance of a micro-plaque - in this case produced by ECHO virus type 7. (X45).

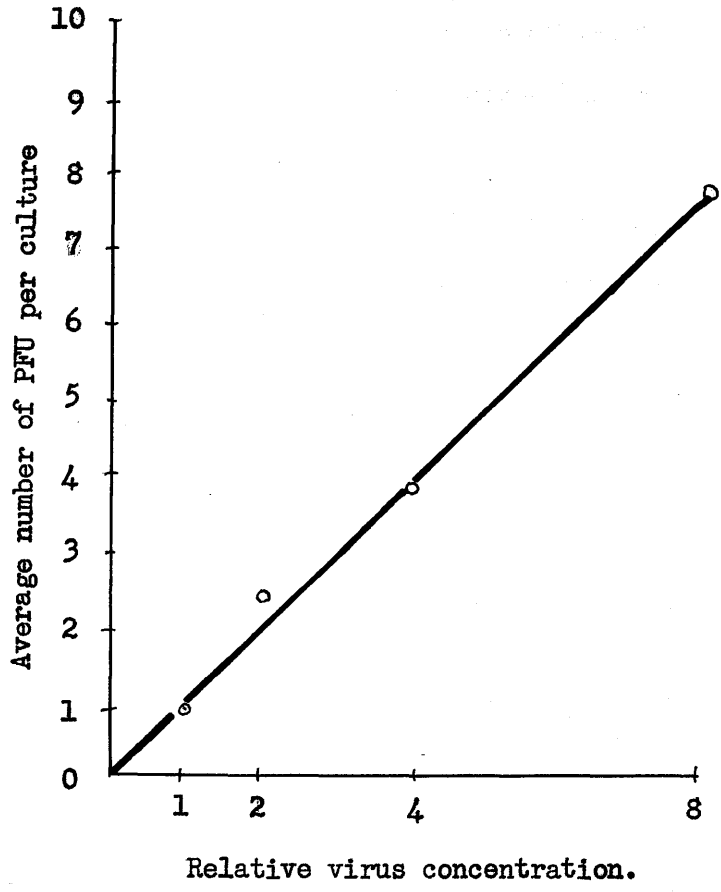


Figure 20

This figure shows the result obtained when a series of dilutions of virus covering 1.2 log steps were inoculated into tube cultures of susceptible monkey kidney cells. The number of micro-plaques increased according to a geometrical progression and a straight line graph was obtained.

A similar observation was made with the foci of degeneration produced by Herpes simplex virus by Farnham (1958).

That the volume of the virus inoculum affects the total number of micro-plaques was demonstrated when it was found that an inoculum of 0.1 ml. gave approximately 1.7 - 1.8 times the number of micro-plaques observed when the same number of ID_{50} was contained in 1.0 ml. This discrepancy is possibly accounted for by lessened opportunity for the virus particles to come into contact with susceptible cells in the layer of fluid contained in the 1.0 ml. inoculum as compared with the shallower layer of 0.1 ml.

The total number of micro-plaques countable in any one tube depended on the type of ECHO virus under observation. With ECHO types 1 and 7 the total number of discrete foci countable was about 30, whereas with ECHO types 2 and 11, 50-60 discrete micro-plaques could be enumerated.

In each instance the micro-plaque titre corresponded closely to the infectivity titre and, with the exception of ECHO type 2 to the plaque titre ascertainable by the method of Hsiung and Melnick (1957).

Figure 21 shows an acceptable variation in numbers of micro-plaques within a series of tubes, all of which have been inoculated with the same amount of virus. This depicts the results of 30 replicate cultures each of which was inoculated

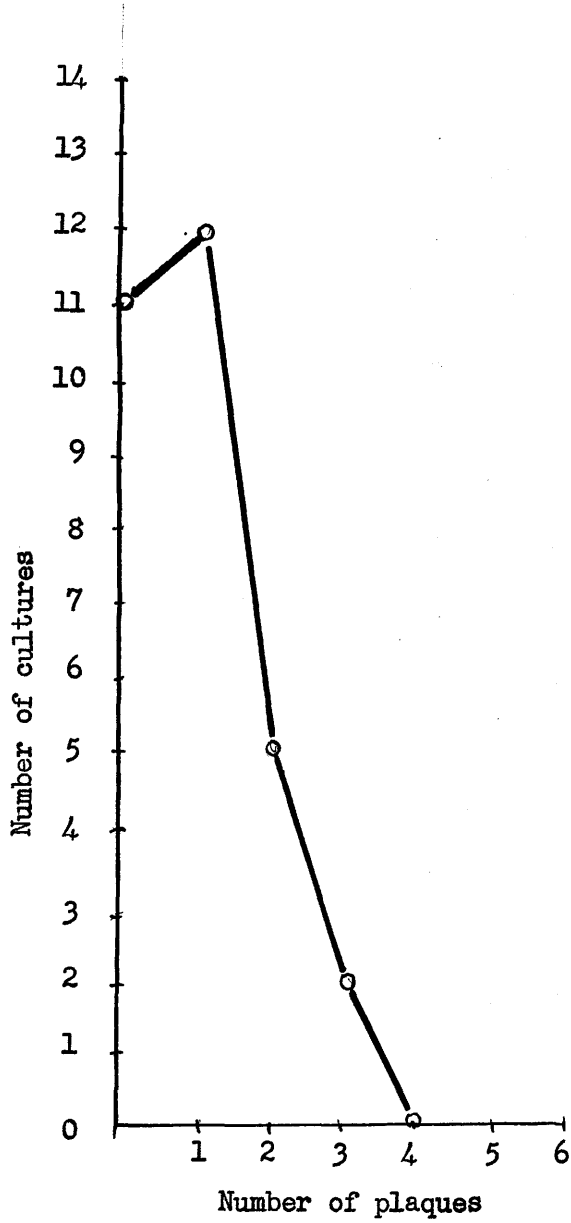


Figure 21

When a series of 30 identical monkey kidney monolayers in test tubes were all inoculated with the same amount of virus the numbers of micro-plaques in each test tube could be arranged in this Poisson type of frequency distribution.

with the same very small dose of ECHO type 2 virus. It will be seen that the scatter of results takes the form of a standard Poisson distribution. By calculation of a X^2 it was found that this distribution did not differ significantly from a theoretical Poisson distribution with the same mean since P = 0.75.

The micro-plaque method for counting virus particles has the advantage of speed and economy over the standard plaque or infectivity titration methods. Also important are the ability to dispense with agar overlays, vital strains and the ability to work in test tubes.

Potential disadvantages to be set against these are the loss of the ability to identify viruses within a group from the size and shape of the plaques, although the appearance of micro-plaques in terms of size and dispersion of degenerate cells within the plaques parallel the size of the standard plaque. The other disadvantage is that the micro-plaque count must be performed microscopically but with practice this ceases to be a disadvantage.

With very little modification this technique can be utilised for studying the rates of attachment of the viruses under study and it has the advantage in this regard that it gives a direct reading of the number of virus particles attaching during a given time.

Preparation of monkey kidney monolayers

For this type of experiment the cells were dispersed in 12 x 100 mm. test tubes which were incubated without variation in GM for 7 days before use.

Plan of the experiment

For each virus under investigation a suitable dilution was prepared which contained in 0.1 ml. approximately the greatest number of micro-plaque forming units (MPFU) which could be counted in one test tube as discrete foci. These were estimated by micro-plaque titration of each virus in several replicate tubes using a 3-hour adsorption time.

The tubes were emptied of GM and washed twice with PBS before inoculation of 0.1 ml. of each virus. All tubes were then restoppered and returned to the 37°C. incubator at a slight slope from horizontal after careful agitation to ensure even dispersal of the inoculum over the monolayers. Groups of five tubes were then washed thoroughly five times at five minute intervals for 30 minutes and at 15 minute intervals thereafter for one hour. This washing removed any unattached virus. One ml. of MM was then added to each tube. The tubes were restoppered and incubated without disturbance for 36 hours at which time the number of micro-plaques in each set of 5 tubes representing each time interval were recorded and averaged. The results obtained are shown graphically in figure 22 and

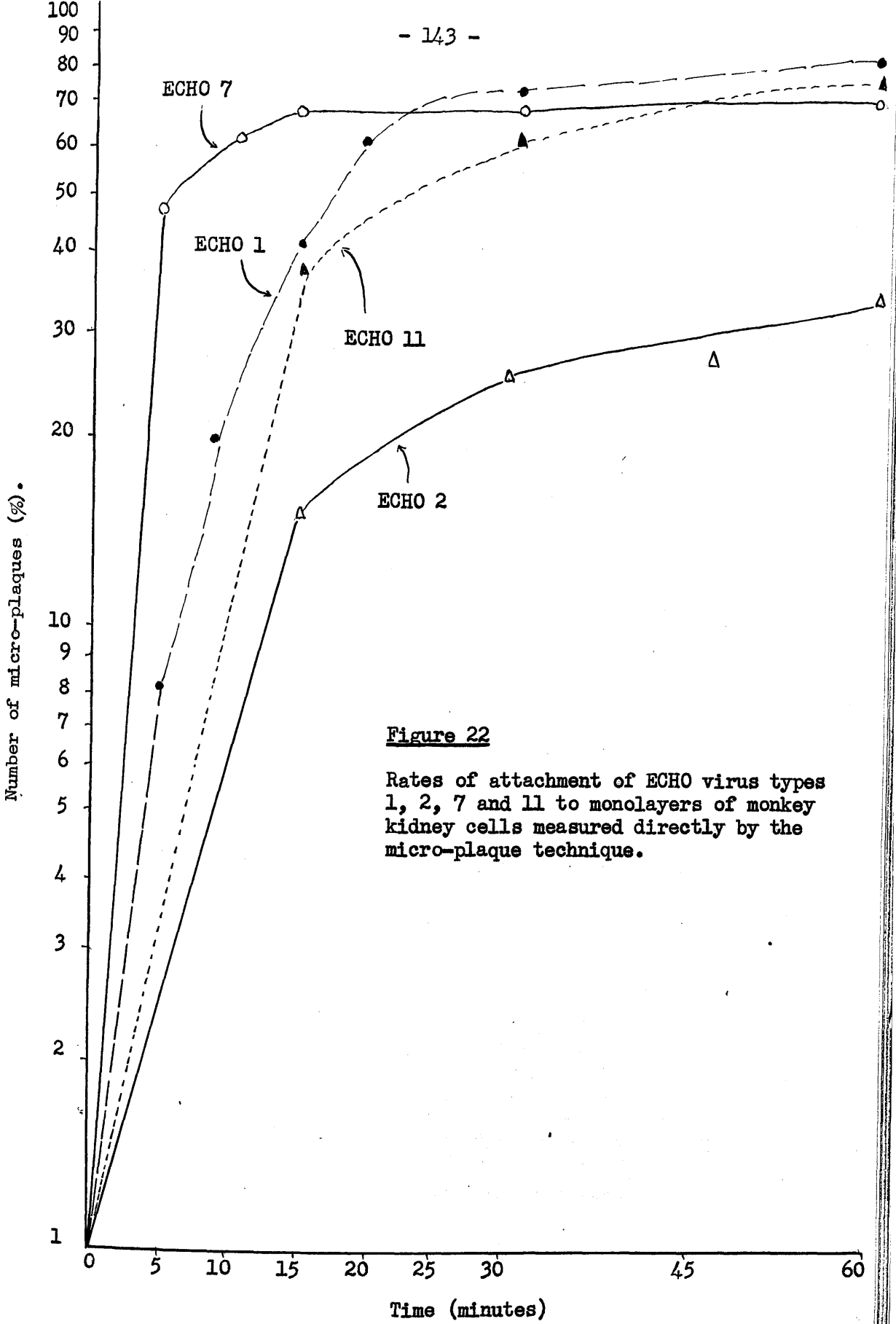


Figure 22

Rates of attachment of ECHO virus types 1, 2, 7 and 11 to monolayers of monkey kidney cells measured directly by the micro-plaque technique.

selected parameters are recorded for each virus in table 23.

TABLE 23

Properties of ECHO virus types 1, 2, 7 and 11 derived from micro-plaque estimations of adsorption rates to monkey kidney cells in tube cultures.

Virus	Average adsorption time. (minutes)	Adsorption at 30 mins. (per cent)	End of rapid adsorption period (mins.)
ECHO 7	4.5	94	15
ECHO 1	15	88	30
ECHO 11	16	77	30
ECHO 2	20	69	60+

Results

It will be seen from figure 22 that each virus adsorbs directly to a monolayer of susceptible cells at a different rate. The rates can be arranged in a descending gradient, as can the shapes of the curves in the figure from left to right, and the gradient is identical to the gradient formed by the differences in the speed with which plaques are formed and the size of plaque eventually produced by the method of Hsiung & Melnick (1957). These conclusions are supported in full by the calculated parameters expressed in table 23. The

exception to this generalisation, of course, is ECHO type 2 which will not produce plaques by the above mentioned method, but this virus clearly takes its place at the lower end of the plaque adsorption gradient and therefore is likely to fit at the lower end of the standard plaque gradient as well.

Discussion

It seems clear that the differences in plaque forming potential of the four viruses under investigation can be explained, with the possible exception of ECHO 2, by the rates of adsorption of the viruses to monkey kidney cells. ECHO type 7 produces very large plaques very quickly (1.0 - 1.2 cm. in 2-3 days) by the standard method and from the results expressed here adsorbs to cells with almost incredible rapidity. ECHO types 1 and 11, from the results expressed here, do not differ very much in their rates of adsorption but perhaps the slight differences noted account for the differences in plaque production. Certainly the differences fall into line in the "adsorption gradient", and parallel those in plaque producing potential.

Some other factors must be invoked to explain the total inability of ECHO type 2 to produce plaques by the standard method. These are open to investigation now that the virus and its behaviour can be studied by micro-plaques and from the

subject matter of a later chapter.

The differences in adsorption rates and the percentages of available virus which adsorb in one hour, even with such small inocula as 60-80 micro-plaque forming units, again demonstrate the inherent error which must be present in all experimental work with these viruses when comparisons are to be made of properties which depend upon an initial adsorption period of one hour.

(3) Indirect measurements of the rate of attachment of virus to monkey kidney cells by observing the length of time required following variable exposure for virus to produce cytopathic effects

Principle

This type of experiment makes no actual measurement of the amount of virus attaching to cells but relies upon the relatively crude measurement of the time taken by a given dose of virus to produce a partial cytopathic effect. The hypothesis is that if in one hour X units of virus fasten to cells and take N days to reach a 2 + (++) stage of degeneration, in a shorter time a smaller number of units of virus will attach to the cells and these will take a proportionately longer time to produce the same degree of cytopathic effect.

Preparation of monkey kidney monolayers

For this type of experiment the cells were grown in 12 x 100 mm. test tubes in GM for 7 days before use.

Plan of the experiment

A suitable dilution was selected for each virus under investigation. In practice serial dilutions were used, optimal results being obtained with multiplicities of infection between 4:1 and 1:1. The GM was discarded from the tubes in which each experiment was to be performed and each monolayer was then washed twice with PBS. The selected virus dilution (containing 4, 2 or 1 ID_{50} per cell of the culture) was then inoculated in 0.1 ml. amounts into each tube in the batch and the tubes were incubated at a slight slope from horizontal. At 15 minute intervals the inoculum was removed from groups of 5 tubes. The monolayers were washed three times to remove any unattached virus and the tubes had 1.0 ml. MM added. Each group of 5 tubes was then re-stoppered and replaced in the incubator at 37°C. This procedure was repeated every 15 minutes with a different group of 5 tubes for 4 hours.

The tubes were examined microscopically at 12 hour intervals and the degree of cytopathic effect was assessed in the usual manner and graded 0 to 4+. The average time taken for all five tubes in each group to reach the stage of 50% degeneration (2+) was recorded and the results were expressed

graphically.

Results

The results which were obtained are depicted in figures 23-26. It will be seen that each graph follows the same general pattern with a more or less steep initial sloping portion and a long horizontal tail. The initial slope covers the samples taken during the first hour of adsorption and the slope itself varies little with the number of virus particles in the inoculum. The greatest slopes are found with ECHO types 2 and 11. After about one hour of adsorption the graphs become horizontal indicating that a greater adsorption time does not increase the length of time required for the virus to destroy 50 per cent of the cells.

A composite graph is depicted in figure 27 in which the results obtained with each virus are superimposed for the sake of comparison. From this figure it can be seen that the indirect adsorption rates can be correlated directly with the plaque producing properties of the viruses. In this graph ECHO type 7 takes the smallest time to produce 50% cytopathogenicity in the cultures and a gradient is present as follows:- ECHO 7 > ECHO 1 > ECHO 11 > ECHO 2. The same gradient exists with regard to plaque producing potential both in terms of the size of plaque and its speed of production.

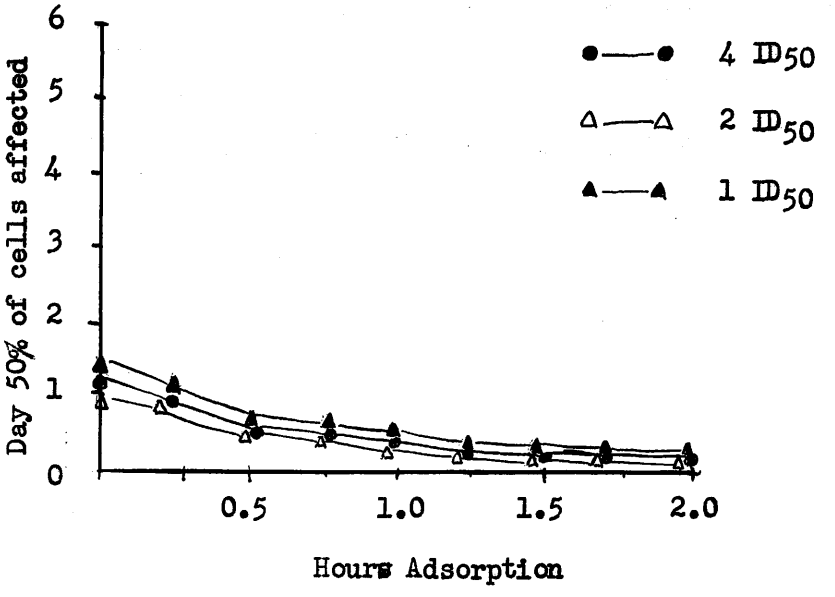


Figure 23

Rate of attachment of ECHO virus type 1 to monolayers of monkey kidney cells measured indirectly as the time required for 50% of the cells to become degenerate after exposure to virus for variable times.
(Three curves are shown representing the rates for 4, 2 and 1 ID_{50} per cell of the culture.)

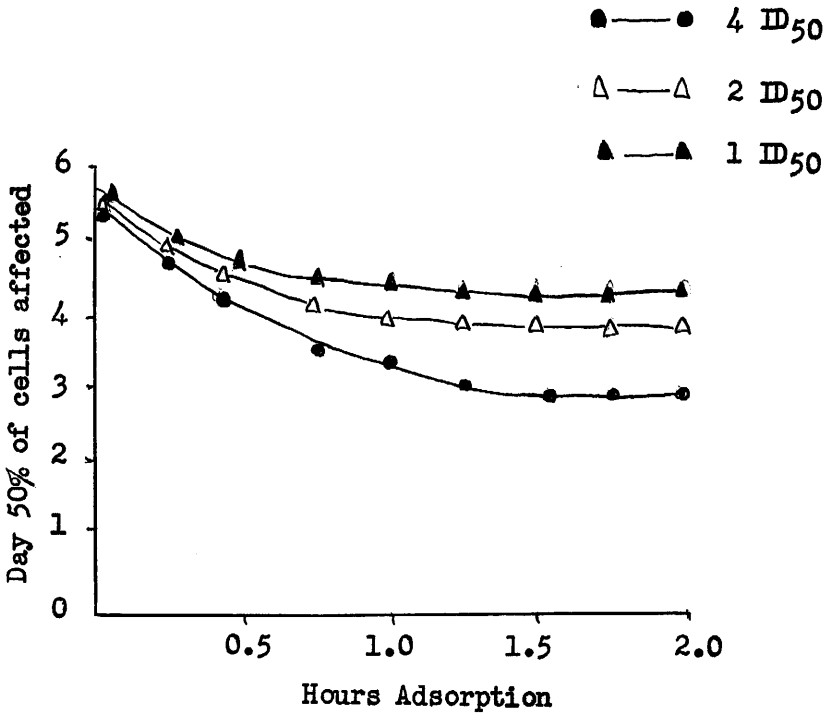


Figure 24

Rate of attachment of ECHO virus type 2 to monolayers of monkey kidney cells measured indirectly as the time required for 50% of the cells to become degenerate after exposure to virus for variable times.
(Three curves are shown representing the attachment rates for 4, 2 and 1 ID_{50} per cell of the culture).

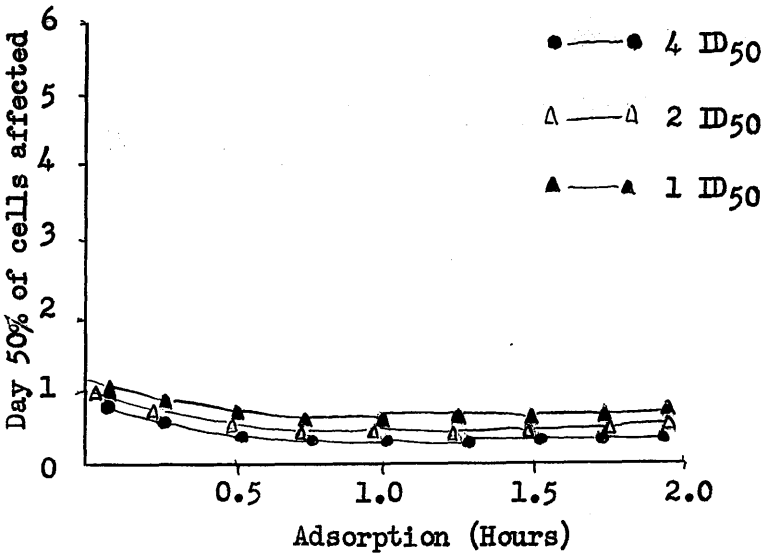


Figure 25

Rate of attachment of ECHO virus type 7 to monolayers of monkey kidney cells measured indirectly as the time required for 50% of the cells to become degenerate after exposure to virus for variable times.
(Three curves are shown representing the attachment rates for 4, 2 and 1 ID_{50} per cell of the culture).

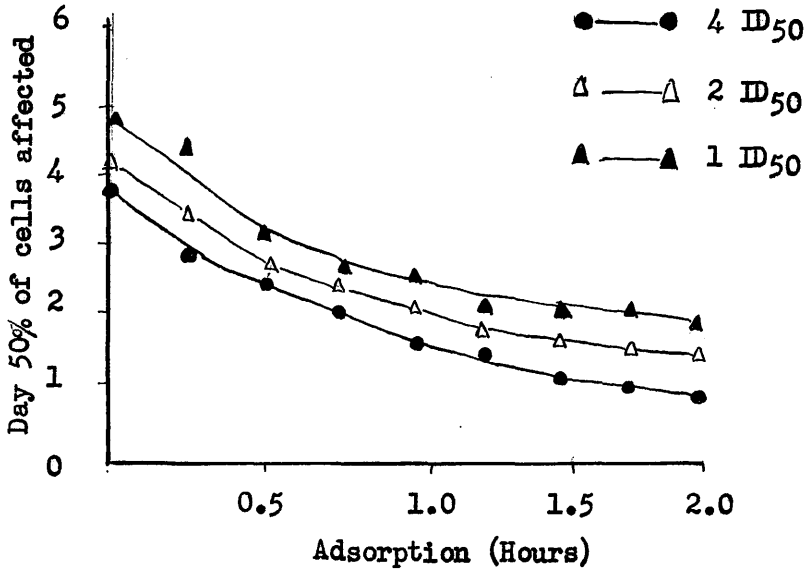


Figure 26

Rates of attachment of ECHO virus type 11 to monolayers of monkey kidney cells measured indirectly as the time required for 50% of the cells to become degenerate after exposure to virus for variable time.
(Three curves are shown representing the attachment rates for 4, 2 and 1 ID_{50} per cell of the culture).

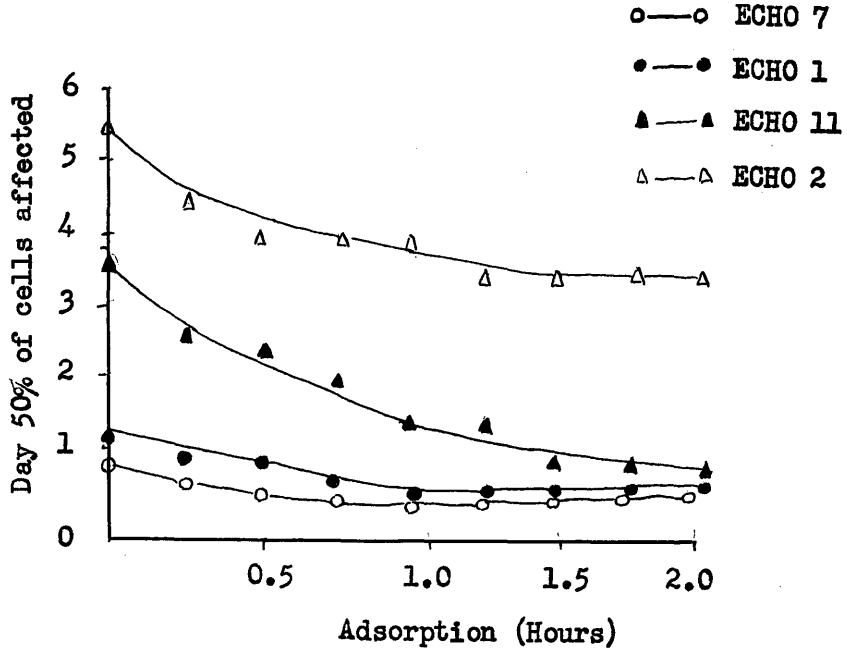


Figure 27

Composite diagram showing the attachment rates for ECHO types 1, 2, 7 and 11 to monolayers of monkey kidney cells. The indirect method of measurement was used and the curves represent the rates of attachment of a dose of 4 ID_{50} of virus per cell of the culture. The four slopes exhibit a gradient which corresponds precisely with the known plaque producing properties of these viruses.

Discussion

Although a positive correlation with the plaque forming ability of the viruses under investigation can be obtained by the indirect adsorption measurement technique it is important to remember that this technique measures only the cumulative effect of a number of factors which have already been measured in individual experiments with each virus. In each experiment of the "indirect" series the total number of cells presented to each virus for destruction was reasonably constant at about 150,000 per tube. In each case samples were taken with the different viruses at the same time intervals. It is known that the length of the growth cycles of each virus is approximately the same, with that of ECHO 2 being probably longer than the others. The existence of a positive correlation was shown between the average adsorption times and the plaque forming ability of the viruses and a similar correlation was demonstrated with the maximum adsorption times as measured by the micro-plaque method. In the present experiments the shortest time taken to produce a cytopathic change in 50 per cent of the cells of the culture was 16 hours with ECHO type 7 after 30 minutes of adsorption initially. In 30 minutes it is known that approximately 80 per cent of the virus is adsorbed to the cells (tables 3 and 5). Two growth cycles will have been completed in 16 hours and the virus

released from cells at the end of the first growth cycle will have attached to other susceptible cells in 2.5 hours with an efficiency of 90%.

With ECHO type 2, on the other hand, after 30 minutes of adsorption between 19 and 38 per cent (tables 21 and 23) of the total available virus will have adsorbed to the same number of cells. This virus begins at a considerable disadvantage, therefore, in terms of amount adsorbed. In the four days required for 50 per cent of the cells to show cytopathic effect approximately 6 growth cycles will have taken place and from table 21 it can be seen that a maximum adsorption of freshly released virus from each growth cycle can be expected at 3 hours with an adsorbing efficiency of 72 per cent. The disturbing factor is that the average yield of virus per cell of the culture, as calculated in the growth cycle experiments in the last chapter (table 20), is 59 for ECHO 7 and 496 for ECHO 2, i.e. 8 times more ECHO 2 is produced than ECHO 7 from each infected cell. It is impossible to determine the part played by this factor in experiments where a number of growth cycles have occurred but it obviously does not mean that ECHO type 2 can attach to 8 times the number of cells in the culture available to ECHO type 7. Perhaps the question of competence discussed by Rubin (1959) in connection with Rous sarcoma virus and chicken cells exists also with ECHO type 2, or perhaps the virus is able to adsorb to

a large number of the cells in a culture without actually penetrating the cell wall, at least for a time and in this way blocking the cell receptors for the attachment of fresh virus and producing a degree of interference.

When the various factors involved in the ECHO virus growth cycles and adsorption rates interact in these "indirect" measuring experiments, however, a positive correlation is produced with the plaque forming potential of ECHO types 7, 1 and 11. The results obtained with ECHO type 2 suggest that its place on the gradient can be assigned below the others with respect to plaques but as these cannot be produced by the standard method with this virus the placing can only be implied. The differences observed in the growth cycle and adsorption experiments cannot be invoked, however, to explain the inability of ECHO type 2 to produce plaques under agar by the standard method described by Hsiung & Melnick (1957) in view of the ease with which micro-plaques can be produced by the method already described here. Some other factor must be responsible in addition to the slow adsorption rate and the series of experiments to be described next was designed to investigate the factor involved.

CHAPTER XII

INVESTIGATION OF THE FACTORS RESPONSIBLE FOR THE INABILITY OF ECHO TYPE 2 VIRUS TO PRODUCE PLAQUES IN MONKEY KIDNEY CELLS UNDER AGAR.

In view of the discovery that ECHO virus type 2 will produce micro-plaques, as described in the previous chapter, despite its inability to produce a "standard" plaque under an agar overlay by the method described by Hsiung and Melnick (1957), and also because no reason was discovered in the growth cycle or adsorption experiments why it should not produce a "standard" plaque, a number of experiments were undertaken to ascertain what factors might be responsible for the non-production of the "standard" plaque.

A comparison of the methods used for the production of a "standard" plaque and of a micro-plaque (see figures 17 and 18) reveals that the essential differences lie in the culture medium used.

In the "standard" method a monolayer of monkey kidney cells, usually in a bottle or petri dish, after infection with virus is covered with a medium consisting of the following:-

A. Earle's saline (10 x concentrated) without phenol red	18.0 ml.
Sterile distilled water	59.4 ml.
Sodium bicarbonate (7.5% W/V)	5.4 ml. (0.225 gm.%)
Neutral red (1:1000)	3.0 ml. (1.6 mgm. %)

A (continued)

Antibiotic mixture	1.0 ml.
Serum (Calf)	3.2 ml.
B. Agar (Difco-Bacto) (2.7% W/V)	90 ml. (1.35%)

For use equal parts of A and B are mixed and held at 43°C. 8.0 ml. of mixture are added to each monolayer.

In the micro-plaque method the same concentrations of Earle's saline, antibiotic mixture and calf serum are used. Phenol red (1:1000) is substituted for neutral red as the identification of micro-plaques is performed microscopically and does not depend upon the lack of vital staining in the plaque as with the "standard" method. The indicator is merely used as a guide to the pH of the culture. The sodium bicarbonate concentration is 0.22 gm %, a reduction of 0.005 gm. %, and agar is omitted altogether.

In all other respects the methods are essentially identical and I thought that one or other of the three differences must account for the inability to produce "standard" plaques. Experiments were accordingly designed so that the effect of each difference could be observed simply by altering the composition of the micro-plaque medium to include each of the three reagents as they would be used in the production of "standard" plaques.

I. The effect of various sodium bicarbonate concentrations on the formation of micro-plaques by ECHO type 2 virus.

The standard method for the production of micro-plaques was used in that 0.1 ml. of a dilution of ECHO type 2 virus containing 50 MPFU's was inoculated into each of 30 test tubes containing monolayers of rhesus kidney cells. After one hour's adsorption time the inoculum was removed and the cells washed twice with PBS. The tubes were then treated in groups of 5 and to each group was added 1 ml. per tube of standard MM altered only in respect of the concentration of sodium bicarbonate. This varied from 0.022 gm.% increasing two-fold for each group of 5 tubes to 0.704 gm %. A group of ten similarly inoculated tubes was used as a virus control and after washing 1.0 ml. of standard MM with the normal sodium bicarbonate concentration of 0.22 gm % was added to each. The tubes were restoppered, incubated in a 37°C. incubator at 5° from horizontal and examined at 36 hours when the number of micro-plaques was counted and averaged for each bicarbonate concentration.

Results

The results obtained are shown in table 24. At the normal concentration of 0.22 gm % $NaHCO_3$ the estimated 50 MPFU per tube produced an average of 48 micro-plaques. The number of micro-plaques countable at the two lowest concentrations is

TABLE 24

The effect of varying concentrations of sodium bicarbonate on micro-plaque production by ECHO type 2 virus.

Concentration of $NaHCO_3$ in normal MM * (Gm. %)	Average micro-plaque count
0.220 (virus control)	48
0.022	3
0.044	3
0.088	28
0.176	55
0.352	56
0.704	D *

* MM. Maintenance medium. - Earle's saline with 0.5% lactalbumen hydrolysate, 5% calf serum and 1% antibiotic mixture.

* D - Cells completely degenerate.

very small indeed. The cultures were uniformly degenerate and many cells had become detached from the glass. This effect was produced, no doubt, by the extremely low pH of the medium at the lowest concentrations of bicarbonate. Similar but slight toxicity was noticed when the bicarbonate concentration was increased to 0.088 gm %. At the highest concentration the medium was light purple in colour and all the cells were necrotic due to the excessively high pH. Within the normal range of bicarbonate concentration the micro-plaque count was uniform

and not significantly different from that observed in the virus control.

Comment

The micro-plaque counts obtained over a concentration range of 0.176 to 0.352 gm % sodium bicarbonate did not vary significantly from the controls. The concentration of bicarbonate used in the "standard" plaque method differs from the normal concentration of 0.22 gm % in MM by 0.005 gm %. Clearly a variation in concentration of this order would have no effect at all upon the production of micro-plaques.

II. The effect of various concentrations of neutral red on the formation of micro-plaques by ECHO type 2 virus.

The method used was essentially similar to that of the sodium bicarbonate experiment. The standard micro-plaque technique was modified only to include several concentrations of neutral red in the nutrient fluid added after one hour's adsorption of virus. The dose of ECHO type 2 was 40 MPFU's in 0.1 ml. per tube to each of 30 tubes for the test and to 10 control tubes. At the end of one hour the inoculum was removed, the tubes were washed twice with PBS in groups of 5 and each group had 1.0 ml. of MM with an added concentration of neutral red (Neutral Red-Fluorchrome, Gurr.) varying from 0.25 mgm % by two-fold steps to

3.0 mgm %. To each of the ten virus control tubes 1.0 ml. of normal MM was added. The tubes were then restoppered and incubated for 36 hours at 37°C at a slope of 5° from horizontal. At the end of this time the micro-plaques were enumerated and an average recorded for each concentration of neutral red.

Results

These are recorded in table 25. The average number of micro-plaques produced in the virus control was lower than anticipated at 30 but at this level the count was still sufficiently high to show differences with varying concentrations of neutral red. A progressive fall in the average count is evident until the concentration of 1.5 mgm % was reached at which point complete suppression of micro-plaques was produced. When the concentration was increased to 3.0 mgm % the cells became rather toxic without showing definite necrosis. They appeared to retain their normal shape but the sheet was disrupted to the extent that each cell had separated from its neighbours. Each was intensely strained with neutral red and the entire culture looked unhealthy. The absence of micro-plaques at this concentration is taken to be an indication of the depression of the cellular activity rather than a specific effect of the dye on virus reproduction. At the other concentrations, on the other hand, the appearance of the cell sheet was completely normal apart from variable intensity of

staining by the dye. When micro-plaques were present most of the cells composing the plaque still retained their deep pink colour, although each was completely rounded and at the stage of 4+ degeneration.

TABLE 25

The effect of varying concentrations of neutral red on micro-plaque production by ECHO type 2 virus.

Concentration of neutral red in normal MM. (Mgm %)	Average micro-plaque count
0 (virus control)	30
0.25	16
0.5	19
1.0	7
1.5	0
2.0	4
3.0	0

Comment

The progressive decrease in numbers of micro-plaques with increasing concentrations of neutral red reached its maximum at 1.5 mgm %. At this level no micro-plaques were produced in 36 hours, by which time an average of 30 had appeared in the virus control tubes. The concentration of neutral red incorporated in the agar overlay of monolayers for the "standard" plaque technique is 1.6 mgm % and this appears to be a very

likely explanation for the inability of this virus to produce "standard" plaques under agar. Unfortunately 1.6 mgm % is the optimal concentration for the visualisation of plaques by the "standard" method. A decrease in this amount renders the foci extremely difficult to see and from the results quoted here the decrease in concentration would have to be to the 0.5 mgm % level before "standard" plaques could be expected to appear. At this concentration of neutral red plaques would not be distinguishable from the surrounding normal tissue.

III. The effect of the addition of agar on the formation of micro-plaques by ECHO type 2 virus.

The third possible difference between the "standard" and micro-plaque techniques was that agar is added to the overlay for the production of "standard" plaques. In this experiment the method was broadly as described before in that 40 MPFU's of ECHO type 2 in 0.1 ml. were added to each of 40 tubes. After one hour the inoculum was removed and the tubes washed twice with PBS. MM was made to double strength and mixed with equal parts of 2.7% agar (Difco-Bacto Agar) to give a final concentration of normal MM in 1.35% of agar. 1 ml. of this mixture was added to each of 20 tubes at43°C, and 1 ml. of normal MM was added to each of the other 20 virus control tubes. The agar was allowed to solidify and the restoppered tubes were returned to the 37°C

incubator at 5° from horizontal for 36 hours. After this incubation time the micro-plaques were enumerated and the average recorded.

Results

These are shown in table 26. The addition of agar made no difference to the micro-plaque count, nor did it affect the ease with which the micro-plaques could be seen or enumerated.

TABLE 26

The effect of agar on the formation of micro-plaques by ECHO type 2 virus.

Concentration of agar in normal MM (Gm %)	Average micro-plaque count
0 (virus control)	33
1.35	30

Discussion

From a consideration of the results expressed here it is apparent that neutral red is capable of suppressing the production of micro-plaques when added to tubes at the same concentration at which it would be used for the production of "standard" plaques in bottles or petri dishes. This observation

explains completely the inability to produce "standard" plaques. No effect on micro-plaque production is evident when agar or sodium bicarbonate are used at the same concentrations as would be used in the production of "standard" plaques although the bicarbonate concentration is critical within fairly narrow limits mainly because of the effect variations in its concentration have upon the pH of the culture fluid. When the pH is too low or too high diminution in the number of micro-plaques probably reflects only the cellular inability to metabolise under unphysiological conditions of pH.

CHAPTER XIII

THE HISTOLOGICAL CHANGES PRODUCED IN MONKEY KIDNEY CELLS DURING THE GROWTH OF ECHO VIRUS TYPES 1, 2, 7 and 11.

During the course of the growth cycle experiments described in chapter 11 the opportunity presented to make a study of the histological changes which are produced in monkey kidney cells by the growth of the ECHO viruses under investigation. No information on these changes is available in the literature to date other than the statement by Ormsbee and Melnick (1957) that these viruses produce histological changes similar to those caused by polioviruses. A previous preliminary study of the changes had given the impression that this was not so and the series of experiments to be described here was accordingly undertaken to clarify the position.

Plan of the experiments

The basis of these experiments was a modification of the growth cycle investigations which have been described already. Second passage monkey kidney cells were grown on 7/8" square coverslips in GM contained in 9 cm. Petri dishes in a humidified atmosphere of 5% CO_2 and air at 37°C. A satisfactory monolayer was produced in between 4 and 7 days. The medium was discarded from 6 plates which were then infected with a small inoculum of

the virus under investigation. This usually consisted of 0.5 ml. virus containing 4 ID_{50} per cell of the culture. The plates were returned to the humidified incubator at 37°C for one hour. During the incubation time they were agitated frequently in two planes to ensure even distribution of the inoculum over the cell sheet. After one hour the inoculum was removed, the plates and their coverslips washed twice in PBS and recovered with 8 ml. of MM before returning to the "gassed" incubator.

At hourly intervals thereafter one plate was opened and two coverslips removed with sterile forceps.

Fixing and staining methods

Several staining methods were used but in practice the best results were obtained with a slightly modified Giemsa's stain.

Modified Giemsa's stain

Wash film in PBS	
Fix in absolute methyl alchol	5 minutes
Remove white precipitate by two rinses in tap water.	
Stain in Giemsa (Gurr 1:20)	2 hours
Wash in running water.	
Differentiate in saturated solution of colophonium resin	30 seconds
Dehydrate, clear and mount in DPX.	

Differentiation is rather difficult to control microscopically with colophonium resin because anything which touches the resin assumes a permanent stickiness. The blue colour is removed quite slowly by this method and no difficulty was experienced, after a little practice, in producing quite satisfactorily stained films.

Heidenhain's Iron Haematoxylin stain.

Another set of coverslips was stained after infection with ECHO type 7 virus by a modification of Heidenhain's Iron Haematoxylin stain. For this stain the coverslips were fixed in Helly's modification of Zenker's fluid and pre-treated after washing with Mallory's bleach. This pre-treatment consisted of immersion in 0.25% potassium permanganate for 5-10 minutes using 3 changes of permanganate, rinsing in water and bleaching with 5% oxalic acid. The staining procedure continued as follows:-

Mordant film in 5% Iron alum	10 minutes
Wash in running water	10 minutes
Stain in saturated alcoholic solution of haematoxylin (well ripened) at a 1:5 dilution.	10 minutes
Wash in running water	5 minutes
Differentiate in 2.5% Iron Alum (controlled microscopically)	2-3 minutes
Blue in alkaline solution	1/2-1 minute
Dehydrate, clear and mount in DPX.	

Heidenhain's Iron haematoxylin stain is excellent for discerning nuclear detail when used in this way with a chrome fixative of comparatively low penetrating power.

In practice these experiments were allowed to run for 8 hours, one or two coverslips being collected hourly; others were collected also at 16 and 24 hours. After staining, each set was systematically examined and the microscopic changes produced by the growth of the viruses observed and recorded.

Results - Giemsa's stain.

With this stain a strikingly uniform series of changes was produced by all four viruses under investigation.

(a) Inclusions

Within one hour, with each ECHO virus, many cells contained a brightly eosinophilic cytoplasmic inclusion body (figure 28). This lay near the periphery in most cells and was comparatively minute. A number of cells in any one area of the culture showed two or perhaps more inclusions, each of which was usually smaller than when only one was present. By two hours the inclusion was considerably larger (figure 29) and its growth continued steadily at the expense of the cytoplasm for between four and six hours. During this time the inclusion stained brightly and was deeply eosinophilic. An inclusion which is the same size as the nucleus is shown in figure 30.

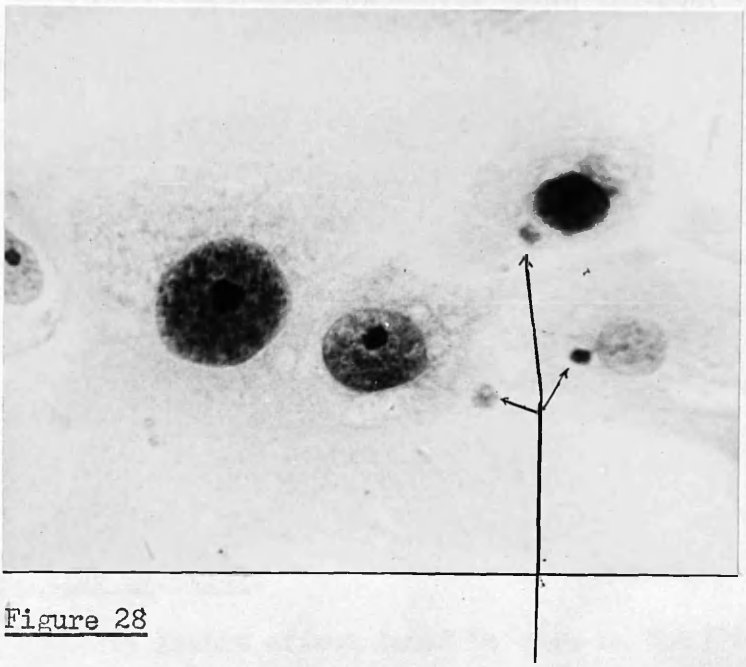


Figure 28

1 hour after infection. A small rounded cytoplasmic inclusion can be seen in 3 cells. (X280) (Giemsa)

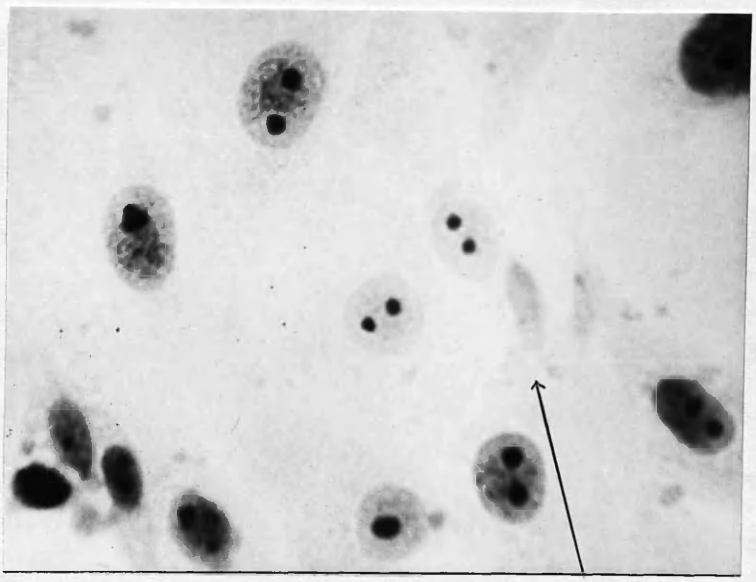


Figure 29

2 hours after infection. A large cytoplasmic inclusion is present in one cell. (X280) (Giemsa)

After this time the colour gradually began to fade. Usually half the cytoplasm was occupied at this stage. The inclusion appeared to push the nucleus to one end of the cell as shown in figure 31 and gradually compressed the nucleus into a little dark staining rim stretched round what by this time was a grey staining inclusion (figure 32).

By the time complete degeneration had occurred and the cell had rounded up most of its bulk was occupied by the large cytoplasmic mass of grey staining inclusion material.

(b) Cellular reaction.

Very little effect could be seen in the form of cellular reaction to the presence of the inclusion body until about half the cytoplasmic space was occupied by inclusion. Up to this time the cell continued to stain quite normally. The reaction invariably took the form of a wrinkling of the nuclear membrane, alteration in size or even coalescence of the nucleoli, loss of nuclear definition and increasing cytoplasmic and nuclear basophilia.

By twelve hours comparatively little nuclear detail could be seen in infected cells and the cytoplasm was deeply basophilic. Usually by this time the nucleus was compressed at one end of the cell, often stretched around the inclusion as a narrow rim of deep blue staining material with the small amount of remaining cytoplasm a lighter shade of blue by comparison.

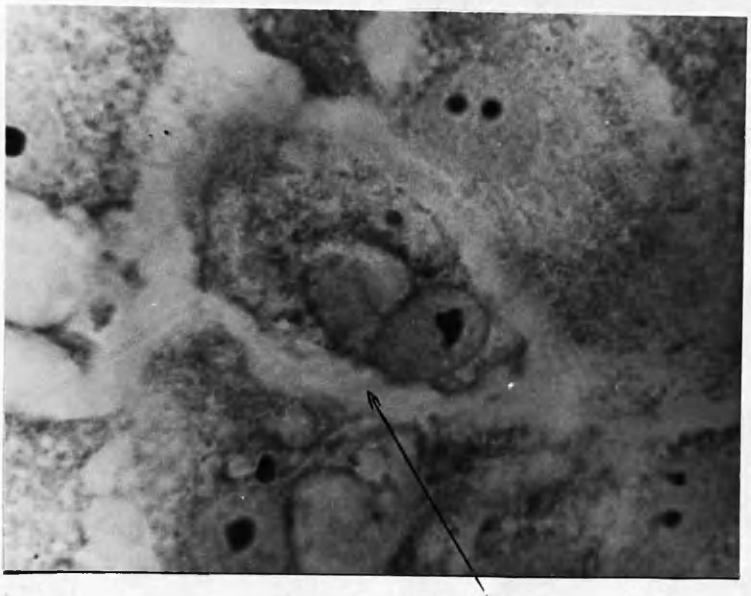


Figure 30

3 hours after infection. The cytoplasmic inclusion is almost as large as the nucleus. (X560)
(Giemsa)

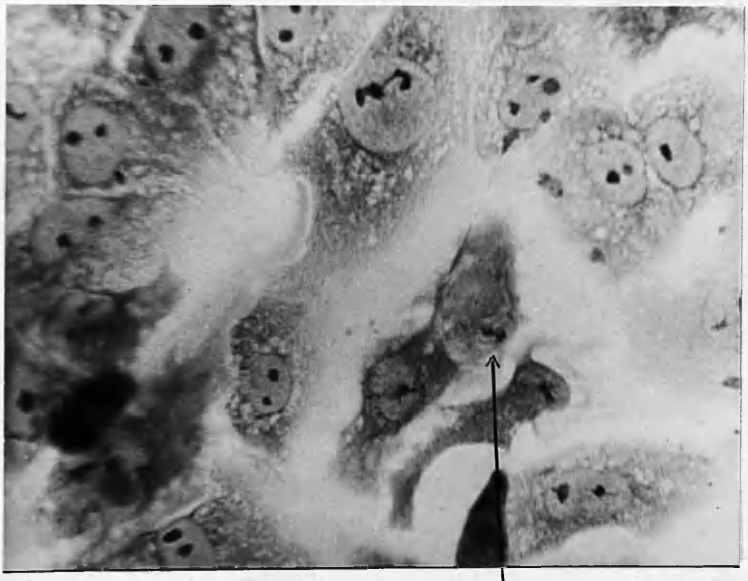


Figure 31

8 hours after infection. The nucleus is being displaced by a very large inclusion body. (X280)
(Giemsa)

At 16 hours after infection a number of cells were fully degenerate, being dark blue rounded spheres with no visible cytoplasmic processes. The grey staining inclusion body was usually quite obvious at this stage. Curiously, these degenerate cells were often seen side by side with apparently normal cells as shown in figure 32.

Comment

Very little variation was distinguishable in this pattern of behaviour from one to another virus. Perhaps the greatest numbers of eosinophilic early inclusion bodies could be seen in cultures infected with ECHO type 2, but the general pattern of inclusion formation, degeneration and the timing of the various stages was remarkably constant with all four viruses.

Results - Heidenhain's Iron Haematoxylin stain.

This stain is particularly useful for the detection of slight alterations in the nucleus and a set of coverslips was stained after infection with ECHO type 7 virus. No counterstain was used.

Changes very similar to those already described were seen at each time interval. The cytoplasmic inclusions stained a lighter shade of grey than the surrounding cytoplasm but the point of most interest was that no nuclear changes could be detected

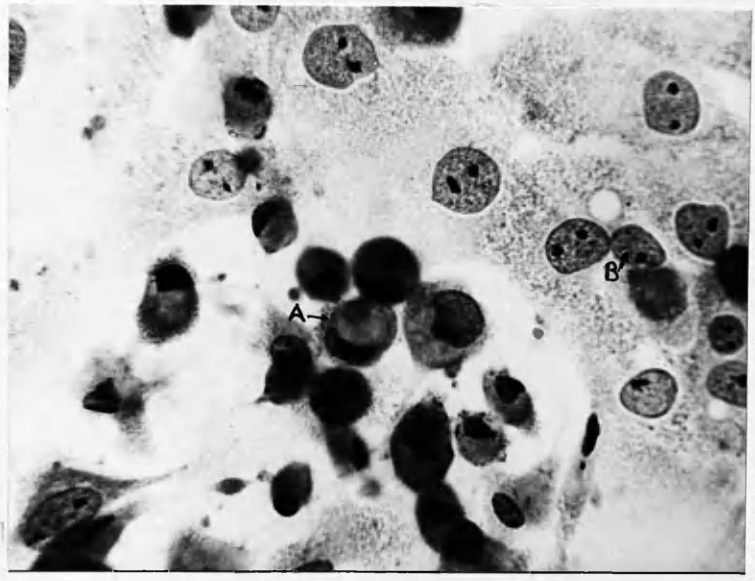


Figure 32

12 hours after infection. In one cell (A) the nucleus is stretched round a large inclusion. Several cells within the field are rounded; nuclear detail has disappeared and the cytoplasm is staining dark blue. This is the final stage of degeneration. Curiously, the degenerate cells are lying side by side with apparently normal cells, but a very large intracytoplasmic inclusion is present in the cell marked (B). (X280) (Giemsa)

until between four and six hours on average when the cytoplasmic inclusion was quite large and perhaps occupied half the cytoplasmic space. Thereafter the changes which took place corresponded precisely to those seen in Giemsa stained preparations.

Discussion

Several points of interest arise from these observations. Without the use of fluorescent antibody studies it is not possible to say that the eosinophilic inclusions in the Giemsa stained films represent ECHO virus aggregates or pre-emergent virus particles. These inclusions have never been seen in un-infected cultures, many of which were used as controls during the experiments, and their regular appearance very shortly after infection with one of the four ECHO viruses is suggestive that they may represent actual virus material within the cell. This suggestion is strengthened by their regular cycle of growth, staining changes and the degeneration which occurs in cells about 15 hours after their first appearance.

The alteration in staining from eosinophilic to neutrophilic at four hours suggests that they are composed of maturing virus material which is altering in character but this change could also reflect merely cellular reaction to their presence.

In any area of an infected culture only a few cells show inclusions despite the relatively high (4:1) multiplicity of infection which was used. This condition holds for the first

seven or eight hours although the numbers of cells showing inclusions increases slightly with time. Thereafter increasing numbers of inclusions can be found until with ECHO types 1, 7 and 11 at 24 hours no normal cells remain in the culture.

Despite the same multiplicity of infection with ECHO type 2, by 24 hours only about 40% of the cells showed changes attributable to the virus; the remainder were normal. In an attempt to represent the development of microscopic changes during infection on a numerical basis figure 33 was constructed. The development in a cell of any of the changes already described and attributable either directly or indirectly to virus growth was scored as "1". 500 cells were examined in each coverslip for each time interval and for each virus and the results expressed as percentages. It will be seen that ECHO types 1, 7 and 11 produce changes in 100% of cells by 24 hours at this multiplicity of infection whereas only 40% of cells showed changes 24 hours after infection with ECHO 2.

When coverslips were stained by Giemsa's stain or by the May-Grunwald-Giemsa method, and differentiated in acetone spirit or acetone-xylol mixture the same type of changes were seen but the eosinophilic inclusions were no longer present. The "inclusions" then appeared as clear cut vacuoles in the cytoplasm. The evolutionary changes were the same with these vacuoles as with the eosinophilic inclusion. The differentiating agents

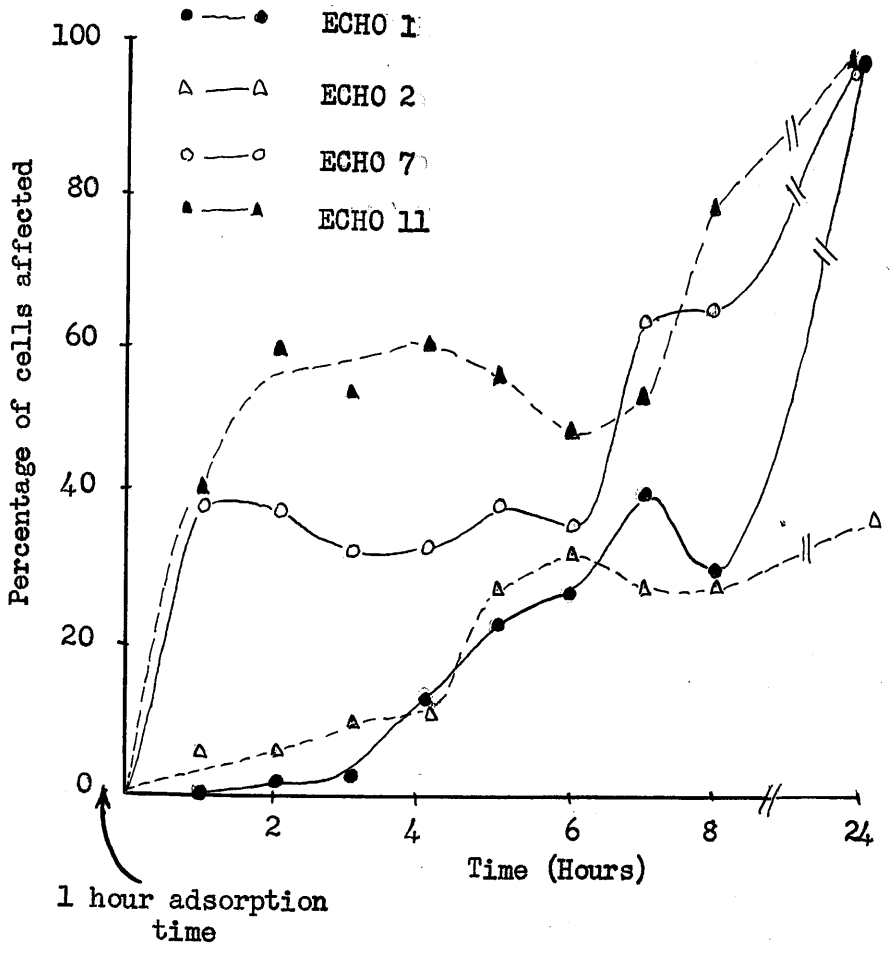


Figure 33

The proportion of monkey kidney cells showing virus induced changes at various time intervals following infection with ECHO virus types 1, 2, 7 or 11.

mentioned have a predilection for the removal of the acid dye whereas blue is removed predominantly by colophonium resin. Such a simple difference as this probably accounts for the different results obtained. The discovery of the eosinophilic inclusions was fortuitous in the first place and was largely due to the preferential use of colophonium differentiator acquired in a previous haematological study.

The absence of nuclear changes in the infected cell as seen by the staining methods used here appears to contradict Ormsbee & Melnick's (1957) suggestion of their presence. These authors used haematoxylin and eosin stained preparations but the absence of nuclear changes in the sensitive Heidenhain stain used here should obviate the difference in the staining method.

CHAPTER XIV

INTERFERENCE EXPERIMENTS INVOLVING ECHO VIRUS TYPES 1, 2, 7 AND 11.

The ability of one virus which is growing within a cell to suppress the growth of subsequently inoculated virus is a well known phenomenon. While most of the work related to interference has been carried out with the influenza viruses a number of reports have appeared recently in which enteroviruses have also shown interference with the growth of each other in susceptible cells. Le Bouvier (1954) showed that an initial infection of monkey kidney cells in vitro with poliovirus type 2 could suppress the growth of a subsequently inoculated Coxsackie B strain whereas Ledinko & Melnick (1954), on the other hand, could demonstrate interference between differing types of poliovirus but not between polioviruses and Coxsackie A strains. A recent paper by Lycke (1958) showed that interference could be demonstrated between polioviruses and Coxsackie types B2, 3 and 4, and also between polioviruses and ECHO types 5, 6 and 7.

In the series of experiments to be described here interference was demonstrated between each and all of the four ECHO viruses under investigation.

Plan of the experiments

Two different types of interference experiment were

performed. In the first type cells were infected with one ECHO virus at a standard dose rate and at varying intervals after infection were challenged with a standard dose of the other 3 viruses. This is referred to as the "time" type of interference experiment.

In the second type the dosage of infecting and challenging viruses were altered but the challenging viruses were always added at exactly one hour after the initial infecting virus was brought into contact with the cells. This type of experiment will be described as the "variable dose" type.

Time type of interference experiment

Large numbers of primary monkey kidney tube cultures were required for each of the four experiments in this series.

For each experiment the tubes were divided into two groups and arranged in sets of 5 within the groups. The first group was emptied of GM and washed once with MM which was then discarded. An inoculum of 0.1 ml. MM containing 4 ID_{50} of virus per cell was added to each tube. (The average number of cells per tube in each experiment was around 150,000). The tubes were re-stoppered and incubated as usual.

The second group of tubes was treated in an exactly similar manner but was inoculated only with 0.1 ml. MM containing no virus.

After incubation for 1, 3 or 5 hours the monolayers in different sets of 5 tubes from both groups were washed thoroughly three times with PBS, and to one set of 5 tubes from each group 1.0 ml. MM containing 4 ID_{50} per cell of one of the other three viruses was added. In this way each challenge virus was inoculated at the same dose into 5 tubes previously infected with a different virus and 5 tubes which had no previous infection and therefore served as virus control tubes for each challenge virus. Two sets of 5 tubes, after washing, were given no challenge virus but had 1.0 ml. MM added to each tube, and thus served as virus controls for the interfering virus. This process was repeated at each time interval. Thereafter the tubes were incubated for 48 hours to allow time for virus growth, before the fluids from each set of 5 tubes were pooled for titration.

Titration was always performed in cells from the same monkey. Before titration an equal amount of high titre antiserum to the interfering virus was incubated for one hour at a suitable dilution with one aliquot of the virus control fluid. An equal amount of the same dilution of an antibody-free normal rabbit serum was incubated for one hour with another aliquot of the virus control. Thereafter each mixture was titrated for infectivity in 0.5 log steps using 5 tubes per dilution.

Every other fluid from each group of 5 tubes was

incubated similarly with the high titre antiserum to the interfering virus and each aliquot, whether interfering virus was present or not, was then titrated for infectivity in 0.5 log steps using 5 tubes per dilution. The titrations were read at 3 days and infectivity titres were calculated as usual by the method of Karber (1931).

This procedure was repeated in turn for each of the 4 viruses as interfering virus and in each case the interference was challenged by the other 3.

Results

The results which were obtained with the "time" type of experiment are shown in tables 27, 28, 29 and 30. Interference was observed with all 4 viruses in each combination of pairs and although the results obtained from the different interfering times with any one virus are rather variable, very little difference was observed between the degree of interference produced at one hour and at five hours. By one hour, in every instance, the titre of the challenging virus was depressed by 2 to 3 log steps.

Proof that the antiserum to the interfering virus produced complete neutralisation of any free particles in the final mixture before titration is available in the second figure in the right hand column of each table, where, at the working dilution of 10^{-2} no growth of interfering virus was obtained.

TABLE 27

Interference experiment - "time type"

Effect of varying the time for which ECHO virus type 1 is allowed to react with monkey kidney cells on subsequent growth of challenge doses of ECHO 2, 7 and 11.

First inoculum		Interfering time (hrs.)	Second Inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50} per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture		Virus	Dose (ID_{50}) per cell of culture		
ECHO 1	4	-	None	-	NRS*	4.16
ECHO 1	4	-	None	-	+E1	<2
None	-	-	ECHO 2	4	+E1	7.16
None	-	-	ECHO 7	4	+E1	7.16
None	-	-	ECHO 11	4	+E1	5.50
ECHO 1	4	1	ECHO 2	4	+E1	4.86
ECHO 1	4	1	ECHO 7	4	+E1	4.86
ECHO 1	4	1	ECHO 11	4	+E1	2.50
ECHO 1	4	3	ECHO 2	4	+E1	4.86
ECHO 1	4	3	ECHO 7	4	+E1	4.50
ECHO 1	4	3	ECHO 11	4	+E1	2.50
ECHO 1	4	5	ECHO 2	4	+E1	5.10
ECHO 1	4	5	ECHO 7	4	+E1	4.86
ECHO 1	4	5	ECHO 11	4	+E1	2.10

* NRS = normal rabbit serum.

TABLE 28

Interference experiment - "time type"

Effect of varying the time for which ECHO virus type 2 is allowed to react with monkey kidney cells on subsequent growth of challenge doses of ECHO 1, 7 and 11.

First inoculum		Interfering time (hrs.)	Second Inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50} per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture		Virus	Dose (ID_{50}) per cell of culture		
ECHO 2	4	-	None	-	NRS*	6.50
ECHO 2	4	-	None	-	+E2	<2
None	-	-	ECHO 1	4	+E2	5.16
None	-	-	ECHO 7	4	+E2	4.16
None	-	-	ECHO 11	4	+E2	5.83
ECHO 2	4	1	ECHO 1	4	+E2	3.50
ECHO 2	4	1	ECHO 7	4	+E2	<2
ECHO 2	4	1	ECHO 11	4	+E2	3.50
ECHO 2	4	3	ECHO 1	4	+E2	3.50
ECHO 2	4	3	ECHO 7	4	+E2	<2
ECHO 2	4	3	ECHO 11	4	+E2	3.16
ECHO 2	4	5	ECHO 1	4	+E2	<2
ECHO 2	4	5	ECHO 7	4	+E2	<2
ECHO 2	4	5	ECHO 11	4	+E2	3.16

* NRS = normal rabbit serum.

TABLE 29

Interference experiment - "time type"

Effect of varying the time for which ECHO virus type 7 is allowed to react with monkey kidney cells on subsequent growth of challenge doses of ECHO 1, 2 and 11.

First inoculum		Interfering time (hrs.)	Second Inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50} per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture		Virus	Dose (ID_{50}) per cell of culture		
ECHO 7	4	-	None	-	NRS*	7.50
ECHO 7	4	-	None	-	+E7	<2
None	-	-	ECHO 1	4	+E7	6.10
None	-	-	ECHO 2	4	+E7	6.50
None	-	-	ECHO 11	4	+E7	7.10
ECHO 7	4	1	ECHO 1	4	+E7	2.70
ECHO 7	4	1	ECHO 2	4	+E7	<2
ECHO 7	4	1	ECHO 11	4	+E7	3.50
ECHO 7	4	3	ECHO 1	4	+E7	2.40
ECHO 7	4	3	ECHO 2	4	+E7	<2
ECHO 7	4	3	ECHO 11	4	+E7	<2
ECHO 7	4	5	ECHO 1	4	+E7	<2
ECHO 7	4	5	ECHO 2	4	+E7	2.80
ECHO 7	4	5	ECHO 11	4	+E7	3.50

* NRS = normal rabbit serum.

TABLE 30

Interference experiment - "time type"

Effect of varying the time for which ECHO virus type 11 is allowed to react with monkey kidney cells on subsequent growth of challenge doses of ECHO 1, 2 and 7.

First inoculum		Interfering time (hrs.)	Second Inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50} per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture		Virus	Dose (ID_{50}) per well of culture		
ECHO 11	4	-	None	-	NRS*	5.16
ECHO 11	4	-	None	-	+E11	<2
None	-	-	ECHO 1	4	+E11	5.16
None	-	-	ECHO 2	4	+E11	7.16
None	-	-	ECHO 7	4	+E11	6.16
ECHO 11	4	1	ECHO 1	4	+E11	2.16
ECHO 11	4	1	ECHO 2	4	+E11	4.50
ECHO 11	4	1	ECHO 7	4	+E11	2.83
ECHO 11	4	3	ECHO 1	4	+E11	3.50
ECHO 11	4	3	ECHO 2	4	+E11	4.50
ECHO 11	4	3	ECHO 7	4	+E11	3.16
ECHO 11	4	5	ECHO 1	4	+E11	2.50
ECHO 11	4	5	ECHO 2	4	+E11	3.80
ECHO 11	4	5	ECHO 7	4	+E11	2.83

* NRS = normal rabbit serum.

In view of the apparent independence of the time of incubation of the interfering virus with the cells with the final degree of interference produced the second type of interference experiment was undertaken. In this the dose of the interfering and challenge viruses was varied in each instance in an attempt to find out whether a greater degree of interference would result when a greater amount of interfering virus was used, relative to the challenge virus.

"Variable dose" type of interference experiment.

In this series the sequence of operations and plan of the experiment was essentially the same as that used in the "time" type, with the differences that 2 dosage levels of virus were used with each of the interfering and challenge viruses in any one experiment. In practice the levels used were 4 and 0.04 ID_{50} per cell of the culture. Either dosage level of each interfering virus was allowed only one hour to adsorb to the cells before the inoculum was removed, washed and challenged with either the high or low dose of one of the other three viruses. The cultures were incubated for 48 hours before harvesting, when the fluids from each group of 5 tubes were pooled.

Before titration each pooled fluid was incubated with high titre rabbit antiserum to the interfering virus for one hour to eliminate any infectious particles of the interfering virus and to ensure that the infectivity titration measured only

the amount of challenge virus present in the fluid. As before the virus control for the interfering virus was incubated separately before titration both in the presence of high titre homologous antiserum and with normal antibody-free rabbit serum. The results of the titrations were read and recorded after 3 days of incubation.

Results

These are contained in tables 31 to 42. In each case the interfering virus grew to a reasonable titre by itself when no challenge inoculum was added and also in each case the interfering virus could be completely neutralized by its homologous antiserum to within the limits of working at the greatest concentration tested, i.e. 10^{-2}. The lower dose of interfering virus produced approximately one-tenth of the amount of infectious virus in the control tubes as compared with the higher dose rate with the exception of ECHO 11 where, curiously, the same titre was recorded with both high and low dose virus controls.

A variable amount of interference was produced with the growth of each challenge virus. This ranged in extent from nil with a low dose of ECHO 1 as interfering virus and a subsequently inoculated high dose of ECHO 2 (table 31), to complete suppression of measurable challenge virus production with ECHO 2 as interfering agent at high or low dosage and high or low dosage of ECHO 1 or ECHO 7 as challenge viruses (tables

TABLE 31 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 1 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 2 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 1	4	None	-	NRS	5.50
ECHO 1	4	None	-	+E1	<2
ECHO 1	0.04	None	-	NRS	4.16
ECHO 1	0.04	None	-	+E1	<2
ECHO 1	4	ECHO 2	4	+E1	4.50
ECHO 1	4	ECHO 2	0.04	+E1	2.16
ECHO 1	0.04	ECHO 2	4	+E1	5.83
ECHO 1	0.04	ECHO 2	0.04	+E1	3.83
None	-	ECHO 2	4	+E1	5.83
None	-	ECHO 2	0.04	+E1	5.50

TABLE 32 — Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 1 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 7 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 1	4	None	-	NRS	5.50
ECHO 1	4	None	-	+E1	<2
ECHO 1	0.04	None	-	NRS	4.16
ECHO 1	0.04	None	-	+E1	<2
ECHO 1	4	ECHO 7	4	+E1	2.83
ECHO 1	4	ECHO 7	0.04	+E1	<2
ECHO 1	0.04	ECHO 7	4	+E1	4.16
ECHO 1	0.04	ECHO 7	0.04	+E1	3.50
None	-	ECHO 7	4	+E1	4.83
None	-	ECHO 7	0.04	+E1	4.83

TABLE 33 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 1 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 11 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 1	4	None	-	NRS	5.50
ECHO 1	4	None	-	+E1	<2
ECHO 1	0.04	None	-	NRS	4.16
ECHO 1	0.04	None	-	+E1	<2
ECHO 1	4	ECHO 11	4	+E1	<2
ECHO 1	4	ECHO 11	0.04	+E1	<2
ECHO 1	0.04	ECHO 11	4	+E1	4.16
ECHO 1	0.04	ECHO 11	0.04	+E1	2.50
None	-	ECHO 11	4	+E1	4.83
None	-	ECHO 11	0.04	+E1	4.16

TABLE 34 — Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 2 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 1 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 2	4	None	-	NRS	6.50
ECHO 2	4	None	-	+E2	<2
ECHO 2	0.04	None	-	NRS	5.83
ECHO 2	0.04	None	-	+E2	<2
ECHO 2	4	ECHO 1	4	+E2	<2
ECHO 2	4	ECHO 1	0.04	+E2	<2
ECHO 2	0.04	ECHO 1	4	+E2	<2
ECHO 2	0.04	ECHO 1	0.04	+E2	<2
None	-	ECHO 1	4	+E2	5.50
None	-	ECHO 1	0.04	+E2	4.16

TABLE 35 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 2 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 1 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 2	4	None	-	NRS	6.50
ECHO 2	4	None	-	+E2	<2
ECHO 2	0.04	None	-	NRS	5.83
ECHO 2	0.04	None	-	+E2	<2
ECHO 2	4	ECHO 7	4	+E2	<2
ECHO 2	4	ECHO 7	0.04	+E2	<2
ECHO 2	0.04	ECHO 7	4	+E2	<2
ECHO 2	0.04	ECHO 7	0.04	+E2	<2
None	-	ECHO 7	4	+E2	5.50
None	-	ECHO 7	0.04	+E2	5.16

TABLE 36 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 2 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 11 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 2	4	None	-	NRS	6.50
ECHO 2	4	None	-	+E2	<2
ECHO 2	0.04	None	-	NRS	5.83
ECHO 2	0.04	None	-	+E2	<2
ECHO 2	4	ECHO 11	4	+E2	3.50
ECHO 2	4	ECHO 11	0.04	+E2	3.50
ECHO 2	0.04	ECHO 11	4	+E2	3.83
ECHO 2	0.04	ECHO 11	0.04	+E2	4.16
None	-	ECHO 11	4	+E2	5.50
None	-	ECHO 11	0.04	+E2	4.83

TABLE 37 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 7 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 1 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 7	4	None	-	NRS	6.1
ECHO 7	4	None	-	+E7	<2
ECHO 7	0.04	None	-	NRS	5.5
ECHO 7	0.04	None	-	+E7	<2
ECHO 7	4	ECHO 1	4	+E7	3.1
ECHO 7	4	ECHO 1	0.04	+E7	2.1
ECHO 7	0.04	ECHO 1	4	+E7	<2
ECHO 7	0.04	ECHO 1	0.04	+E7	<2
None	-	ECHO 1	4	+E7	4.8
None	-	ECHO 1	0.04	+E7	4.5

TABLE 38 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 7 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 2 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 7	4	None	-	NRS	6.1
ECHO 7	4	None	-	+E7	<2
ECHO 7	0.04	None	-	NRS	5.5
ECHO 7	0.04	None	-	+E7	<2
ECHO 7	4	ECHO 2	4	+E7	2.8
ECHO 7	4	ECHO 2	0.04	+E7	2.1
ECHO 7	0.04	ECHO 2	4	+E7	3.8
ECHO 7	0.04	ECHO 2	0.04	+E7	3.8
None	-	ECHO 2	4	+E7	5.1
None	-	ECHO 2	0.04	+E7	4.5

TABLE 39 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 7 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 11 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration.	Virus titre (log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 7	4	None	-	NRS	6.1
ECHO 7	4	None	-	+E7	<2
ECHO 7	0.04	None	-	NRS	5.5
ECHO 7	0.04	None	-	+E7	<2
ECHO 7	4	ECHO 11	4	+E7	2.8
ECHO 7	4	ECHO 11	0.04	+E7	N.T.*
ECHO 7	0.04	ECHO 11	4	+E7	4.5
ECHO 7	0.04	ECHO 11	0.04	+E7	4.1
None	-	ECHO 11	4	+E7	4.5
None	-	ECHO 11	0.04	+E7	3.8

* N.T. = not tested.

TABLE 40 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 11 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 1 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 11	4	None	-	NRS	4.16
ECHO 11	4	None	-	+E11	<2
ECHO 11	0.04	None	-	NRS	4.16
ECHO 11	0.04	None	-	+E11	<2
ECHO 11	4	ECHO 1	4	+E11	<2
ECHO 11	4	ECHO 1	0.04	+E11	<2
ECHO 11	0.04	ECHO 1	4	+E11	<2
ECHO 11	0.04	ECHO 1	0.04	+E11	<2
None	-	ECHO 1	4	+E11	5.16
None	-	ECHO 1	0.04	+E11	3.50

TABLE 41 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 11 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 2 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 11	4	None	-	NRS	4.16
ECHO 11	4	None	-	+E11	<2
ECHO 11	0.04	None	-	NRS	4.16
ECHO 11	0.04	None	-	+E11	<2
ECHO 11	4	ECHO 2	4	+E11	2.50
ECHO 11	4	ECHO 2	0.04	+E11	<2
ECHO 11	0.04	ECHO 2	4	+E11	4.50
ECHO 11	0.04	ECHO 2	0.04	+E11	4.83
None	-	ECHO 2	4	+E11	5.50
None	-	ECHO 2	0.04	+E11	5.16

TABLE 42 Interference experiment - "variable dose type"

The effect of varying the dosage of ECHO 11 as interfering virus on the degree of interference with the growth of subsequently inoculated ECHO 7 in monkey kidney cells.

First inoculum		Second inoculum		Type of serum added at time of titration.	Virus titre (Log 10 ID_{50}) per 0.1 ml.
Virus	Dose (ID_{50}) per cell of culture	Virus	Dose (ID_{50}) per cell of culture		
ECHO 11	4	None	-	NRS	4.16
ECHO 11	4	None	-	+E11	<2
ECHO 11	0.04	None	-	NRS	4.16
ECHO 11	0.04	None	-	+E11	<2
ECHO 11	4	ECHO 7	4	+E11	2.16
ECHO 11	4	ECHO 7	0.04	+E11	2.50
ECHO 11	0.04	ECHO 7	4	+E11	3.16
ECHO 11	0.04	ECHO 7	0.04	+E11	4.16
None	-	ECHO 7	4	+E11	5.50
None	-	ECHO 7	0.04	+E11	5.16

34 and 35). Similar complete suppression of measurable challenge virus production occurred also with high dosage of ECHO 1 challenged with ECHO 7 at low dosage, with high dosage of ECHO 1 challenged with ECHO 11 at high or low dosage, with low dosage of ECHO 7 challenged with high or low dosage of ECHO 1, with high or low dosage of ECHO 11 challenged with high or low dosage of ECHO 1, and with high dosage of ECHO 11 challenged with low dosage of ECHO 2.

As was espected, in general, the lower dosage of challenge virus in each case produced less infective virus after 3 days than did the greater inoculum contained in the higher dosage.

Conversely and also as expected the amount of challenge virus produced was greater at either the high or low dosage levels of input when the dosage of the interfering virus was at its lower level.

Discussion

The results obtained from both series of experiments ("time" and "variable dosage" types) show beyond doubt that interference can be demonstrated in every combination tested between the four ECHO viruses under investigation. The degree of interference is variable from one to another instance and the variability is no doubt mediated partially by experimental error in that the interference was measured on the pooled fluid

obtained from 5 test tubes each representing "one experiment" in every case. The titrations of infectivity performed in 0.5 log steps and using 5 tubes per step are also liable to incorporate an inherent error which is not able to be overcome without the use of a very large increase in the number of tubes and consequently of monkeys.

The degree of interference was of the same order as observed by Lycke (1958) who used poliovirus type 1 as his interfering agent which was subsequently challenged with one or other of several ECHO virus types. In Lycke's experiments, however, optimal inhibition was obtained when the poliovirus was allowed between 8 and 22 hours of contact with the cells before the addition of the challenge virus. The growth cycle of poliovirus is known to be completed in approximately 8 hours and it did not therefore seem surprising that he was able to show marked interference after between one and 3 growth cycles. Indeed the surprising point of his work lay in his ability to demonstrate any growth of challenge virus at all.

The experiments which have been described here were all designed to overcome this difficulty by keeping well within the known duration of one growth cycle for any of the viruses under present investigation. I have previously shown that the growth cycles are all complete in approximately 8 hours after infection of the monolayer and the 5 hour end point was chosen

therefore as the greatest length of time permissable between the addition of interference and challenge viruses.

Mature consideration of my results has however revealed the disadvantage of this otherwise advantageous procedure. The level of virus input at 4 ID_{50} per cell of the culture is certainly not adequate to infect all the cells of the culture after one hour's incubation. At best the proportion of cells infected will vary from 90% for ECHO 7 to about 40% for ECHO 2, and when this relatively short interfering time is used the interference may simply represent the diminished number of cells in the culture which have not already been infected by the interfering virus. A smaller number of cells infected with virus would produce a smaller amount of total infectivity than a larger number of cells infected with the same number of virus particles because in the latter instance more virus particles can act as infectious units and initiate their individual growth cycles.

This, of course, is a form of indirect interference but what was intended to be measured - the ability of a cell previously infected with one virus to support the growth or otherwise of a second virus - would not be completely achieved.

A much higher proportion of interfering virus would be adsorbed by 3 or 5 hours - indeed it is likely that maximum adsorption occurs at about 3 hours - and as a consequence the results obtained from the 3 and 5 hour interfering time experiments

are probably reflecting true interference, if one assumes that every cell in the culture is in the physiological state necessary for adsorption to itself of a particle of interfering virus.

In the "variable dose" type of interference experiment a number of interesting results was obtained. In particular the ability of high or low dosage of ECHO type 2 virus to suppress the growth in measurable amount of ECHO types 1 and 7 at either high or low dosage is of importance. This suppression occurred after one hour's adsorption of ECHO type 2 - by which time 40% of the 4 ID_{50} per cell inoculum would be absorbed, as measured in the earlier series of adsorption experiments. It is therefore difficult to explain the ability of this virus under these circumstances to depress the infectivity titre of either ECHO 1 or ECHO 7 by 3.5 log steps.

The ability, in general, of a high dosage of interfering virus to suppress partially the growth of a smaller dosage of challenge virus as has been demonstrated is perhaps to be expected. If the high dosage of interfering agent does not utilise all the cell receptors, or even utilise any of the cells themselves, then the challenge virus will still be able to produce some new infectious particles in the remaining uninfected cells. This ratio could also hold good if the cells were not the substrate of prime importance for the virus. Ledinko & Melnick (1954) and Ziegler & Horsfall (1944) who were studying interference

phenomena between influenza viruses proposed that, when two "related" viruses interfere with the growth of each other in cells in which both can grow, the interference is due to competition within the cells for a substance which exists in a certain fixed amount. When interference is produced by small amounts of interfering virus the necessary substrate is rendered unavailable by the multiplication of the interfering virus. If large amounts of the challenge virus are inoculated the advantage in terms of numbers, would be shifted in favour of the challenger. It seems likely that such an explanation would necessitate the simultaneous multiplication of two different viruses in one cell, at least in some instances. This is perhaps a rather unlikely event which would require a very large amount of experimental investigation to prove.

CHAPTER XV

AN EXAMINATION OF THE PROPERTIES OF 24 LOCALLY ISOLATED STRAINS OF ECHO VIRUS TYPE 7.

In the course of the surveys of the distribution of enteroviruses in the stools of patients admitted to hospital with the diagnosis of aseptic meningitis or diarrhoea which were described in part one, 24 viruses belonging to ECHO type 7 were isolated during 1956 and 1957. The manipulations which were required for the identification of these viruses revealed differences in the time taken to produce complete cytopathic effects when the same number of ID_{50} of each virus were inoculated into identical tubes of monkey kidney cells. This varied between 2 and 5 days. In view of the fact that these variations could not be accounted for by alterations in the infectivity titre I thought it possible that other fundamental differences might be definable experimentally which would allow classification of at least some of these strains as "prime" variants of ECHO type 7. A precedent existed in that the Committee on Enteroviruses (1957) had already described "prime" variants of ECHO type 6 and also variants of ECHO types 5, 7, 9, 10, 11 and 13 which exhibited only partial antigenic crossing with their respective prototype strains. The investigations to be described were undertaken with a view to classifying my

locally isolated strains in the light of these potential differences.

Plan of the experiments

The methods I selected for the examination of the strains can be listed as follows.

(a) Neutralization Tests. Two types of test were used in which the locally isolated strains were neutralized by high titre stock rabbit antiserum prepared against ECHO type 7 prototype (Wallace) strain. Each measured different functions of the neutralization reaction and each type of test produced a series of results which could be recorded graphically as a "neutralization slope".

(b) Plaque production in monkey kidney cells with regard to the time of first appearance and the size of plaque produced in a standard time.

(c) Mouse pathogenicity after inoculation of virus by the intraperitoneal or intracerebral routes.

(d) Haemagglutination titre. The titre produced by each virus after a standard time of incubation in monkey kidney cells.

The methods used for each test will be described in detail in the Materials and Methods section which follows, and which is included at this point because these differed significantly from the standard methods used throughout the work.

MATERIALS AND METHODS

Viruses

Each strain of ECHO type 7 was originally isolated in tube cultures of either cynomolgous monkey kidney (CMK) or human amnion cells. In 21 instances the virus was isolated from a child admitted to Ruchill Hospital, Glasgow, with a diagnosis of either "clinical dysentery" or "Gastro-enteritis"; 2 were isolated from children admitted to the same hospital with acute respiratory symptoms and the other came from an adult female admitted to King's Cross Hospital, Dundee, with a diagnosis of aseptic meningitis. Each virus was isolated from a stool specimen collected between November 1956 and February 1958. The technique used for isolation have already been described in part one.

After identification by the conventional neutralization test a stock of each strain was prepared by inoculating it into 20 tubes of CMK cells. The fluids from each group of tubes were pooled when complete degeneration occurred. The cell debris was removed by centrifugation after one cycle of freezing and thawing and each strain was then stored frozen at -40°C. in aliquots of 2.0 ml.

Preparation of stock high titre ECHO 7 prototype (Wallace) antiserum.

After 3 passages at limiting dilution, 200 ml. bulk

stock of ECHO type 7 (Wallace) was prepared in CMK cells. Cell debris and protein were removed by centrifugation and by 2 extractions with a mixture (S.G. 1.3) of fluorocarbon and n-heptane. By these combined procedures the protein content of the suspension was reduced to one tenth of its original value. A tenfold concentration of the virus was subsequently achieved by centrifugation at 40,000 r.p.m. for 2 hours in a "spinco" ultracentrifuge. Pellet formation was aided by the addition of 5% monkey serum free from ECHO 7 antibody. The infectivity titre of this preparation after treatment was $10^{7.58}$ ID_{50} per ml.

Three rabbits were then immunized by intravenous injection of 1.0 ml. of the concentrated preparation of ECHO 7 on three occasions at 4 day intervals. Each rabbit was bled by heart puncture 7 days after the last injection. The sera were pooled, inactivated and stored frozen at -20°C. An 0.1 ml. aliquot of this antiserum neutralized 20,000 ID_{50} of the stock ECHO 7 (Wallace) strain.

Neutralization Tests

Two types were employed:-

(a) Checker-board type.

In this test serial dilutions containing approximately 10^4, 10^3, 10^2, or 10 ID_{50} of each virus in 0.5 ml. were mixed with 0.5 ml. of antiserum diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} or 10^{-5}. The mixtures were incubated for 1 hour at 20°C. Residual

infectivity after 1 hour was estimated for each mixture by titration in CMK cells and the results obtained were plotted graphically to obtain the "neutralization slope".

(b) Time type.

In 4 tubes approximately 10^4 ID_{50} of each virus in 0.1 ml. was mixed with 0.1 ml. undiluted ECHO 7 (Wallace) antiserum. The mixtures were incubated at 20°C. and at intervals of 0, 5, 10 and 15 minutes 0.1 ml. of mixture was diluted 1:100 in chilled PBS. These dilutions were kept in an ice bath until the end of each experiment when each was titrated for residual infectivity in CMK cells. The results were again plotted graphically to obtain the "neutralization slope".

Plaque production.

The "standard" plaque technique of Hsiung & Melnick (1957) was used. Each virus was tested at several dilutions, usually 10 and 1 ID_{50} per ml. Plaques were produced usually in 4 oz. "medical flat" bottles, and ECHO 7 antibody-free monkey serum was incorporated in the agar overlay. The number of days to first appearance and the mean diameter of 10-20 plaques measured after 2 days were recorded.

Mouse pathogenicity.

Each virus was inoculated in the form of cell free tissue culture fluid by the intracerebral and intraperitoneal

route into suckling albino mice of a virus sensitive strain less than 24 hours old. The actual amount of virus varied with the titre of the preparation under investigation. The inoculum was 0.1 ml. intraperitoneally and 0.025 ml. by the intracerebral route.

Haemagglutination Tests

Each virus was titrated for haemagglutination in plastic plates by mixing 0.3 ml. of doubling dilutions prepared in sodium veronal buffer (NaVB) at pH 7.3 with an equal amount of 1% suspension in NaVB of human group O red cells. These latter had previously been washed three times in NaVB. The tests were read by the pattern produced at 1 hour, the titre being recorded as the reciprocal of the final dilution of virus producing 50% haemagglutination.

RESULTS

Neutralization Tests

(a) Checker-board type.

ECHO type 7 (Wallace) produced a straight line graph with slope 0.70 against its own antiserum in this type of test. A straight line was also obtained with all 24 isolates and the results are shown in table 43. The arithmetic mean ($\bar{x}$) of slopes was 0.77 and the standard deviation (σ) corrected for the small

size of the sample population, 0.34. The range covered by plus or minus two standard deviations from the mean ($\bar{x} \pm 2\sigma$) is therefore 0.09 to 1.45 and only two viruses were observed to give a slope greater than the upper extreme of this range. Isolates nos. 10 and 24 respectively gave slopes of 1.50 and 1.53, but these do not differ significantly from the mean since $P > 0.05$.

(b) Time type

With this type of experiment ECHO 7 (Wallace) produced a slope of 0.73 when tested against its own antiserum. The mean slope ($\bar{x}$) obtained from the 24 isolates was 0.74 with a corrected standard deviation (σ) of 0.163. The range ($\bar{x} \pm 2\sigma$) was therefore 0.414 to 1.066. Only one isolate produced a slope which lay outside this range (No. 11), but the slope (0.40) again does not differ significantly from the mean since $P > 0.05$. The results obtained from individual viruses are recorded in table 43.

Plaques

With all 24 isolates and with ECHO type 7 (Wallace) these were first observed between 3 and 4 days after seeding. The average time to first appearance was 3.2 days. Remarkably homogeneity is shown in the size of plaques produced since in each instance the average mean diameter falls within plus or minus two standard deviations from the mean of 2.54 mm. These

results are also contained in table 43.

Mouse pathogenicity

No effects were observed following intracerebral or intraperitoneal inoculation of each agent into suckling mice which were less than 24 hours old at the time of inoculation.

Haemagglutination Tests

The prototype strain of ECHO 7 (Wallace) was shown to produce haemagglutination with 1% washed human group O red cells after one hour of incubation at room temperature, 37°C. or 4°C. As is shown in table 43 only 8 of the 24 isolates were able to induce haemagglutination. The haemagglutinin titres varied from 12 to 128 and no correlation with infectivity titre or temperatures of incubation could be demonstrated. The remaining 16 viruses did not cause haemagglutination when the temperature of incubation was varied from 4 through 37°C., or when red cells from several donors belonging to group O were tested.

Discussion

Despite the differences which were observed in the speed with which complete degeneration was produced in tissue cultures, the twenty-four agents under consideration form a remarkably homogeneous group in respect of neutralization by type-specific antiserum, plaque formation and absence of mouse

pathogenicity. The only major characteristic which could be used to separate the viruses into two groups was the presence or absence of the ability to cause haemagglutination. This ability appears to be independent of infectivity titre and not related to the number of passages which the virus has undergone. In each instance the haemagglutinin could be inhibited by high titre antiserum prepared against the prototype ECHO 7 (Wallace).

A similar variation in ability to cause haemagglutination has been described by Lahelle (1958) for ECHO virus type 6, and I have recently found two strains of Coxsackie type A9 which, contrary to accepted standards, are able to agglutinate human group O red cells (Sommerville & McIntosh, 1959).

Viruses of simian origin have been noted by Hull, Minner and Smith (1956) to be capable of causing haemagglutination with human group O red cells, and the possibility that some of the strains under consideration may have become contaminated by a simian agent must be considered. To be set against this possibility, however, are the facts that agent 5 of the present series, which is haemagglutinin positive, prior to the passage in cynomolgous monkey kidney cells for "stock building" purposes had been isolated and passaged only in human amnion cells, and also that all agents were passaged in the same batch of monkey kidney cells for "stock building" purposes. I have not been

aware of simian viruses or other agents causing spontaneous degeneration or the formation of inclusion bodies within monkey kidney cells in any cultures during the last three years in this laboratory.

Goldfield, Srihongse & Fox (1957) were able to demonstrate haemagglutinin-inhibiting antibody in humans infected with ECHO type 7 or with a variety of different enteroviruses all of which are capable of causing haemagglutination. This helps to strengthen the likelihood that the haemagglutinin is not necessarily associated with a contaminating simian virus. I think the evidence in the present context, however, strongly favours the theory that the haemagglutinin is not simian in origin.

Despite the inability to separate strains on the basis of neutralization slope into prototypes and "prime strains" the differences in behaviour with regard to haemagglutinin production seem to be permanent for any given virus within this series. This represents a most important marker mechanism for the differentiation of otherwise identical agents and considerably strengthens the possibility that "prime" strains of ECHO 7 do exist.

TABLE 43

A comparison of the measurable characteristics of 24 locally isolated strains of ECHO virus type 7 with prototype ECHO 7 (Wallace)

Strain	Disease from which isolated.	Tissue culture type and pass number	Titre Log_{10} ID_{50} per 0.1 ml.	Days to complete CPE (*) (10^3 ID_{50}) inoculum	Neutralization Slopes		Plaques		Haemagglutination with human group "O" cells.
					Checker board	Time	Days to first appearance	Mean diam. at 2 days (MM)	
ECHO 7 (Wallace)		Monkey kidney (MK) 13	6.45	2	0.70	0.73	3	2.85	+
1	Diarrhoea	MK 5	4.75	2	0.87	0.85	3	2.55	-
2	Diarrhoea	MK 9	4.55	4	0.90	0.90	3	2.16	-
3	Diarrhoea	MK 4	2.25	3	0.55	0.75	4	2.00	-
4	Diarrhoea	MK 4	5.45	3	0.33	0.85	4	2.00	-
5	Diarrhoea	Amnion (AM) 6	5.25	2	0.50	0.85	3	2.83	+
6	Diarrhoea	MK 2	5.95	2	0.30	0.55	3	3.10	+
7	Diarrhoea	MK 6	5.95	2	0.67	0.85	3	2.80	+
8	Diarrhoea	MK 5	5.35	4	0.50	0.85	4	2.00	-
9	Diarrhoea	MK 4	6.35	3	0.74	0.85	3	3.10	-
10	Diarrhoea	MK 4	5.85	3	1.50	1.05	3	2.45	-
11	Diarrhoea	MK 5	6.15	3	0.67	0.40	3	2.60	-
12	Diarrhoea	AM 2	6.65	3	0.83	0.60	3	2.50	-
13	Diarrhoea	AM 2	6.55	3	0.60	0.85	3	2.40	-
14	Diarrhoea	AM 2	6.05	3	0.77	0.70	3	3.10	-
15	Respiratory	MK 3	5.85	3	1.25	0.67	3	2.80	-
16	Diarrhoea	MK 2	6.35	3	0.50	0.67	3	2.40	+
17	Diarrhoea	MK 2	5.25	2	0.83	1.00	3	2.53	+
18	Diarrhoea	MK 2	6.15	2	1.17	0.75	3	2.00	+
19	Diarrhoea	MK 2	5.25	2	0.50	0.75	4	2.45	+
20	Diarrhoea	MK 3	5.25	2	0.67	0.60	3	2.48	-
21	Respiratory	MK 3	6.25	3	0.50	0.47	3	2.73	-
22	Diarrhoea	MK 2	5.85	3	0.55	0.65	3	2.90	-
23	Diarrhoea	MK 2	6.45	3	1.17	0.57	3	2.50	-
24	Aseptic meningitis	MK 3	5.75	2	1.53	0.75	3	2.80	+

(*) Cytopathic effect

CHAPTER XVI

GENERAL DISCUSSION AND SUMMARY

The studies which have been reported here were undertaken during my tenure of the Sir Maurice Bloch Medical Research Fellowship in Virology and have allowed me an outlet for my thoughts in two directions - towards the patient in hospital and towards the laboratory in the realm of pure research.

In a work of this nature, however, it has been possible to cover only certain facets within the general topic of "enteroviruses" and I am left at the end of my three-year study only too well aware that my contribution to the body of knowledge about these agents is but a scratch on the surface of a very much larger problem. My studies have uncovered a number of observations however, which I have felt were worthy to be recorded and which point the way for future investigations.

As the thesis was written I have realised on countless occasions how favoured I have been in obtaining excellent clinical cooperation during the epidemiological investigations which are described in parts 1 and 2. Without this full measure of cooperation such epidemiological research becomes extremely hazardous. Perhaps the best demonstration of what can be achieved under ideal conditions is the story of the outbreak of aseptic meningitis due to ECHO virus type 9 in Dundee, where

by careful clinical selection of cases, almost 100% of persons examined were proved to be infected with this virus. This result is most striking when it is compared with the rather disappointing overall pattern of laboratory results from cases of aseptic meningitis from the remainder of my series - cases which came from scattered hospitals in sporadic outbreaks and from which an aetiological agent was seldom isolated.

In general the results which I obtained from cases of paralytic poliomyelitis and aseptic meningitis parallel the experience of workers in many other parts of the world in recent years. I found that poliovirus is easy to isolate from a paralysed patient whereas an aetiological agent is difficult to find in a sporadic case of aseptic meningitis; other agents were not encountered in paralysed patients whereas poliovirus was not infrequently grown from the stool of a patient with aseptic meningitis. My results also demonstrate the curious way in which the type of poliovirus present in the community changes from year to year so that no two years are exactly alike.

It remains for the future to assess what the effect of mass inoculation with poliomyelitis vaccine may be in terms of virus prevalence and age group affected. It may even happen in the foreseeable future that a sufficiently large part of the population will have been protected artificially in this way so

that it will become possible to administer one of the oral live-virus vaccines with complete safety to the entire population at regular intervals. This would serve as a boosting mechanism, easily and safely given, which might well make the appearance of paralytic poliomyelitis as rare an event as an attack of diphtheria is today.

As poliomyelitis disappears, however, fresh problems will arise to take its place and it seems very likely indeed that aseptic meningitis, albeit a mild infection, will be one such problem. My own results as well as those of many others who have tried to isolate viruses from sporadic cases are a pointer to the difficulties likely to be encountered. These difficulties probably lie in our imperfect knowledge of the agents likely to be responsible, many of which may not yet have been isolated because of inadequate culture methods. Advances in technique and extensions of our knowledge about tissue culture may be necessary before improvement can be expected in this field of study.

The work which has been described in part 3 all grew from a simple curiosity - a desire to find out the reasons for the differences in plaque producing potential of four members of the ECHO virus group. This curiosity led me into considerably deeper water than I anticipated but produced the satisfaction of finding a solution and thereby adding a little to the

growing body of knowledge about this interesting group of agents which appear to share so many properties in common. Had I started upon this investigation in 1959 I would have had little doubt that the reason for the inability of ECHO virus type 2, or indeed any of the others like it, to produce plaques in monkey kidney cells under agar lay in the addition of the vital dye neutral red at the same time as the agar. Plaque techniques in general have now been modified to allow staining with this dye after the plaque has been produced. In the elucidation of this problem, however, I was able to perfect the micro-plaque technique for use with the ECHO viruses which dispenses with petri dishes or agar and gives reproducible counts within definable limits with any ECHO virus. This discovery is of potential importance in that it allows a considerable speeding up of virus work, both diagnostic and research.

My study of the growth cycles of the four ECHO viruses which were examined produced some interesting results although not the answer to the problem I was investigating in relation to plaques. Perhaps the most interesting finding is the relatively low efficiency of attachment of each of these ECHO viruses, and type 2 in particular, to susceptible cells during one hour of adsorption time even with a ratio of 4 virus particles from a recent, viable preparation to each cell of the

culture. Also of interest is the curiously variable yield of virus of each type from the infected culture after a standard time of 8 hours.

The answer to my problem about the plaque producing potential lay in the speed with which the viruses attached to susceptible cells and was also linked with their efficiency of attachment. The rates of attachment proved to be very difficult subjects for experimentation however and many unsuccessful attempts were made before reproducible results could be obtained.

An example of the use which can be made of the information obtained from growth cycle and rate of attachment experiments is contained in the chapter on interference in which each ECHO virus in turn was allowed to fasten on to susceptible cells and the amount of blocking of growth of each of the other three was measured. A possible imperfection of the system was found to be the relative inefficiency with which these agents attach to cells, but within limits considerable interference was produced by all four viruses.

The opportunity also presented to examine a number of viruses which all belonged to ECHO type 7 and which yet showed curious differences in their rate of growth in monkey kidney cells under standardised conditions. These were grown from the patients in the investigations in parts 1 and 2 of my thesis and I examined them in the expectation that at least

some of the differences might be permanent and could be used to demonstrate the existence of "prime" strains. A number of curious results were obtained - non-haemagglutinating strains of ECHO type 7 were discovered for the first time - but the principle of neutralization of each agent by type specific serum prepared against the prototype strain failed to produce differences between the strains which would allow the definite classification of prime strains. Differences in haemagglutinating ability of other ECHO viruses have now been reported in the literature and for the future it will be of considerable interest to know whether the haemagglutinin forms an integral part of the virus particle or is a distinct entity perhaps genetically determined in its own right.

My researches have brought me to the frontiers of new skills and disciplines in biochemistry, biophysics and genetics and advances await the application of new techniques in these fields particularly in regard to the nature of the enterovirus particle and the fascinating series of changes brought about in itself and its host cell during the complex process of infection.

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