<u>CULTURES  OF  HUMAN  TISSUES</u>

<u>IN  VIROLOGY</u>

<u>A study of their application in diagnostic virology</u>

<u>with particular reference to a previously unrecognised</u>

<u>virus associated with aseptic meningitis</u>

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by

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## ACKNOWLEDGEMENTS

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# INTRODUCTION

# INTRODUCTION

The work reported in this thesis had its beginnings in a fairly minor technical problem. Early in 1959 when I took over for a time the work of the tissue culture section of the University Virus Laboratory at Ruchill Hospital, it seemed that for administrative reasons there was likely to be in the near future a shortage of monkeys from which to obtain kidney tissue for preparing tissue cultures. At that time monkey kidney tissue culture was the sole type of culture in use in the laboratory for the isolation of enteroviruses, and it seemed of immediate practical importance to evaluate alternative types of tissue cultures. Human amnion was one fairly obvious choice, and a report which appeared just at this time by Pulvertaft and his colleagues (1959) suggested that surgically removed human thyroid might be another useful source of tissue cultures.

I first established methods of growing these two tissues in culture in adequate amounts for routine needs. The method of growing amnion was based on one I had myself seen used in Canada, and that for thyroid followed the method described by Pulvertaft et al. With some modifications both were successful. I then determined the range of viruses which were cytopathogenic in human thyroid cultures and found that it included a large proportion of the prototypes of the enteroviruses. Human thyroid and human amnion were therefore used in parallel with monkey kidney cultures for the isolation of viruses from stool specimens for one year's trial period. Human thyroid was also compared with HeLa cells for adenovirus isolation and with the fertilised hen's

egg for the isolation of herpes simplex virus.

The year of the trial of these two types of tissue culture turned out to be most unusual in that the viruses causing aseptic meningitis and paralytic disease were quite different from those encountered in Scotland in previous years. Clinically there was a small amount of paralytic poliomyelitis and a large epidemic of aseptic meningitis. The virus isolations which I made in tissue cultures showed, however, that poliomyelitis was almost entirely absent from the community and that many of the cases of aseptic meningitis and a few of those of paralytic disease were due to enteroviruses other than poliovirus. My own investigations were limited to those viruses which are cytopathogenic for tissue cultures and in the thesis only these are reported. Dr. N.R. Grist carried the investigation of these diseases further by inoculating specimens into suckling mice, and showed that most of the paralytic disease and some of the aseptic meningitis was caused by Coxsackie type A7 virus.

Apart from various Coxsackie and ECHO viruses which I was able to identify I isolated from patients with aseptic meningitis many viruses which I was unable to type. I proved serologically that these were strains of the same virus which I called Frater virus, the name being that of one of the early patients. It proved particularly fortunate for the investigation of this epidemic of aseptic meningitis that I was inoculating stool specimens to human thyroid and amnion as well as to monkey kidney tissue cultures. Only 26 isolations of Frater virus were made in monkey kidney out of a total of 75 made altogether. I was able to show a clear aetiological association of

Frater virus with aseptic meningitis and I investigated the epidem-
iology of Frater virus in the community by serological means. Finally
I made a full investigation of the properties of Frater virus and
found that they were those of an ECHO virus but that Frater virus did
not correspond to any of the recognised ECHO types. I therefore sent
the virus to Dr. J.L.Melnick for consideration by the Enterovirus
Committee as a possible new enterovirus.

These investigations, although they all formed part of the same
piece of work and although they followed logically one from another,
seemed to fall under three general headings. I therefore wrote my
thesis in this form. Part 1 consists of the laboratory investigation
of human amnion and human thyroid as alternatives to monkey kidney
tissue cultures in a routine diagnostic laboratory. Part 2 describes
the epidemiology of aseptic meningitis and paralytic disease in Scot-
land in 1959 with the major emphasis on the epidemic of disease
caused by the new Frater virus. Part 3 gives an account of the pro-
perties and attempted classification of Frater virus. The work might
be described in summary as the investigation of a technical problem
of a diagnostic laboratory, which chanced to provide the means of
fully unravelling the epidemiology of an outbreak of disease caused
by an unusual virus, and hence led to the discovery that the virus
was in fact a previously undiscovered viral pathogen.

# PART 1

## CULTURES OF HUMAN AMNION AND HUMAN
## THYROID IN DIAGNOSTIC VIROLOGY

# CHAPTER I

## REVIEW OF THE LITERATURE

The literature of tissue culture in many ways closely parallels that of virology itself. Both subjects had their effective beginnings in the first decade of this century, and after a steady but fairly slow progress over the next 40 to 50 years, advanced with unprecedented speed during the last dozen years. During the earlier years the study of viruses depended largely on the use of susceptible laboratory animals. A very real advance was possible with the introduction by Woodruff and Goodpasture in 1931 of the chick embryo for the propagation of viruses. However, the greatest advances in virology have come from the widespread use of the relatively simple methods of tissue culture which were introduced following the discovery in 1949 by Enders, Weller, and Robbins that poliovirus will grow in cultures of other human tissues than nervous tissue.

Because so much of the work reviewed belongs to the last decade, it is convenient to discuss under one heading the general history of tissue culture and its relation to virology up to 1950, with only a brief mention of the main lines of advance which in the next few years led to routine modern methods of tissue culture in virology. Under separate individual headings are considered various aspects of recent work such as methods of culture, culture media, and types of cells employed.

## History of tissue culture

Although rather primitive attempts to carry out tissue culture, such as that of Beebe and Ewing (1906) had been made previously, the

first completely successful performance of true tissue culture is
generally accepted to have been in 1907 when Harrison maintained
embryonic frog tissues with continuation of function for several
weeks in a hanging drop of coagulated frog lymph. In 1913 Steinhardt,
Israeli, and Lambert following the tissue culture methods of Harri-
son, were able to grow vaccinia virus in cultures of rabbit and
guinea pig corneal tissue. This was probably the first genuine
cultivation of a virus in tissue culture, because the earlier growth
of this virus in a rabbit cornea which was kept after removal in a
saline-moistened chamber can hardly be classified as true tissue
culture (Aldershoff and Broers, 1906). Soon after Harrison's original
experiments Burrows (1910) introduced the plasma clot technique to re-
place the frog lymph used by Harrison, and with this improved method
Carrel and Burrows (1910) grew Rous sarcoma in tissue culture. These
workers also discovered the stimulating effect on tissue cultures of
adding embryo extracts to the plasma medium (Carrel, 1912). Thus by
1912 the basic methods had been developed which were to be used with
only minor modification for almost 40 years.

Successful tissue culture at this time was made possible only by
a most meticulous aseptic technique such as that which Carrel brought
with him to his tissue culture work from his earlier researches in
experimental surgery. Tissue culture was therefore a difficult skill
to master and for many years was carried on in a limited number of
centres. Among the best known of these groups were Carrel and his
associates at the Rockefeller Institute and Strangeways and Honor Fell
at Cambridge. The work of the Carrel school was particularly concerned

with the growth over long periods of actively multiplying cells, and it laid the foundation of later work with continuous cell lines which are nowadays widely used in virology. Fischer (1925), who worked for a time with Carrel, obtained the first permanent epithelial cell line in tissue culture. Carrel himself grew the Rous sarcoma virus in tissue culture (Carrel, 1926), and was an early advocate of pure cell lines for virus culture in order to reduce the variables inherent in the use of experimental animals (Carrel, 1928). At Cambridge the early work was done by Strangeways (1924), and has now been carried on for many years by Honor Fell who has studied the organised growth in culture of tissues such as bone under conditions which permit them to grow in the same way as they would inside the animal body (Fell, 1951).

During the 2 decades up to 1939 tissue cultures were used from time to time for the growth of viruses. Parker and Nye (1925) grew vaccinia virus in explants of rabbit testis and showed an increase in virus in the eleventh generation tissue culture of 51,000 times the original inoculum. This was the first irrefutable proof of the multiplication of a virus in tissue culture. Maitland and Maitland (1928) grew vaccinia virus in what was a new type of tissue culture although they themselves at the time did not consider it to be true tissue culture. It consisted of finely chopped hen's kidney suspended in Tyrode's solution and hen's serum. In a review article on the cultivation of viruses Sanders states that by 1939 only 24 mammalian viruses had been propagated in tissue culture. Half of these were human pathogens. By 1950 the total number of mammalian viruses had

reached 38 definitely grown in tissue culture and another 5 probably grown (Robbins and Enders, 1950). Of the 43, 25 were human pathogens.

From 1949 onwards progress was very rapid. Many known viruses were discovered solely through the use of tissue culture. This progress resulted mainly from three major innovations; the use of antibiotics to prevent bacterial contamination, the discovery that a cytopathic effect in tissue culture was an adequate criterion of the presence of a virus, and the use of trypsin for the dispersion of cells. This last method greatly facilitated the preparation of large numbers of cell cultures for virological investigations.

As they came into general use in medicine both sulphonamides and antibiotics were added to tissue culture media to prevent bacterial contamination. Sulphonamides, which were first tried, proved somewhat toxic to the tissue cultures (Sanders and Huang, 1944) but penicillin was both highly effective and non-toxic (Rose, Molloy and O'Neill, 1945). Rose et al. experienced some contamination with Gram-negative bacilli such as strains of Proteus and Pseudomonas pyocyanea. This was avoided by later workers by adding streptomycin as well as penicillin to their tissue culture media (Enders, Weller, Robbins, 1949; Weller, Robbins and Enders, 1959). Soon afterwards it was found that even such grossly contaminated material as faeces could be directly inoculated into tissue cultures for virus isolation if high concentrations of penicillin and streptomycin were added to the solution in which the faecal extract was made and if the extract was also centrifuged to remove the bulk of the bacteria.

Earlier workers (Topacio and Hyde, 1932; Huang, 1942) had previously observed what is now known as the cytopathic effect of a virus in tissue culture. However it was not until the outstanding work of Enders, Weller, and Robbins on the propagation of poliovirus in tissue culture that it was fully realized that the cytopathic effect in tissue culture was as adequate a criterion of the presence of a virus as the infection of a susceptible laboratory animal (Robbins, Enders, and Weller, 1950). This discovery was the basis of the most commonly used method of virus isolation in the modern diagnostic laboratory.

Trypsin was brought into common use by Scherer, Syverton, and Gey in 1953 to obtain suspensions of HeLa cells for passage to fresh cultures. It had been introduced as a means of dispersing cells by Rous and Jones in 1916 and used subsequently by surprisingly few workers (Vogelaar and Erlichman, 1934, 1939; Gey and Bang, 1939). Also in 1953 Fritsch and Jentoft used trypsin to obtain the inoculum for secondary cultures of fibroblasts growing out from explants of monkey testis. The following year Dulbecco and Vogt (1954) and Youngner (1954a) described methods of obtaining by trypsinisation a suspension of monkey kidney cells for culture. This type of procedure has been used ever since for the preparation of primary cultures of fresh human and animal tissues.

### Modern methods of tissue culture

The term tissue culture is widely used as a general name for methods of growing both portions of tissue and dispersed cells. More correctly the two techniques are often separately named tissue culture and cell culture. Cell cultures have very largely replaced the older types

of tissue culture in virological work. This short review of modern
methods, therefore, will concentrate largely on methods of cell cul-
ture and will deal very briefly with the culture of true tissues.

The standard methods of tissue culture by the plasma clot tech-
nique in tubes and Carrell flasks are well described by Parker (1950).
These methods were formerly most valuable in virology though they are
little used to-day, and as recently as 1953 were employed by Rowe and
his colleagues in the work which led to the discovery of the adenovi-
ruses. Some methods used in the culture of tissues were later adapted
to the culture of cells. The use of roller tubes was introduced by
Gey (1933) who states that he later found that the method had previously
been suggested by Carrell. Gey and Bang (1939) used roller tube cul-
tures of human thyroid cells to grow the virus of lymphogranuloma in-
guinale. The roller tube technique was widely used by many workers for
the growth of cells such as HeLa and monkey kidney. Another method of
even greater importance in advancing cell culture for virological pur-
poses was the discovery that tissue fragments would grow on glass with-
out the support of a plasma clot. Gey and Gey (1936) were able to grow
several continuous lines of human sarcoma cells in this way. More re-
cently in the antibiotic era Morann and Melnick (1953) showed that
large numbers of tube cultures could be prepared with monkey kidney
tissue fragments grown directly on glass. Cultures of suspensions of
chopped tissue of the kind developed by Maitland and Maitland (1928)
were the forerunners of modern spinner cultures of cells. In addition
Maitland-type cultures have found an important use in recent years in
the production of the large amounts of poliovirus required for the

manufacture of vaccine (Farrell et al., 1955).

The culture of cells depends on there being a satisfactory means of dispersion of the cells to be cultured.  The introduction of trypsin for this purpose has already been discussed.  Since Dulbecco and Vogt (1954) first used trypsin to prepare cultures of monkey kidney cells, monolayers of these cells prepared by trypsinisation of kidney tissue have become the most frequently used tissue culture in the diagnostic virus laboratory.  Youngner (1954a) introduced a method of repeated extractions of the kidney tissue with trypsin, and Bodian (1956) greatly simplified the whole process of trypsinisation.  Other workers have introduced simple methods of obtaining larger volumes of kidney cell suspensions with the minimum of trauma to the cells (Bishop, Smith and Beale, 1960).  Trypsin has also been used to obtain cells for culture from a variety of tissues including human amnion (Zitcer, Fogh, and Dunnebacke, 1955) and human thyroid (Pulvertaft, Davies, Weiss, and Wilkinson, 1959).  Enzymes other than trypsin have been used to free cells from tissue.  Hinz and Syverton (1959), for example, found collagenase much superior to trypsin for the release of cells from lung tissue.  Chelating agents as well as enzymes will free cells into suspension, particularly from monolayer cultures when cells are required for preparing fresh cultures.  Versene was introduced by Zwilling (1954) to dissociate cells from chick embryos, using a solution of versene in a calcium and magnesium free saline described by Moscona and Moscona (1952) for making trypsin solutions.

Simple methods of counting cells in suspension in the standard haemocytometer chamber and of dispensing cells in culture medium by

means of the Cornwall pipette (Syverton, Scherer, and Elwood, 1954) greatly facilitated the setting up of large numbers of tissue culture tubes.

Glass test tubes sealed with rubber stoppers or sometimes screw caps have been used most commonly for growing cell cultures in large numbers for virus isolation. Stock cultures of continuous lines of cells have been grown in bottles of many shapes and sizes. For special purposes certain more specialised methods have been devised. Salk, Youngner, and Ward (1954) introduced a method of titrating poliomyelitis antibodies in serum by adding serum-virus mixtures to monkey kidney cell suspensions in stoppered tubes and observing the colour change produced by the cell metabolism in those tubes in which the virus was neutralized by antibody. This test was simplified ( Melnick and Opton, 1956) by placing the cell suspensions and other reagents in cups in a plastic plate under layers of oil. The simplified method has also been used for the titration of adenovirus antibodies using HeLa cells (Johnston, Grayson and Loosli, 1957).

Dulbecco and Vogt (1954) described the extremely valuable method of producing plaques of virus growth in monolayers of cells growing under a layer of agar in Petri dishes. The monolayers were grown from trypsinised monkey kidney cells and after the virus inoculum had been applied to the monolayer a layer of agar containing medium was poured on top. This method was used by Dulbecco and Vogt to prove that one virus particle could initiate an infection. It provides an excellent direct method of counting viruses and of isolating pure strains of virus. Plaques were also produced by a similar method in monolayers

grown in stoppered bottles (Hsiung and Melnick, 1955). Another application of the plaque method arose from the discovery (Porterfield, 1959; Henderson and Taylor, 1960) that some arthropod-borne viruses may fail to produce cytopathic effects in ordinary monolayers and yet produce definite plaques under agar overlays. Hsiung (1959a) extended this work further by isolating from clinical specimens viruses which only produced cytopathic changes under an agar overlay.

The cell cultures which were originally employed consisted of heterogeneous cell populations. For some investigations an entire cell population derived from a single parent cell is desirable, and various methods of obtaining such a line or "clone" of cells have been devised. As early as 1916 a method was described of plating out cells in the same way as bacteria are streaked on the surface of a solid medium (Rous and Jones, 1916). More recently Sandford, Earle and Likely (1948) obtained clones of L cells by separating out single cells with a capillary pipette and growing cultures from these cells in a medium which had previously been conditioned to support growth more satisfactorily by growing L cells in the medium and then removing all the cells. This method was less successful in the hands of other workers, and a more reproducible method was described by Puck, Marcus, and Cieciura (1956). They were able to clone HeLa cells by two methods. In one, a HeLa cell suspension was allowed to become fixed to the glass of a Petri dish and then the cells were irradiated to prevent multiplication. This formed the so-called feeder layer which in some way encouraged the growth of colonies of HeLa cells from single cells in a dilute suspension later inoculated into the Petri dishes. In the other method,

cloning was performed without a feeder layer by using as inoculum a dilute cell suspension which had received the minimum of handling. Cloned strains of HeLa cells prepared by these workers, such as the S3 strain, are used in many American virus laboratories.   Leidy et al. (1959) used the Puck method to obtain a number of clones of HeLa cells from which they selected one which was more sensitive than the parent strain in producing plaques with poliovirus.

The cell cultures which have been discussed so far are all fixed cultures.  Suspended cell cultures have also been brought into use in recent years.  Cooper (1955) grew several types of cells dispersed in agar and nutrient medium and was able to demonstrate plaques of viral growth.  This method, however, requires large numbers of cells and has not been widely used.  Cultures of cells kept in suspension in fluid medium have found a wider application in virology.  A full description of this method is given by McLimans et al. (1957) who kept L cells and HeLa cells in continuous suspension in culture fluid by agitation with a rotating plastic-covered magnet.  The same group of workers were able to modify this spinner culture method for the growth of very large volumes of cells (McLimans, 1957).  However, although suspended cell cultures are valuable for some procedures in virology the fixed mono- layer cell culture technique is at present the most widely used method in diagnostic virus laboratories.

### Tissue culture media

The basis of most modern media for tissue and cell culture is a balanced salt solution.  This must contain the essential inorganic ions required by living cells, must be in osmotic equilibrium with the cells,

and must be at the correct pH. Balanced salt solutions were intro-
duced first for physiological experiments. Ringer's saline, contain-
ing the chlorides of sodium, potassium and calcium, was the simplest
and earliest balanced salt solution. Tyrode (1910), in the course of
pharmacological experiments with excised portions of intestine, intro-
duced a more elaborate solution containing also magnesium and glucose,
and phosphate and bicarbonate to give a buffering action. This solu-
tion was widely used for many years for tissue culture media. To-day,
however, probably the most generally useful and most popular balanced
salt solutions are those of Hanks (1948) and Earle (1943). Earle's
solution is the better buffered of the two. It contains more sodium
bicarbonate than Hanks' and requires a gaseous environment of air plus
5% carbon dioxide.

Serum has long been used as a component of tissue culture media
(Carrel, 1926 ; Maitland and Maitland, 1928). Indeed a mixture of
balanced salt solution and serum is an excellent medium and one which
is so easy to prepare that it is very widely used by virologists.
Extracts of embryonic tissue which used to be added to media to stim-
ulate growth have largely been replaced in cell culture work in viro-
logy by simpler substances. Melnick and Riordan (1952) found that
lactalbumin hydrolysate was a convenient substitute for embryo extract,
and Melnick (1955) later used it in growth medium for primary cultures
of monkey kidney. Yeast extract has also been used to supplement bal-
anced salt plus serum media (Robertson, Brunner and Syverton, 1955).
Some workers have used bovine amniotic fluid instead of balanced salt
solution as the main component of culture media (Enders, 1953). Bovine

amniotic fluid is much more satisfactory than bovine allantoic fluid
(Malherbe, 1954). In the more commonly used media based on balanced
salt solutions, the buffering action of the medium depends on a bi-
carbonate buffer. The bicarbonate buffer may be replaced by tris
buffer (Swim and Parker, 1955) for purposes where bicarbonate is
unsuitable.

Media containing sera and such additions as lactalbumin hydro-
lysate are very convenient to prepare and are most useful in viro-
logy. However, serum and protein digests are extremely complex sub-
stances chemically, and their exact composition is uncertain. For
some investigations such media are unsuitable and chemically more
well-defined media are required. One of the first such defined media
to be prepared was mixture number V605 of Fischer and his colleagues
(1948) which was devised by mixing all the biologically important
substances known to be present in animal tissues. A medium, fairly
similar to Fischer's V605, was introduced by Morgan, Morton and Parker
(1950) under the laboratory number 199. Medium 199 is probably the
best known defined medium used in virus laboratories, possibly because
it is used in the preparation of Salk poliomyelitis vaccine (Salk,
1953; Farrell et al., 1955). There is no doubt that medium 199 con-
tains substances which are not absolutely essential for cell growth
and simpler defined media have been introduced. That of Eagle (1955a)
is a good example. However for good growth of cells Eagle's medium
usually has to be supplemented with serum. Medium MB752/1 of Waymouth
(1959) lies between medium 199 and Eagle's medium in complexity and
will support the growth of L cells without other added nutrients.

## Types of cells used in virology

Cultures of very many different types of cells have been intro-
duced for the cultivation of viruses in the last decade. Some viruses,
such as those of measles and chickenpox, have been grown in the labora-
tory for the first time by the inoculation of these cultures. Many
viruses, such as the ECHO group, adenoviruses, para-influenza viruses
and other respiratory viruses, have been discovered entirely through
their use. Human and monkey cells have been found most useful for the
growth of the commoner human viruses and these types of cells will be
discussed almost exclusively.

At present, there are two sources of cells for culture. Primary
cell cultures are prepared from cells dispersed from fresh tissue, and
continuous cell lines maintained through many generations are kept
readily available in the laboratory. Each of the two types has its
own advantages and disadvantages. Primary cultures tend to be more
variable than a well-established continuous cell line although the
component cells of a continuous line which is not a pure clone may
vary markedly in their susceptibility to viral infection (Leidy et al.,
1959). Certain viruses such as the ECHO viruses which are readily
isolated in primary cell cultures are cytopathogenic for relatively
few continuous cell lines. Some primary cell cultures are not infre-
quently contaminated with viruses from the animal whose cells were
used. Monkey kidney cultures may be contaminated with virus B
(Farrell et al., 1955) or a wide variety of simian orphan or ECMO
viruses (Kalter, 1960). Such contamination has not proved a problem
in primary cultures of human cells. In continuous cell lines

troublesome contamination has been encountered, but by bacteria rather than viruses. Continuous lines may be lost entirely in a laboratory by chance contamination. In addition to occasional contamination by more common bacteria, some lines have been shown to be contaminated continuously over many generations by pleuropneumonia-like organisms or bacterial L forms (Robinson, Wichelhausen, and Roizman, 1956; Collier, 1957). Theoretically a stable continuous cell line, free from contamination and with a wide spectrum of viral susceptibility, would be the most biologically constant host system for virus isolation. A really adequate cell line of this nature is not yet available and in the meantime primary cultures continue to be used very widely in diagnostic virology.

Of the primary cultures in common use, monkey kidney was the first to be introduced and still remains the most widely used. Dulbecco and Vogt, and also Youngner used monkey kidney cultures in 1954, and Bodian (1956) described the method of preparation commonly used with only minor modifications to-day. Monolayers of monkey kidney were found to be more sensitive in the detection of poliovirus than explant cultures of monkey kidney (Youngner, 1954b). Coxsackie viruses of type B were found to be cytopathogenic for monkey kidney cultures and were isolated from faeces in these cultures by Sickles, Feorino, and Plager (1955). Only a few type A Coxsackie viruses are cytopathogenic for monkey kidney cultures. Type A9 (Melnick, 1955) and certain epidemic strains of A16 (Robinson, Doane and Rhodes, 1958) are among the cytopathogenic types. The ECHO group of viruses are all cytopathogenic for monkey kidney cultures and were in fact originally isolated in these cultures (Melnick, 1955; Com-

mittee on the ECHO Viruses, 1955). Adenoviruses are also cytopatho-
genic for monkey kidney cells, the cytopathic effects resembling
those seen in HeLa cells (Prier and Lebeau, 1958). Kidney cells from
various species of monkey have been used for culturing viruses but
rhesus and cynomolgus monkey cells have been most commonly employed.
The cells from different species differ in virus susceptibility, par-
ticularly to the ECHO viruses. All the ECHO viruses are cytopatho-
genic for rhesus and cynomolgus monkey cells, but only types 7, 10
and 12 among the first 14 types of ECHO viruses are cytopathogenic
for patas monkey cells (Hsiung, 1961). Cells of the African green
monkey are even more susceptible to ECHO viruses than rhesus monkey
cells (Hsiung and Melnick, 1957).

Monkey kidney cells have been used largely as a matter of con-
venience but it would seem logical to try to make use of human cells
rather than primate cells for the cultivation of human viruses. The
main difficulty with human cells is that of obtaining an adequate
supply of fresh tissue, but despite this various types of human tis-
sues have been used. Hsiung (1959b) grew cells from human kidneys re-
moved surgically and at post mortem. He found that kidney cells from
infants were susceptible to the same types of enteroviruses as were
rhesus monkey kidney cells. Adult human kidney cultures however, though
fully susceptible to polioviruses were very inconsistant in showing cyto-
pathic changes with Coxsackie A9, Coxsackie B viruses, and ECHO viruses.

Human kidney which is fresh enough to give satisfactory cultures
is available in only very meagre quantity. The possibility of using
more readily available human tissues has therefore been explored. The

human tissues which can be obtained in the largest amounts are placentas and membranes from hospital obstetrical departments, and both amnion and chorion cells can be grown in culture. Amnion cells have proved very much the more valuable of the two. Zitcer, Fogh, and Dunnebacke first described the preparation of cultures of human amnion in 1955, and showed that poliovirus was cytopathogenic for amnion cells. Since then methods of preparing amnion tissue cultures have been described by virologists in many countries including the United States (Weinstein et al., 1956; Takemoto and Lerner, 1957), Canada (Wilt, Stanfield, and Leindl, 1956; Beale, Doane, and Ormsby, 1957), Norway (Lahelle, 1956), Germany (Witt, 1961), Britain (Ferguson and Tobin, 1958), and Australia (Lehmann-Grube, 1961a). In all these methods cells were freed from the membrane with trypsin, but different workers used different temperatures for trypsinisation. Room temperature was used by the earlier workers, Lahelle found $30^{o}C$ most satisfactory, and Ferguson and Tobin obtained good results with trypsinisation at $37^{o}C$. Amnion cells show cytopathic effects with many of the enteroviruses including poliovirus types 1, 2, and 3, ECHO viruses types 1, 3, 5, 6 and 7 (Bernkopf and Rosin, 1957), ECHO type 9 (Mc Lean and Melnick, 1957), and Coxsackie types A9, B1, B3, B5 and some strains of B2 (Takemoto and Lerner, 1957). Herpes virus and adenoviruses types 1 to 8 are also cytopathogenic in amnion cells (Takemoto and Lerner, 1957). All 3 types of poliovirus will produce plaques in amnion cultures (Fogh and Lund, 1955). Because it is so readily available and has a fairly wide range of susceptibility to enteroviruses human amnion has become after monkey kidney cells probably the most commonly used type of primary culture

for the isolation of viruses from faecal specimens. Chorion cells
by contrast have been used by very few workers. Lerner, Takemoto,
and Shelokov (1957) found that chorion cultures showed cytopathic
changes with polioviruses, Coxsackie viruses, and adenoviruses just
as did amnion, but that chorion was susceptible to only 5 of the first
14 types of ECHO viruses.

Among the various organs and portions of tissue which are re-
moved surgically from human beings, thyroid tissue is one which con-
sists largely of epithelial cells and would therefore seem a likely
source of human cells for culture. Gey carried out some early exper-
iments with tissue cultures of human thyroid in 1938. In the fol-
lowing year Gey and Bang (1939) grew cells from human thyroids, fore-
shadowing some of the methods of tissue and cell culture employed
today in their use of roller tubes and of trypsin for freeing cells
for serial passage. They grew the virus of lymphogranuloma inguinale
in their thyroid cultures and performed neutralization tests with
human sera. Twenty years later Pulvertaft, Davies, Weiss and Wilk-
inson (1959) introduced a simple method of preparing primary cultures
of human thyroid from tissue removed at partial thyroidectomy opera-
tions. These workers studied the metabolism of the thyroid cells and
tested them for susceptibility to viruses. They found that poliovirus,
Coxsackie types A9 and B1, ECHO types 6 and 9, and adenovirus types
1 to 11 were all cytopathogenic for primary thyroid cultures. With
poliovirus, cytopathic effects were variable and the virus sometimes
multiplied in the cells without producing a visible effect. Pulver-
taft's group have also reported the use of thyroid cell cultures in

the investigation of Hashimoto's disease (Pulvertaft, Doniach, Roitt and Hudson, 1959). Hovel and Schneider (1960) have described a somewhat more complicated method of preparing primary cultures of human thyroid. They obtained similar titres of all 3 types of poliovirus when titrated in thyroid and monkey kidney cultures, and were more successful than Pulvertaft and his colleagues in making serial passes of poliovirus in thyroid cultures. Hart (1959) was able to isolate a type 1 para-influenza virus from throat washings in thyroid cultures, and Parson et al., (1960) isolated strains of Coe virus from throat washings and faeces. Few other types of tissue removed surgically from human beings have been used for tissue culture in virology. Among those which have been tried is tissue from the Fallopian tube which Pizarro and Perez-Rebelo (1956) grew in culture and used for the cultivation of poliovirus.

Because they are more readily available than human and monkey tissue, kidney cells from various domestic and laboratory animals have been investigated by some virologists. Lenahan and Wenner (1960) compared the virus spectrum of kidney cells of the calf, lamb, pig, hamster, dog, cat, rabbit and mouse with that of monkey kidney and human amnion cells. They concluded that dog, cat, rabbit and mouse cells supported the growth of too few human viruses to be useful in the diagnostic laboratory. Calf, lamb, pig, and hamster cells were susceptible to the Coxsackie B viruses and all except lamb to the adenoviruses. Calf kidney showed cytopathic changes with ECHO types 4 and 9 and pig kidney with ECHO type 4. ECHO virus type 10 was cytopathogenic for all except rabbit cells but this virus is not now

classified as an ECHO virus (Sabin, 1959). Hsiung (1961) presents
findings similar to those of Lenahan and Wenner. Barron and Karzon
(1959) found that a few strains of ECHO type 9 were cytopathogenic
for hamster kidney but that many other strains were not.

The virologist's alternative to preparing primary cultures of
fresh tissue as they are required, is to maintain continuous lines of
cells in the laboratory. Many cells have now been adapted to con-
tinuous culture. The catalogue of one biological supply laboratory
in the United States, for example, lists 25 lines of these cells
which are commercially available (Microbiological Associates, 1961).
It is convenient to divide continuous lines of cells into two groups,
those derived from normal tissues and those from malignant. In the
first group probably the best known is the L strain of mouse fibro-
blast (Earle, 1943) which has been in continuous cultivation since
October 1940. The L cell is seldom used in virology but does show
a cytopathic effect with herpes virus (Scherer, 1953).

Soon after the introduction of primary cultures of human amnion
a strain of amnion was adapted to continuous cultivation and is
known as the FL cell after the originators, Fogh and Lund (1957).
FL cells are highly susceptible to infection by polioviruses and
adenoviruses (Fogh, Lund and McClain, 1959). Human thyroid cells
were grown in continuous culture by Fulvertaft, Davies, Weiss and
Wilkinson (1959) who found them susceptible to infection by polio-
virus, Coxsackie B virus and adenovirus but not to infection by
ECHO virus. Cells from human conjunctiva, liver, kidney and appendix
were adapted to growth as continuous cell lines by Chang (1954).

Chang's conjunctiva cell and liver cell are used in diagnostic
virology particularly for the isolation of adenoviruses. A con-
tinuous line of monkey heart cells (Salk, 1957) may also be used
for the isolation of adenoviruses (Gutenkust and Heggie, 1961). In
this country Westwood, Macpherson, and Titmuss (1957) obtained con-
tinuous lines of cells from human liver, monkey kidney, monkey lung,
and rabbit kidney. All of these were transformed lines which resembled
HeLa cells with the exception of one line of monkey kidney, MK1. The
MK1 line has been shown to differ markedly from most continuous lines
of cells in showing cytopathic changes with all of the first 20 types
of ECHO viruses except type 4 (Flewett, 1959). A continuous line of
human kidney cells, strain HK/55, has recently been originated (Guerin
and Kitchen, 1961) which shows cytopathic changes with all 3 polio-
viruses, the 6 Coxsackie B viruses and 18 of the 28 types of ECHO
viruses, ECHO types 4, 10, 14, 15, 16, 18, 21, 22, 23, and 27 not
being cytopathogenic.

Among continuous lines of cells derived from malignant tissues,
by far the best known is the HeLa cell which was first grown by Gey
in February 1951 from a carcinoma of cervix (Scherer, Syverton and
Gey, 1953). These workers showed that all 3 types of poliovirus were
cytopathogenic for HeLa cells, which became the first continuous line
used for the isolation of poliovirus. It was soon found that herpes
virus and vaccinia virus (Scherer and Syverton, 1954), Coxsackie B
viruses (Crowell and Syverton, 1954), and adenoviruses (Rowe, Hartley
and Huebner, 1956) were cytopathogenic for HeLa cells. However ECHO
viruses are not cytopathogenic for HeLa cells except for some lab-

oratory strains which have been specially adapted to grow in HeLa cells
(Archetti, Weston, and Wenner, 1957). HeLa cells are now mainly used
in diagnostic virology for the isolation of adenoviruses. A number of
other cell lines have been obtained from human malignant tissues. These
include H.Ep.1 from carcinoma of cervix, H.Ep.2 from carcinoma of larynx,
H.Ep.3 from metastatic buccal carcinoma (Moore, Sabachewsky, Toolan,
1955), K.B. from carcinoma of mouth (Eagle, 1955b), Detroit 6 from metast-
atic lung carcinoma in bone marrow (Berman, Stulberg and Ruddle, 1955),
and Maben from adenocarcinoma of lung (Frisch et al., 1955). The K.B.
cell shows cytopathic changes with poliovirus, herpes virus, vaccinia
virus and adenovirus (Eagle et al., 1956). Detroit 6 cells are sus-
ceptible to poliovirus, herpes virus, and Coxsackie B virus (Stulberg,
Berman, and Ruddle, 1955). The Maben cell gives cytopathic changes with
poliovirus, herpes virus, and vaccinia virus (Frisch et al., 1955).

In general it may be said that continuous lines of malignant
cells show cytopathic changes with the pox viruses, herpes viruses,
adenoviruses, polioviruses, and some of the Coxsackie B viruses but not
with ECHO viruses. Many of the cell lines derived from normal tissues
have changed in the course of adaptation to continuous culture to re-
semble the malignant cell lines in their morphology and growth char-
acteristics, a process sometimes known as transformation. These cells
tend to resemble malignant cell lines in the viruses to which they are
susceptible. They differ from certain other cell lines from normal
tissues, such as the MK1 and HK/55 lines, which resemble more closely
the cells of the normal parent tissue and retain the parent cell's
susceptibility to infection by ECHO viruses.

# CHAPTER II

## MATERIALS AND METHODS

Most of the media and many of the methods employed in the work described in Part 1 of this thesis were also used in the investigations reported in Parts 2 and 3. These will be described in full only once in this chapter. The materials and methods referable to Part 2 are described in Chapter IX, and methods of performing particular experiments as distinct from general methods used repeatedly are discussed in the appropriate chapters.

The preparation of tissue cultures of human amnion and human thyroid is not mentioned in this chapter because entire chapters are later devoted to these subjects. The method of making trypsin solution is discussed in the chapter on amnion tissue culture as the details of making this solution were found to be of great importance in obtaining successful tissue cultures.

### Phosphate buffered saline (PBS)

| | |
|---|---|
| Sodium chloride | 8.0 g. |
| Potassium chloride | 0.2 g. |
| Di-sodium hydrogen phosphate | 1.15 g. |
| Potassium di-hydrogen phosphate | 0.2 g. |
| Phenol red solution (0.4%) | 4.0 ml. |
| Water | to 1000 ml. |

Sterilized by autoclaving at 10 lb. per sq. in. for 15 minutes.

## Hanks' solution + lactalbumin

### Stock solution A

| | |
|---|---|
| Sodium chloride | 40.0 g. |
| Potassium chloride | 2.0 g. |
| Magnesium sulphate ($MgSO_4 \cdot 7H_2O$) | 0.5 g. |
| Magnesium chloride ($MgCl_2 \cdot 6H_2O$) | 0.5 g. |
| Calcium chloride | 0.7 g. |
| Water | to 250 ml. |

### Stock solution B

| | |
|---|---|
| Di-sodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) | 0.76 g. |
| Potassium di-hydrogen phosphate | 0.3 g. |
| Dextrose | 5.6 g. |
| Water | to 250 ml. |

### Complete medium

| | |
|---|---|
| Solution A | 50 ml. |
| Solution B | 50 ml. |
| Lactalbumin hydrolysate | 5 g. |
| Phenol red solution (0.4%) | 5 ml. |
| Water | 870 ml. |

Sterilized by autoclaving at 10 lb. per sq. in. for 15 minutes.

### Concentrated Earle's solution (x10)

| | |
|---|---|
| Sodium chloride | 68.0 g. |
| Potassium chloride | 4.0 g. |
| Magnesium sulphate $(MgSO_4.7H_2O)$ | 2.0 g. |
| Sodium di-hydrogen phosphate $(NaH_2PO_4.H_2O)$ | 1.4 g. |
| Glucose | 10.0 g. |
| Calcium chloride | 2.0 g. |
| Phenol red solution (0.4%) | 25.0 ml. |
| Water | 950 ml. |

Sterilized by autoclaving at 10 lb. per sq. in. for 15 minutes

### Lactalbumin solution

| | |
|---|---|
| Lactalbumin hydrolysate | 5.0 g. |
| Water | 1000 ml. |

Sterilized by autoclaving at 10 lb. per sq. in. for 15 minutes

### Sodium bicarbonate solution

| | |
|---|---|
| Sodium bicarbonate | 4.4 g. |
| Phenol red solution (0.4%) | 0.5 ml. |
| Water | 100 ml. |

Saturated with carbon dioxide until orange in colour.

Containers tightly capped.

Sterilized by autoclaving at 10 lb. per sq. in. for 15 minutes

### Antibiotic solutions

#### PSN solution

| | |
|---|---|
| Penicillin G | 2 million units |
| Streptomycin | 2 g. |
| Mycostatin ("Nystatin") | 500,000 units |

Hanks' solution                                        200 ml.

Stored at -20°C until used

PS solution

This consisted of PSN but without mycostatin

### Serum for tissue culture media

Calf

In the earlier part of the work fresh calf blood was

obtained from the slaughter house.  The serum was

separated from the clot by centrifugation, Seitz

filtered, inactivated at 56°C for 30 min., and stored

at -20°C.

Later calf serum was obtained commercially; Oxoid Calf

Serum was used.

Human

Human serum was obtained from the West of Scotland Blood

Transfusion Service.  This serum was Seitz filtered, in-

activated at 56°C for 30 min., and stored at -20°C.

### Earle's solution, calf serum medium

Concentrated Earle's solution                          10 ml.

Lactalbumin solution                                   80 ml.

Calf serum                                             5 ml.

Sodium bicarbonate solution                            4.ml.

PSN solution                                           1 ml.

## Versene Solution

### Stock solution

1% versene (sequestric acid, disodium salt) in water

stored at room temperature.

### Solution as used (1:20,000)

| | |
|---|---|
| 1% versene stock solution | 0.1 ml. |
| PBS | to 20 ml. |

This is made up immediately before use.

## Virus stock strains

### Polioviruses

1 - Mahoney and Brunenders

2 - MEF 1

3 - Saukett

### Coxsackie viruses

A7 - Monkey kidney tissue culture adapted strain of Habel
   and Loomis (1957)

A9 - PB.50546

| | |
|---|---|
| B1 - PO.49683 | B4 - JVB.51196 |
| B2 - Ohio Red.50207 | B5 - 53112 |
| B3 - Nancy.50531 | B6 - 1-51-21 |

### ECHO viruses

| | |
|---|---|
| 1 - Farouk | 14 - Tow |
| 2 - Cornelis | 15 - Charleston 96-51 |
| 3 - Morrisey | 16 - Harrington |
| 4 - Pesascek | 17 - CHHE HML-1 |
| 5 - Noyce | 18 - Metcalf D-3 |

| | |
|---|---|
| 6 – D'Amori | 19 – Burke D-4 |
| 7 – Wallace | 20 – 200155 |
| 8 – Bryson | 21 – Farina |
| 9 – a strain, pathogenic for suckling | 22 – Harris |
| mice, isolated in Glasgow by Dr. | 23 – Williamson |
| R.G. Sommerville | 24 – De Camp |
| 10 – Lang | 25 – ATCC 01037 |
| 11 – Gregory | 26 – 11-3-6 |
| 12 – Travis 2-85 | 27 – 1-36-4 |
| 13 – Hamphill 2-188 | |

Adenoviruses

| | |
|---|---|
| 1 – Ad.71 | 9 – Hicks |
| 2 – Ad.6 | 10 – J.J. |
| 3 – G.B. | 11 – Slobitski |
| 4 – RI-67 | 13 – A.A. |
| 5 – Ad.75 | 14 – DeWit |
| 6 – Ton.99 | 15 – Ch.38 |
| 7 – Gomen | 16 – Ch.79 |
| 8 – Trim. | 17 – Ch.22 |

Herpes simplex virus

Strain HFEM

Influenza viruses

A – PR8, A/Scotland/1/53, A/Singapore/4/57

B – B/Scotland/28/55

C – 1233

## Ovine Abortion virus

Strain originally obtained from Dr. Stamp, Edinburgh

## Pox viruses

Cowpox strain originally obtained from Professor A.W.

Downie of Liverpool.

Vaccinia strain from Evans' vaccine lymph.

### Diagnostic antisera

Antisera to each of the 3 types of poliovirus were obtained from

the Virus Reference Laboratory, Colindale. These sera were pre-

pared by immunising monkeys.

Antisera to each of Coxsackie viruses A9 and B1 to 5, ECHO viruses

types 1 to 20 except 10 and 16, and adenoviruses types 1 to 7 were

prepared in this laboratory by immunising rabbits with live virus

seed grown in human amnion, monkey kidney, or HeLa tissue cultures.

### Monkey kidney tissue culture

A single kidney was aseptically excised from a rhesus monkey.

These operations were performed by Dr. R.G. Sommerville and Dr. N.R.

Grist, to whom I am most grateful for their help.

The capsule and medullary tissue were removed and the cortex finely

chopped with sterile scissors. The minced tissue was washed in PBS

and then added to 100 ml. of 0.25% trypsin (Difco 1:250) dissolved

in Hanks' solution at pH 7.6. This was agitated at $37^{O}$C. in a

closed flask with a magnetic stirrer for 30 min. when the fluid was

decanted off the chopped tissue. A fresh batch of 100 ml. trypsin

solution was added to the tissue and the mixture agitated at $37^{O}$C.

until the tissue disintegrated forming a turbid suspension of cells.

This happened after approximately 2 hours. The kidney cells were
centrifuged out of suspension, washed in Hanks' solution, and re-
suspended at a concentration of $1.5 \times 10^5$/ml. in the following pro-
pagating medium:-

| | |
|---|---|
| Hanks' solution + lactalbumin | 78 ml. |
| Calf serum | 10 ml. |
| Human serum | 10 ml. |
| Sodium bicarbonate solution | 1 ml. |
| PSN solution | 1 ml. |

This cell suspension was dispersed in 0.8 - 1.0 ml. volumes to
$4 \times \frac{1}{2}$ in. test tubes which were closed with rubber stoppers and in-
cubated in stationary racks at $37^{\circ}$C. until monolayers formed in the
tubes. This took 6-7 days. If the medium became acid during growth
the propagating medium was changed. As soon as monolayers formed
the medium was replaced by Earle's solution, calf serum medium for
maintenance.

## HeLa cell culture

### Stock cultures

Stock cultures of HeLa cells were maintained continuously in 14 or
16 oz. flat bottles at $37^{\circ}$C. Each bottle was reseeded with cells
every 7-10 days. When a bottle required reseeding the medium was
removed and the monolayer washed twice with 20 ml. of PBS. Then
20 ml. of 1:20,000 versene solution was added to the bottle which
was stoppered and kept at $37^{\circ}$C. until the monolayer separated and
the cell sheet disintegrated. The versenated cells were centrifuged

out of suspension and used to reseed the original bottle and, if
necessary, to prepare tube cultures. The stock bottle was reseeded
with $0.5 \times 10^6$ cells in 25 ml. of the following medium:-

| | |
|---|---|
| Hanks' solution + lactalbumin | 78 ml. |
| Human serum | 20 ml. |
| Sodium bicarbonate solution | 1 ml. |
| PSN solution | 1 ml. |

The medium was changed at 3 days and a monolayer had usually formed
at 4 days.

Tube cultures

Cells obtained by versenating monolayers in stock bottle cultures
were centrifuged from versene suspensions and resuspended at a con-
centration of $10^5$ per ml. in the following medium:-

| | |
|---|---|
| Hanks' solution + lactalbumin | 94 ml. |
| Calf serum | 4 ml. |
| Sodium bicarbonate solution | 1 ml. |
| PSN solution | 1 ml. |

This cell suspension was dispensed into $4 \times \frac{1}{2}$ in. test tubes, each
tube receiving $0.8 - 1.0$ ml. The tubes were incubated at $37^{\circ}C$ in
stationary racks and a monolayer formed in 3-4 days. The medium
was changed at 2-3 day intervals as dictated by colour changes of
the phenol red indicator in the medium.

### Titrations of virus

In comparative titrations of seed viruses 3 tubes of each type
of culture were used and the virus was titrated in 10-fold dilutions.
Wild viruses in stool extracts were titrated in half log. steps and

2 tubes of each tissue were used for each dilution. The medium was
not changed in tubes of amnion, thyroid, and kidney during the period
of incubation. The medium in HeLa cell tubes became acid much more
quickly and had to be changed every 3-4 days. All these titrations
were read after 14 days' incubation except those of prototype strains
of enteroviruses which were read after 7 days, since early in the
work it was found that cytopathic changes in a tube infected with
prototype enterovirus seed had always become apparent within a week.

Tissue culture seed of freshly isolated viruses was titrated in
10-fold dilutions using 2 tubes per dilution of amnion cultures or
kidney cultures if the isolate was not cytopathogenic for amnion.
These titrations were read after 3 days' incubation for complete
degeneration of the cell layer.

Control uninoculated tubes of each type of tissue culture were
always incubated in parallel with cultures used for titrations. All
tubes were incubated at 37°C. The virus inoculum per tube was 0.1ml.
and this volume was added to 0.9 ml. of medium already in the tube.
The $TCD_{50}$ of virus in all titrations was calculated by the method of
Kärber.

## Neutralisation tests

The virus to be tested was diluted in PBS so that 100 $TCD_{50}$
was contained in 0.1 ml. volume. This volume of diluted virus was
mixed in a 3 x $\frac{1}{2}$ in. test tube with 0.1 ml. of a suitable dilution
in PBS of antiserum which had been previously inactivated. As a
control, 0.1 ml. virus dilution was mixed with 0.1 ml. PBS in a
second tube. The mixtures were carefully shaken and the tubes were

kept at room temperature for 1 hour after which the 0.2 ml. contents

of each tube were transferred to a tube of tissue culture. Amnion

cultures were used for all enteroviruses cytopathogenic for amnion:

monkey kidney cultures were used for the remainder. Adenoviruses

were tested in thyroid or HeLa cell cultures. Control tubes were

read daily until complete degeneration of the monolayer was observed -

usually about the third day. Antiserum-containing tubes were read

24 hours later and neutralisation was considered to have occurred

if the monolayer was intact.

## Complement fixation tests

Complement fixation tests were carried out in perspex haemagglu-

tination plates. All reagents were added in 0.1 ml. volumes. One

volume each of viral antigen, serum, and complement were mixed. 0.1 ml.

of complement contained twice the 50% haemolytic dose. The plate was

kept overnight at 4°C. After warming the plate the following morning

to 37°C. 0.1 ml. of sensitised sheep cells were added. The plate was

reincubated for 30 min. and readings made several hours later when

the cells had settled. Serum, antigen, and complement controls were

included with every test.

## Virus isolation from stools

Stool extracts were made by emulsifying 1 - 1.5 ml. of faeces

in 7ml. PBS containing 2.5% PSN. The mixture was well shaken and

later centrifuged at 3,000 r.p.m. for 30 min. The supernatant con-

stituted the stool extract which was stored at -40°C. until inoculated

to tissue culture.

Every stool extract was inoculated to 2 tubes of each type of

tissue culture used. To the 1 ml. of Earle's solution, calf serum medium already in the tube 1 ml. of stool extract was added. The tube was restoppered and left in a sloping position at 37°C. for 1 hour. The fluid was then removed and 1 ml. of Earle's solution, calf serum medium added to the tube.

Tubes were examined daily for 14 days for cytopathic changes in the monolayer. If the layer degenerated due to age before 14 days a second pass was made to fresh tissue culture tubes. Uninoculated controls of each batch of tissue cultures were incubated along with the inoculated tubes. If cytopathic changes took place in a tube, the monolayer was allowed to become completely infected and then 0.1 ml. of the culture fluid was inoculated to 2 fresh tubes of human amnion tissue culture. If the amnion cultures were infected several passes were made until complete cell degeneration took place in 1 to 2 days. This seed was then titred and used for neutralisation tests. If the virus failed to infect amnion cultures the same procedure was carried out in the tissue in which the virus was primarily isolated. If the cytopathic changes in the original tube resembled those of an adenovirus, passes were made in HeLa or thyroid cultures. In this case the tissue cultures were frozen and thawed 4 times before 0.1 ml. tissue culture fluid was inoculated to a fresh tube of tissue culture.

## Identification of virus isolates

When the isolate had been passed until it caused rapid cytopathic changes, seed of the last pass was titrated. Neutralisation tests were then performed with 100 $TCD_{50}$ doses of virus against

antisera to poliovirus types 1 to 3, Coxsackie A9 and B1 to 5, and ECHO viruses types 1 to 20 except 10 and 16. As a first stage only 1 tube of tissue culture was used for each antiserum, and 2 tubes of virus without antisera were included as controls. If one antiserum prevented degeneration of the monolayer, a repeat test was performed with this antiserum using at least 2 antiserum containing tubes and 2 virus controls. These tests were all done in amnion tissue cultures if the isolate was cytopathogenic for amnion.

A virus which showed cytopathic effects like those of an adenovirus was first tested against adenovirus antiserum in a complement fixation test. Virus was heated at 60°C. for 30 min. Doubling dilutions of this antigen were mixed with a constant known optimal dose of antiserum and a complement fixation test performed. If this test showed the isolate to be an adenovirus, neutralisation tests were carried out in thyroid or HeLa cultures against antisera to adenovirus types 1-7 in a manner similar to that employed with the enteroviruses.

## CHAPTER III

## PREPARATION OF HUMAN AMNION TISSUE CULTURES

When I decided to compare the relative efficiency of human

amnion and monkey kidney tissue cultures for enterovirus isolation,

a method of preparing human amnion cultures had not yet been esta-

blished in the laboratory.  As I had previously seen a satisfactory

method employed in the laboratory of Dr. A.J. Rhodes, then virologist

at the Toronto Hospital for Sick Children, I decided to follow the

method in use there.  Although I made a number of modifications,

which are fully described in this chapter, tne basic method proved

very satisfactory.  In all, I personally prepared cultures from 55

amnions and the results reported here are based on this series.

Because the first 8 cultures were unsuccessful, detailed analysis

was made only of the series of 47 successful cultures.  When the

method had been working satisfactorily for some months I passed on

the details to my colleague Dr. E.J. Bell who has since then used the

method routinely.  In cooperation Dr. Bell and I have published a

paper on human amnion tissue culture in which we reported the com-

bined results obtained with my series of 55 and with 53 done by Dr.

Bell (Duncan and Bell, 1961).  The results obtained with this larger

number were substantially the same as those with my smaller personal

series reported in this chapter.

### Method used at Toronto Hospital for Sick Children

The method is described in detail because several changes were

introduced by the Toronto workers after their original published

description of the method (Beale, Doane, and Ormsby, 1957).

A placenta delivered between midnight and 9 a.m. was placed immediately in a sterile 2 litre beaker containing 400 ml. Hanks' solution. The beaker was covered and left at room temperature until it was taken to the laboratory. In the laboratory the placenta was suspended from a retort stand by a pair of sterile Spencer-Wells forceps attached to the umbilical cord, and the amnion separated from the chorion by blunt dissection. After 4 washings in Hanks' solution the amnion was placed in 100 ml. of 0.25% Difco (1:250) trypsin at pH 7.6 in a closed flask at room temperature. After 1 hour this solution was discarded and replaced by 100 ml. of fresh trypsin which was kept at room temperature for about 5 hours until the amnion cells were released into suspension. The cell suspension was filtered through sterile gauze. The remaining tissue was rinsed with Hanks' solution and this suspension also filtered through gauze. The two suspensions were pooled and centrifuged at 1000 r.p.m. for 15 min. The supernatant was discarded and the packed amnion cells were resuspended in propagating medium to a volume of 10 ml. The cells in this suspension were counted in a haemocytometer chamber, and the suspension then diluted to give a concentration of $3.5 \times 10^5$ cells per ml. of propagating medium. The medium consisted of Hanks' solution + 0.5% lactalbumin hydrolysate + 20% human serum + penicillin and streptomycin. This was dispensed in 1 ml. amounts for primary cultures in 6 x 5/8 in. tubes or in 60 ml. amounts in bottles for later preparation of secondary cultures. The propagating medium was changed every 3-4 days, and in 10-14 days when a solid sheet of cells had formed, the medium was changed to a maintenance medium consisting

of Earle's solution + 0.5% lactalbumin hydrolysate + 0.1% yeast
extract + 1% of 10% glucose solution + 2% horse serum + penicillin
and streptomycin.

Second generation cultures were prepared by washing the mono-
layer in a bottle culture with Hanks' solution, then adding 40 ml.
of 0.25% trypsin, and incubating the bottle at 37°C. for 30-45 min.
The cell suspension obtained in this way was centrifuged at 1000 r.p.m.
for 5 min. and the supernatant discarded. The cells were resuspended
to a concentration of 1.0 - 1.5x10$^5$ per ml. in propagating medium,
which was the same as the propagating medium used for primary cultures
except that 20% horse serum was substituted for 20% human serum.
Volumes of 1 ml. were dispensed in tubes which were incubated at 37°C.
The medium was changed after 3 days and the cultures were ready for
use by 4-5 days.

### Effect of method of preparing trypsin solution
### on success of amnion tissue cultures

Attempts to grow the cells from the first 8 amnions were uni-
formly unsuccessful. This may be seen from Table 1; 2 were contam-
inated, 4 failed to grow at all, and 2 gave only a few cultures with
sparse monolayers after prolonged incubation. Amnions processed in
exactly the same way as I had seen done in Toronto did not yield
their cells into suspension in adequate numbers after 5 hours but
only after overnight trypsinisation. Trypsinisation was attempted
at 37°C. and again cells were not released in adequate numbers in
less than about 16 hours except with amnion 5. After these prolonged
periods of trypsinisation the cells appeared to be unable to grow.

No immediate inquiry was made into the potency of the solutions
of trypsin employed because the same solutions were being used in the
laboratory with satisfactory results for the trypsinisation of rhe-
sus monkey kidney tissue. However, after I had failed to obtain suc-
cessful trypsinisation of the first 8 amnions by any method attempted
I decided to change the method of preparing trypsin solutions lest
this were responsible for the failures. The method then in use was
to add trypsin powder to 1-2 litres of Hanks' solution in a flask and
to dissolve by periodic shaking over the course of the day, keeping
the flask at room temperature throughout. The solution was stored at
$4^{o}$C. overnight and filtered in bulk over a period of 3-4 hours the
following day. It was evident that this long period at room tempera-
ture would permit considerable inactivation of the enzyme before the
sterile solution could be bottled and frozen on the second day.

I therefore introduced a new method of preparing a series of
batches, each of only 200 ml., over the course of the day. 0.25 g.
of trypsin was added to 100 ml. of Hanks' solution which was agitated
at $37^{o}$C. in a magnetic stirrer. The trypsin had dissolved completely
in 30 min., and the solution was immediately filtered in a small
Seitz filter. A second batch of 100 ml. trypsin solution was then
prepared in the same way, and as soon as the solution was complete
this was passed through the same Seitz filter. When the complete
batch of 200 ml. was filtered it was placed in a bottle and stored
at $-20^{o}$C. until it was used. In this way none of the trypsin solution
was in a warm atmosphere for longer than $1\frac{1}{2}$ hours. Seitz filtration
and storage at $-20^{o}$C. did not reduce the activity of trypsin solutions.

## TABLE 1

## Details of preparation of tissue cultures from

## 55 amnions

| Reference number of amnion | Stored over-night before process-ing | Trypsinisation time in hours (After first $\frac{1}{2}$ hr.) | Yield in ml. $(3.5 \times 10^5$ cells) ml. | Propor-tion of tubes contam-inated | Number of days until monolayer |
|---|---|---|---|---|---|
| 1 | no | 4 | 10 | all | |
| 2 | no | 21 | 300 | none | failed to grow |
| 3 | no | various times tried | 200 | none | failed to grow |
| 4 | no | various times tried | 500 x | none | 14 |
| 5 | no | 6 | 200 | all | |
| 6 | no | 6 | 10 | none | failed to grow |
| 7 | no | 16 | 300 x | none | 9 |
| 8 | no | 21 | 400 | none | failed to grow |
| 9 | no | $4\frac{1}{2}$ | 200 | none | 4 |
| 10 | no | $3\frac{1}{4}$ & $4\frac{1}{4}$ | 530 | none | 4 |
| 11 | no | 5 | 600 | none | 6 |
| 12 | yes | 4 | 500 | none | 5 |
| 13 | no | $4\frac{1}{2}$ | 700 | none | 7 |
| 14 | no | $4\frac{1}{2}$ | 400 | none | 7 |
| 15 | no | 4 | 1000 | 1/6 | 5 |
| 16 | no | 4 | 570 | none | 5 |
| 17 | no | $5\frac{1}{2}$ | 180 | none | 6 |
| 18 | no | $4\frac{1}{2}$ | 500 | very few | 7 |
| 19 | no | 4 | 550 | none | 8 |
| 20 | no | ... | 500 | all | |
| 23 | yes | 4 | 750 | 1/10 | 6 |
| 24 | yes | $3\frac{1}{2}$ | 900 | none | 7 |
| 25 | yes | ... | 550 | none | 7 |
| 26 | yes | ... | 500 | none | 8 |
| 27 | yes | $2\frac{1}{2}$ | 400 | none | 7 |
| 28 | yes | 4 | 700 | all | |
| 29 | yes | 3 | 150 | none | 9 |
| 30 | yes | 3 | 450 | none | 10 |

x   only a small proportion of cultures grew

...   not recorded

## TABLE 1 (Continued)

### Details of preparation of tissue cultures from

### 55 amnions

| Reference number of amnion | Stored over-night before process-ing | Trypsinisation time in hours (After first $\frac{1}{2}$ hr.) | Yield in ml. ($3.5 \times 10^5$ cells) ml. | Propor-tion of tubes contam-inated | Number of days until monolayer |
|---|---|---|---|---|---|
| 31 | no | ... | 700 | none | 7 |
| 32 | no | ... | 800 | none | 6 |
| 33 | yes | $4\frac{3}{4}$ | 400 | none | 8 |
| 57 | no | 4 | 450 | none | 10 |
| 58 | yes | $4\frac{1}{2}$ | 180 | all | |
| 60 | yes | ... | 200 | all | |
| 61 | no | ... | 200 | none | 8 |
| 63 | no | ... | 230 | very few | 6 |
| 65 | no | $4\frac{1}{2}$ | 450 | none | 7 |
| 67 | no | $4\frac{1}{2}$ | 500 | 1/11 | 7 |
| 68 | yes | ... | 300 | very few | 7 |
| 71 | no | ... | 600 | none | 10 |
| 73 | no | $4\frac{1}{2}$ | 180 | all | |
| 74 | yes | ... | 150 | none | 12 |
| 78 | yes | 4 | 70 | none | 13 |
| 79 | no | $4\frac{1}{2}$ | 550 | none | 12 |
| 80 | no | $4\frac{1}{2}$ | 420 | none | 7 |
| 83 | no | 4 | 550 | 1/10 | 7 |
| 85 | no | 5 | 400 | none | 7 |
| 87 | yes | $4\frac{1}{2}$ | 700 | none | failed to grow |
| 95 | no | ... | 30 | | not cultured |
| 96 | yes | $4\frac{1}{2}$ | 370 | 1/7 | 11 |
| 97 | yes | $4\frac{1}{2}$ | 500 | all | |
| 99 | yes | $4\frac{1}{2}$ | 200 | none | 10 |
| 100 | no | $4\frac{1}{2}$ | 300 | none | 6 |
| 103 | no | 4 | 950 | none | 7 |
| 104 | no | 5 | 600 | none | 6 |

... not recorded

This was demonstrated by an experiment with thyroid number 9 which is reported in Chapter IV.

Trypsin prepared in this way was used in the preparation of cultures from amnion number 9 onwards. With the new trypsin large numbers of cells were released after 4-4$\frac{1}{2}$ hours' trypsinisation at 37°C. and the cells gave excellent yields of satisfactory tissue cultures.

The difference in potency between trypsin solutions prepared by the two methods was shown in an experiment with amnion number 10. This amnion was divided equally; one half was trypsinised with old trypsin solution and the other half with new. The old trypsin released 1.8x $10^8$ cells in 4$\frac{1}{4}$ hours and the new trypsin 2.8x $10^8$ cells in 3$\frac{1}{4}$ hours, the trypsinisation in each case having been allowed to continue until the fluid became turbid.

## Modifications in the original method of preparing
## amnion cultures

### Overnight storage of amnion

At first only amnions from placentas delivered during the night were used, as the processing of the tissue could then be done during normal laboratory working hours and without a long period of storage after delivery. As this considerably limited the chance of obtaining an amnion when it was required, a method was evolved of overnight storage of amnions from placentas delivered during the day. The amnion was stripped from the chorion, washed in Hanks' solution, and stored at room temperature in a tightly stoppered bottle containing 100 ml. of complete propagating medium. The following morning the amnion was washed twice in Hanks' solution to remove any serum present in the

storage medium, and then trypsinised in the usual way.

The yield of tissue cultures from amnions which had been stored overnight before trypsinisation was no less than that from amnions processed at once. When the cultures which failed to form a mono-layer were omitted from the calculations the results were:-

Average yield from 13 amnions stored overnight      560 ml.

Average yield from 34 amnions trypsinised at once   397 ml.

## Temperature of trypsinisation

Among many variations in methods made in an attempt to obtain successful cultures from the first few amnions, different portions of amnion 4 were trypsinised at room temperature and at 37°C. Cells were released much more rapidly at 37°C. and they produced tissue cultures no less successful than those obtained by room temperature trypsinisation. Accordingly trypsinisation of the next few amnions was done at 37°C. and when trypsin solutions made by the new method came into use it was apparent that trypsinisation at 37°C. in no way harmed the cells. It was therefore adopted as the routine method. On average, 4 hours' trypsinisation after the $\frac{1}{2}$ hour preliminary trypsinisation released the cells into suspension from the amnion. For both preliminary and final trypsinisation 200 ml. of trypsin solution was used routinely because the 100 ml. originally employed seemed insufficient for the bulkier amnions.

The effectiveness of trypsinisation with active trypsin solution at 37°C. was demonstrated by histological examination of amnion number 80. During trypsinisation amnion tissue changes in appearance from a number of discrete pieces of thin white membrane to a tangled

mass of transparent gelatinous material. Samples taken before and
after trypsinisation were fixed in formol saline. Paraffin sections
were made and stained with haematoxylin and eosin. Microscopically
the untrypsinised amnion consisted of a continuous layer of cuboidal
epithelial cells, one cell thick. Counts were made of the number of
cells in several straight lengths of membrane stretching across the
diameter of one high power field. The average was 41. The trypsin-
ised amnion consisted of a very thin eosinophilic membranous lamina
with scanty cuboidal cells lying at irregular intervals along it.
These cells did not differ in appearance from the cells of the un-
trypsinised amnion. There was an average of 4.6 cells on each high
power field length of the eosinophilic lamina. These figures suggest
that the trypsinisation procedure freed roughly 90% of the cells from
the amnion tissue.

Culture media

No change was made in the medium for propagation of primary
cultures. The maintenance medium was changed to Earle's solution,
calf serum medium as a matter of convenience because this medium was
already in routine use in the laboratory for the maintenance of monkey
kidney tissue cultures. It proved very satisfactory for maintaining
human amnion tissue cultures.

Secondary cultures

Primary cultures in Roux bottles were made from some of the ear-
lier amnions. The inocula varied from 75 to 100 ml. per bottle, the
cell concentration being $3.5 \times 10^5$ per ml. in all except the case of
amnion 10 where it was $6 \times 10^5$ per ml. Secondary tube cultures were

prepared later by trypsinising the monolayers in the Roux bottles.
The method was not continued because fewer tube cultures were obtained
as secondary cultures than would have been produced if the primary
inoculum had been dispensed in tubes instead of in Roux bottles.
This is shown in Table 2.

## TABLE 2

### Comparative yields from primary and secondary amnion cultures

| Reference number of amnion | Volume of culture in ml. | |
| --- | --- | --- |
| | Used to inoculate Roux bottle primary cultures | Available to inoculate tubes from trypsinisation of Roux bottles |
| 10 | 80x | 100 |
| 11 | 100 | 80 |
| 14 | 160 | 125 |
| 15 | 220 | 70 |

x   Contained $6 \times 10^5$ instead of the usual $3.5 \times 10^5$ cells per ml.

### Results obtained with human amnion tissue cultures

This analysis of the results obtained with the method of tissue
culture adopted and the factors influencing the success or failure of
the cultures, is based on the findings with the 47 amnions, from
amnion number 9 onwards, which were trypsinised with fully active
solutions of trypsin.

### Yields of cultures

There was great variation in the yields of tissue cultures
from different amnions, from as little as 30 ml. and 70 ml. to as
much as 1000 ml.  The total yield from 47 amnions was 21,610 ml.,
but of this total 2,720 ml. of cultures were lost by contamination
and 700 ml. failed to produce satisfactory monolayers.  This repre-

sents an average of 387 ml. of satisfactory cultures from each

amnion. If none of the cultures had been lost by contamination the

average per amnion, omitting only those which failed to form a mono-

layer, would have been 445 ml. However, much of the bacterial and

fungal contamination seemed to arise not in the laboratory but at

the time of delivery. This was suggested by bacteriological cultures

taken from a number of the amnions as soon as they reached the lab-

oratory. The figure of 387 ml. rather than that of 445 ml. would

therefore be a better representation of the average volume of tissue

culture which may be obtained in practice from each amnion. Because

4 x $\frac{1}{2}$in. tissue culture tubes were in general use in the laboratory,

they were used in preference to 6 x 5/8 in. tubes used in the origi-

nal Toronto method. The number of individual cultures in 4 x $\frac{1}{2}$ in.

tubes was always greater than the number of millilitres of tissue

culture, because rather less than 1 ml. - on average 0.85 ml. - was

found to give adequate monolayers in this size of tube. From each

amnion, therefore, an average of 455 satisfactory tube cultures was

obtained. The figure for loss by contamination was 13% and for

failure to grow was 3% of the total cultures in the whole series.

Time required to form monolayers

The average time between the setting up of the cultures and the

formation of a complete monolayer was 7 days for the 39 amnions which

grew satisfactorily. The range was from 4 days to 13 days. Propagat-

ing medium was usually changed at 3 to 4 days and the change to

maintenance medium made when the monolayer was complete at 7 days.

With the more slowly growing tissue cultures additional changes of

propagating medium were required.

## Influence of condition of amnion on success of tissue culture

An attempt was made to assess the effect of a number of variable factors in the amnion itself on the success of tissue culture. It seemed that there might possibly be a fall in the proportion of viable amnion cells when the interval between delivery of the placenta and processing of the amnion was greater. The exact time of delivery was known for only 19 amnions and the range of times between delivery and processing was from $\frac{1}{2}$ to 6 hours. Although the numbers in the different groups are too small to permit accurate comparisons to be made, it would seem probable from the results given in Table 3 that there was no great loss of viability of amnion cells with a delay of up to 6 hours.

## TABLE 3

### Interval between delivery of amnion and trypsinisation and yield of cultures

| Interval in hours | Number of amnions in group | Average yield per amnion in ml. |
|:---:|:---:|:---:|
| 0 - 1 | 9 | 457 |
| 2 - 3 | 7 | 480 |
| 4 - 6 | 3 | 800 |

Some amnions were firmly adherent to the placenta and chorion and others were practically free except for an attachment at the insertion of the umbilical cord. This factor did not have any influence on the success of tissue culture. The average yield from 8 firmly

attached amnions was 459 ml. and from 10 loose amnions 497 ml.

Neither did the presence of much mucus on the membranes appear
to affect the success of tissue cultures, although it made the centri-
fugation of cells out of the trypsinised suspension much more difficult.

## Microscopic appearances of tissue cultures

### Monolayers in tubes

Growing cultures in 4 x $\frac{1}{2}$ in. tubes were examined with a mono-
cular microscope fitted with a x8 eyepiece and a x3.5 objective and
without a condenser. With this system the gross morphology and ar-
rangement of cells was obvious but intra-cellular detail was not
clearly seen.

The first day after the cultures were set up a number of cells
were seen sticking to the tube. These were fairly large round cells
of the same appearance as cells examined immediately after trypsini-
sation. On the second day the cells had not changed in appearance
and twice as many were sticking to the tube as on the previous day.
Similarly on the third day twice as many were adherent to the tube
as on the second day and almost all the cells still looked the same.
A very few had become spindle shaped or triangular. Very few more
cells stuck to the glass after the third day and therefore the propa-
gating medium was usually changed at this time. By about the fourth
day approximately $\frac{3}{4}$ of the cells had changed in shape from round to
irregularly triangular or rhomboid, the shape being determined by
pressure of neighbouring cells. In this way small islands of mono-
layer were formed. By the fifth day few round cells remained and
the areas of monolayer were larger. These areas had joined to form

a complete monolayer by the seventh or eighth day.

The fully formed monolayer consisted almost entirely of irregularly shaped cells with clear cytoplasm and of uniform appearance. There were in addition scanty round cells which were less translucent and which from their morphology seemed to be cells which had failed to change from the original form in which they were released by trypsinisation. The monolayer of human amnion gave the appearance of a very even layer of uniform cells. This allowed ready recognition of the cytopathic effect when a culture was infected with virus. The monolayer remained unchanged in appearance for about 3 weeks. Then over a period of a few days the cells became separated from each other. Some became small and round but most became more obviously triangular with much sharper and more jagged angles than before. The cells gradually separated from the glass until none were left sticking to the wall of the tube.

## Stained coverslip preparations

Cultures of amnion number 19 were prepared by the "flying coverslip" method. Coverslips 4 x 1 cm. were used inside 4 x $\frac{1}{2}$ in. test tubes. The coverslip cultures were fixed in Rhodes' fixative (94 volumes methyl alcohol, 5 volumes 40% formaldehyde, 1 volume acetic acid) and stained with toluidine blue. Cultures ranging by intervals of 1 day from 1 to 8 days of age were examined.

All the cells seen in the 1 and 2 day old cultures and most in the 3 day culture were round cells with eccentrically placed nuclei. The diameter of the nucleus was about $\frac{1}{2}$ that of the cell. The cytoplasm stained uniformly pale blue and nucleus was darker and showed

a fine reticulation. A few cells had double nuclei. In the 3 day

old culture a few groups of 4 - 10 neighbouring cells had developed

triangular or irregular shapes forming tiny areas of monolayer and

scanty solitary cells had become spindle shaped. The cytoplasm of

the cells which had changed shape was finely vacuolated. Only about

1/3 of the cells were still round in shape in the 4 day old culture,

and areas of monolayer consisted of 15 - 20 cells. In the 5 day old

culture the monolayer was almost complete, scanty cells had 3,4,5 or 6

nuclei, and for the first time very rare mitotic figures were seen.

The monolayer in the 7 day culture was complete. It consisted of

irregularly shaped cells closely apposed to one another to cover the

whole surface of the glass and having fairly obvious cell margins.

A few round cells with non-vacuolated cytoplasm staining more darkly

than that of the other cells and with dense nuclei showing no struct-

ural detail were also present. They seemed to be lying on the mono-

layer and not forming part of it. Mitotic figures were very rare

though rather more were seen than in the 5 day culture.

### Growth of amnion cells in culture

The microscopic examination of cultures suggested that the tryp-

sinised amnion cells remained in a latent phase for about 3 days af-

ter inoculation to culture medium, during which time a proportion of

them became attached to the glass wall of the container. Those which

had stuck to the glass then grew out until they made contact with

surrounding cells and thus eventually formed a monolayer. The mono-

layer had largely formed by this process before any mitotic figures

were observed. It thus seemed likely that monolayer formation de-

pended mainly upon redistribution of the cell inoculum rather than active multiplication of cells. I therefore attempted to test this hypothesis by making counts of cells during the process of monolayer formation in tubes. For comparison, similar counts were made of HeLa cells growing to form monolayers in tubes because these cells are known to multiply in culture.

Daily counts from the time of inoculation to monolayer formation were made of the number of cells lying free in the medium and also of the number attached to the glass in each of 6 tube cultures. Cells on the glass were counted after removal of the medium and versene treatment of the monolayer. Similar counts were done with both human amnion and HeLa cell cultures. The results for amnion cultures are shown in Table 4. In calculating the total number of cells the number which must have been removed at previous changes of medium were added to those in the fluid and those on the glass. The total represents therefore the inoculum plus any increase by multiplication, less any loss by autolysis. It appears that only about $\frac{1}{2}$ the inoculum stuck to the glass in 2 days and that there was no increase in the number of cells during the growth of the monolayer. The disappearance of about 1/5 of the inoculum during the first two days may have been due to the autolysis of cells damaged during trypsinisation and centrifugation. Table 5 shows that there was a considerable increase in the number of HeLa cells under the same experimental conditions. The marked difference in the nature of growth of the two types of cells in culture is graphically depicted in Figure 1.

The results of all the experiments suggest that the formation

of a monolayer of human amnion cells is due to the redistribution

of the actual cells of the inoculum with little contribution from

cells formed by the multiplication of inoculum cells.

## TABLE 4

### Numbers of cells in growing cultures of human amnion

(Counts expressed as multiples of $10^5$)

| Day | 0 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Cell inoculum | 5.0 | | | | | |
| Cells in fluid | | $2.3^m$ | 0.5 | $0.5^m$ | 0.1 | 0.4 |
| Cells attached to tube | | 1.5 | 1.4 | 1.3 | 1.2 | 1.2 |
| Total cells[c] | 5.0 | 3.8 | 4.2 | 4.1 | 4.1 | 4.4 |
| Stage of growth | | Islands of growth | Islands joining | Almost joined | Complete monolayer | |

m – medium in all tubes changed just after this count.

c – total includes cells in fluid, cells attached to tube, and any cells removed at previous changes of medium.

## TABLE 5

### Numbers of cells in growing cultures of HeLa cells

(Counts expressed as multiples of $10^5$)

| Day | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| Cell inoculum | 1.0 | | | | | | | |
| Cells in fluid | | 0.2 | 0.2 | 0.2 | 0.1 | 0.2$^m$ | 0.3 | 0.5 |
| Cells attached to tube | | 1.3 | 1.6 | 2.1 | 2.8 | 3.1 | 3.9 | 4.7 |
| Total cells[c] | 1.0 | 1.5 | 1.8 | 2.3 | 2.9 | 3.3 | 4.4 | 5.4 |
| Stage of growth | | | Islands of growth | Islands joining | Almost joined | Complete monolayer | | |

m - medium in all tubes changed just after this count.

c - total includes cells in fluid, cells attached to tube, and any cells removed at previous changes of medium.
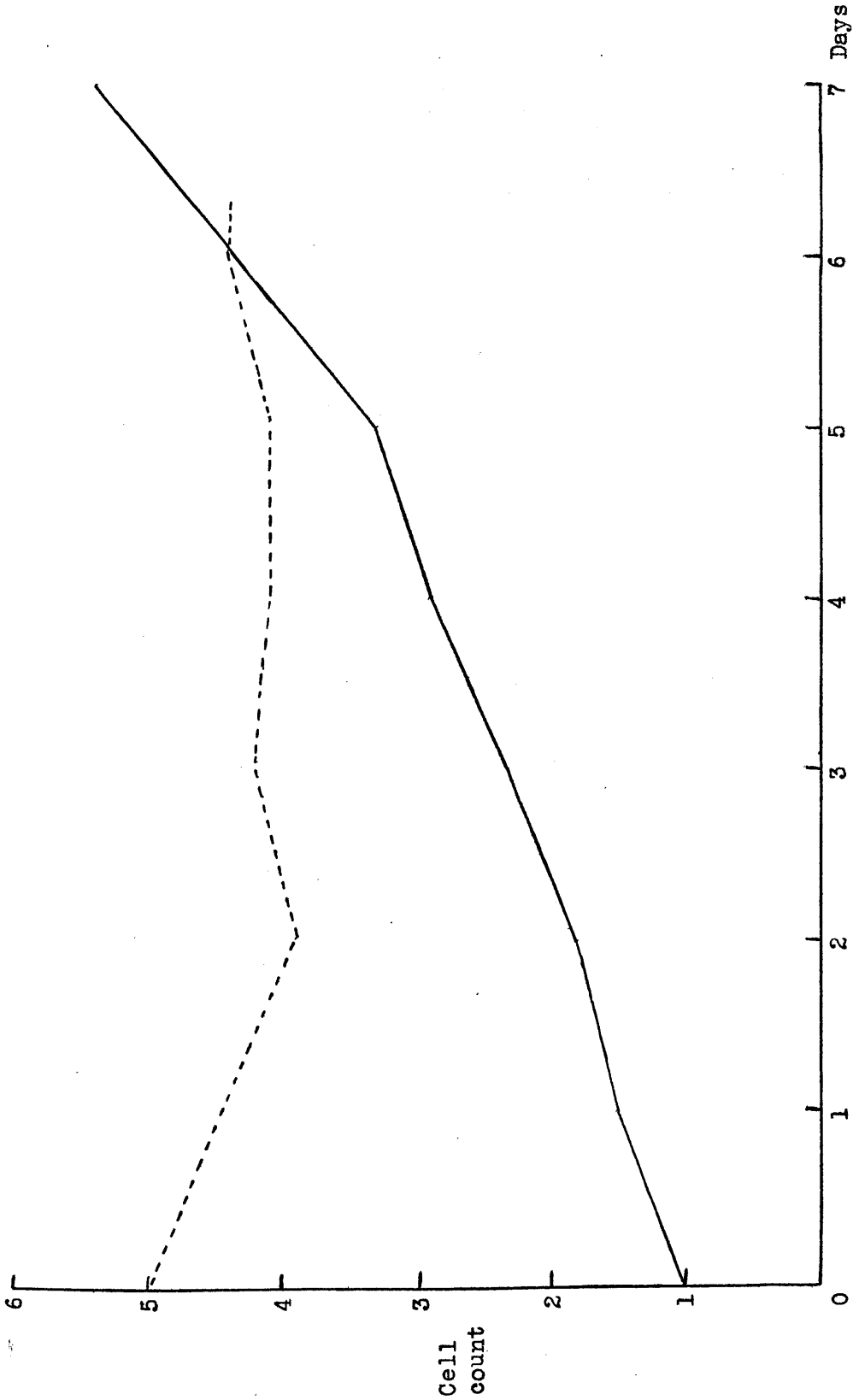
<u>Figure 1</u>   Failure of amnion cells to increase as compared with rapid multiplication of HeLa cells. The cultures were set up on day 0 and had formed monolayers by day 5. Cell counts expressed as multiples of $10^5$.

HeLa ————        amnion ------

CHAPTER IV

PREPARATION OF HUMAN THYROID TISSUE CULTURES

This chapter deals only with the preparation of tissue cultures
of human thyroid. Their use for virus isolation as compared with other
types of tissue culture is described in Chapters V and VI. The modi-
fications which I made in the original published method for the culture
of thyroid tissue are given in full, and an analysis is reported of
my results with tissue cultures prepared from 34 thyroids between
February 1959 and May 1960.

## Method of culture

### Published method

The method described by Pulvertaft, Davies, Weiss and Wilkinson
(1959) consisted basically of the digestion of chopped thyroid tissue
with trypsin only until such time as clumps of cells were released,
trypsinisation being stopped before the clumps were dispersed to in-
dividual cells. The clumps were dispensed in medium in various sized
glass containers.

Thyroid tissue was collected in a container of P.B.S. and on
arrival at the laboratory cut into fine pieces. The chopped tissue
was well washed and 5 g. placed in 100 ml. of 0.25% trypsin solution
at 37°C for 30 minutes. The suspension was then shaken well, the
tissue was allowed to settle, and the supernatant fluid discarded and
replaced by 75 ml. of fresh trypsin. The suspension was further in-
cubated at 37°C and shaken vigorously every 15 minutes for 1 - 3 hours.
When microscopic examination of a drop showed adequate release of
cell aggregates the fluid was filtered through gauze and centrifuged

for 3 minutes at 1500 r.p.m. The cells were resuspended in medium
to a concentration which showed several cell aggregates per field of
the x10 objective when a drop was examined under a coverslip. The
media employed were Hanks' solution with 10% human serum and in
addition lactalbumin hydrolysate, yeast extract, or trypsinised human
serum. Cultures were grown in bottles of several sizes and in special
chambers for photomicrography.

## Method of trypsinisation

The first 5 thyroids, like the first 8 amnions, were trypsinised
with trypsin solutions of uncertain strength. This was fully dis-
cussed with reference to amnion in Chapter III. As may be seen from
Table 6 thyroid number 1 was an excellent source of tissue cultures
but the next 4 thyroids yielded few cells on trypsinisation, almost
certainly due to lack of activity of the trypsin used. Trypsin solu-
tions prepared by the improved method described in Chapter III were
used for the trypsinisation of all thyroids from number 6 onwards
and with few exceptions the trypsinisation process proved successful.
Tests of the efficiency of digestion of thyroid number 9 were made
with trypsin prepared by the improved method, trypsin made in this
way but used without being frozen at $-20^{\circ}C$, and trypsin made in this
way but used without being Seitz filtered or frozen. All proved
equally effective and therefore filtration and storage at $-20^{\circ}C$ were
routinely employed.

In order to find out if better results would be obtained with
a higher concentration of trypsin than the 0.25% advocated by Pul-
vertaft et al. for thyroid and used in the laboratory for the tryp-

## TABLE 6

## DETAILS OF PREPARATION OF TISSUE CULTURES FROM 34 THYROIDS

| REFERENCE NUMBER OF THYROID | WEIGHT (g.) OF EXCISED THYROID TISSUE | WEIGHT (g.) USED TO PREPARE CULTURES | TRYPSINISATION TIME IN HOURS (AFTER FIRST ½ HOUR) | INOCULUM OF CELL CLUMPS PER CUB. M.M. OF MEDIUM | YIELD (ML.) OF SUCCESSFUL CULTURES TOTAL | PER g. OF THYROID TISSUE | NUMBER OF DAYS UNTIL MONOLAYER |
|---|---|---|---|---|---|---|---|
| 1 | 60 | 0.4 | 5 | 10 | 120 | 300 | 1 |
| 2 | 18 | 11 | 4½ | 5 | 0 | 0 | FAILED TO GROW |
| 3 | 20 | 10 | 4½ | 5 | 0 | 0 | FAILED TO GROW |
| 4 | 10 | 10 | 18 | NO CELLS RELEASED BY TRYPSIN | | | |
| 5 | 17 | 17 | 5 | NO CLUMPS | 30 | 2 | 10 |
| 6 | 12 | 10 | 3 | 30 | 220 | 22 | 3 |
| 7 | 50 | 30 | 3 | NO CLUMPS | 0 | 0 | FAILED TO GROW |
| 8 | 50 | 15 | 5 | 20 | 100 | 7 | 6 |
| 9 | 60 | 30 | 1½ | 15 | 200 | 7 | 3 |
| 10 | 20 | 20 | 2 | 20 | 80 | 4 | 2 |
| 11 | 30 | 15 | 3 | 10 | 70 | 5 | 3 |
| 12 | 2.5 | 2.5 | 2¼ | <5 | 6 | 2 | 5 |
| 13 | 25 | 20 | 1½ | 15 | 190 | 10 | 3 |
| 14 | 10 | 10 | 2½ | 10 | 50 | 5 | 4 |
| 15 | 12 | 12 | 1½ | 10 | 120 | 10 | ... |
| 16 | 15 | 15 | 2 | 20 | 65 | 4 | 2 |
| 17 | 20 | 20 | 1¾ | 30 | 300 | 15 | 3 |
| 18 | 50 | 50 | 1¼ | 20 | 400 | 8 | 6 |
| 19 | 20 | 20 | 2¼ | <5 | 0 | 0 | FAILED TO GROW |
| 20 | 70 | 70 | 1 | 60 | 1100 | 16 | 1 |
| 21 | 18 | 18 | 2 | <5 | 0 | 0 | FAILED TO GROW |
| 22 | 50 | 50 | 1 | 20 | 600 | 12 | 2 |
| 23 | 70 | 40 | 1 | 30 | 250 | 6 | 3 |
| 24 | 60 | 60 | ... | 30 | 700 | 12 | 2 |
| 25 | 40 | 22 | 1½ | 30 | 450 | 20 | 2 |
| 26 | 90 | 45 | 1½ | 15 | 110 | 2 | 4 |
| 27 | 20 | 20 | 1½ | 25 | 400 | 20 | 2 |
| 28 | 40 | 18 | 1¼ | 20 | 100 | 6 | 2 |
| 29 | ... | ... | ... | NO CELLS RELEASED BY TRYPSIN | | | |
| 30 | 28 | 28 | 1½ | 40 | 350 | 13 | 1 |
| 31 | ... | ... | ... | 25 | 250 | ... | ... |
| 32 | 18 | 18 | 1½ | 25 | 380 | 21 | 2 |
| 33 | 35 | 35 | 2 | 20 | 200 | 6 | 2 |
| 34 | 27 | 27 | 1¾ | 15 | 300 | 11 | 2 |

... NOT RECORDED

sinisation of other types of tissue, thyroid number 6 was divided

into 3 aliquots each of which was processed with a different concen-

tration of trypsin. Solutions of 1% and 0.5% trypsin released no

more clumps into suspension than the 0.25% solution and all 3 digested

the tissue at the same speed. Accordingly 0.25% solutions of trypsin

were used routinely.

The time of exposure of the tissue to trypsin was found to be

critical if yields of tissue cultures adequate for routine require-

ments were to be obtained. In less than about 1 hour following the

preliminary $\frac{1}{2}$ hour period very few cell clumps had been released.

After $2\frac{1}{2}$ - 3 hours the released clumps began to be digested further

to single cells. Since the yield of tissue cultures depended on the

number of clumps, the shortest period of trypsinisation which would

free a large number of cell clumps was desirable. This optimum time

had to be assessed for each thyroid by examining drops of fluid re-

moved periodically during trypsinisation, and deciding largely as a

matter of experience when the number of cell clumps was sufficient.

The average period of trypsinisation for the series was 2 hours after

the preliminary $\frac{1}{2}$ hour.

During the process of trypsinisation thyroid tissue changed in

gross appearance from numerous chopped fragments into a single bulky

mucinous mass. Histological sections were made of the mass left

after the trypsinisation of thyroid number 30 and compared with

sections of the gland before trypsinisation. Haematoxylin and eosin

stained sections of the untreated tissue showed many large acini and

some small ones, all filled with well-stained colloid and lined by

flattened epithelium. Sections of the trypsinised tissue stained with H and E showed some very small acini almost all empty of colloid, long epithelial chains one cell thick, cell clumps, single epithelial cells, and erythrocytes. These were quite irregularly arranged in a pale pink fibrillary stroma. Mallory's stain of the section showed that the fibrillary stroma consisted of course fibres of fibrin and thinner fibrous tissue fibres in approximately equal numbers. It is clear from these appearances that trypsin did not release into suspension all the epithelial cells available. Earlier experiments with thyroids number 9 and 10 had also demonstrated this. The tissue left after trypsinisation of each of these thyroids was again chopped up and retrypsinised. Enough cell clumps were released in each case to provide half as many tissue cultures as were obtained from the original trypsinisation processes.

Presence of erythrocytes in thyroid cell suspensions

Many red blood cells were always present in surgically excised portions of thyroid gland. A variable number of these cells were always mixed with the thyroid cells dispensed in culture medium into tubes, and by sedimenting in a mass in the lowest part of the sloped culture tube they prevented the growth of a monolayer on that part of the tube. In order to obtain a complete monolayer it was therefore necessary to get rid of these red cells. It was found that this was best done by a combination of two methods.

In the first place cutting the thyroid into the finest pos-
sible pieces followed by a most thorough series of washes of the
tissue in repeated changes of PBS reduced the number of red cells
present. The minced tissue was first swirled in a beaker with 4 or
5 changes of PBS and then washed twice by centrifugation. Further
red cells were released during the trypsinisation process and were
therefore dispensed with the thyroid cells into the culture tubes.
The second essential point was to remove these red cells from the
tubes before growth of the monolayer was complete and after the
thyroid cell clumps had stuck to the glass. This was achieved by
gently agitating the tubes and then immediately changing the culture
medium 18 - 24 hours after the cultures were set up. Once the red
cells had been removed, redistribution of thyroid cells produced a
complete monolayer which included the lowest part of the tube.

Size of cell inoculum

It was evident from the beginning of the work that attempts to
assess the inoculum of thyroid cells for cultures by counting single
cells gave most irregular results. I found, as Pulvertaft et al.
had stated, that counts had to be made in terms of clumps of aggre-
gated cells. Unlike these workers, however, I found that accurate
counts made in a haemocytometer chamber were necessary rather than
rough counts on an ungraduated slide. It soon became obvious that
too small an inoculum of clumps failed to produce a monolayer. Iso-
lated patches of monolayer formed but they failed to coalesce and
the cells rapidly degenerated. Later it also became clear that too
large an inoculum was unsatisfactory because the layer in the lowest

part of the tube where the cells were most densely packed peeled off the glass soon after the monolayer formed.

The upper and lower limits of inoculum giving satisfactory results were demonstrated by making a concentrated suspension of thyroid cells in propagating medium and from this suspension making 6 more dilute suspensions differing by twofold steps. From each suspension 5 tubes were inoculated, 1 ml. volumes being dispensed to each tube. A count of clumps of cells in each suspension was made before it was dispensed. The results obtained with these various inocula are shown in Table 7. The failure of any cells to stick to the glass in the 5 tubes with the heaviest inoculum was probably due to the large number of red blood cells which covered the glass. The range of clumps per cub.mm. which yielded successful cultures appeared to be from about 7 to about 30 or 40. The results of this experiment corresponded well with the finding that the average number of clumps per cub.mm. of inoculum was 22 for the 26 thyroids which gave successful cultures.

Although the monolayers grown from different sized inocula covered similar areas of glass surface it was very obvious microscopically that the layers grown from small inocula consisted of fewer cells and that each cell covered a larger area than was the case with monolayers grown from larger inocula. By removing the cells of some of the monolayers from the glass with versene it was possible to prove that monolayers covering the same area might consist of very different numbers of cells. The 5 cultures grown from the inoculum of 66 clumps per cub.mm. and the 5 from the 7 clump inoculum

## TABLE 7

### Effect of varying inoculum of thyroid cells

| Inoculum (clumps/cub.mm.) | Days to monolayer | Extent of growth of monolayer |
|---|---|---|
| 210 | ... | No cells stuck to tube |
| 133 | 2 | Layer only above lower 2 cm. of tubes; lower ends peeled |
| 66 | 2 | Complete monolayer in 3 tubes: lower end peeled in 2 |
| 27 | 3 | Complete monolayer |
| 10 | 4 | Complete monolayer |
| 7 | 5 | Complete monolayer |
| 4 | ... | Patches of monolayer formed and then degenerated |

were versenated. The monolayers from the first group consisted on average of $2.6 \times 10^5$, and those from the second group of $0.6 \times 10^5$ cells. Moreover the rounded up cells freed by versene were considerably smaller in the first than in the second group. It appears that thyroid cells can change considerably in size as well as in mutual orientation as they become redistributed to form a monolayer on glass.

## Culture media

Several types of propagating media were compared. Hanks' saline plus 10%, 5%, 2%, or 1% human serum or 5% calf serum, and Earle's saline plus 10% human serum or 5% calf serum were all tried. These media also contained 0.5% lactalbumin hydrolysate and antibiotics.

Hanks' saline plus 10% human serum gave a perfect monolayer in the shortest time although Hanks' plus 5% human serum was almost as good. All the other media gave monolayers rather more slowly and Hanks' plus 1% human serum and Earle's plus 5% calf serum caused most delay in monolayer formation. Hanks' plus 10% human serum was used as the routine propagating medium for all thyroids from number 5 onwards.

Earle's saline plus 5% calf serum was used routinely as the maintenance medium. This proved very satisfactory and was particularly convenient as it was the medium also used to maintain monkey kidney and human amnion tissue cultures. Thyroid cultures frequently retained a satisfactory pH for 2 weeks or more in this medium without a change of medium. By contrast, Hanks' saline plus 5% calf serum when used experimentally as maintenance medium had to be changed every 3 - 4 days because of the fall in pH of the medium. It was found essential to change from Hanks' plus 10% human serum to maintenance medium immediately the monolayer was complete or virtually complete. If the change was delayed for even a day the monolayers generally peeled from the lower part of the tube.

## Storage before trypsinisation

Most of the thyroids were trypsinised within a few hours of the excision of the tissue. Sometimes it was inconvenient to do this and experiments were carried out on the storage of thyroid tissue. In an early experiment a large portion of thyroid was left in the PBS in which it was collected, and processed the next day. The cells failed to stick to the glass although a similar inoculum of cells trypsinised from the same gland on the day of collection gave excel-

lent cultures. Overnight storage at room temperature of finely chopped thyroid tissue in a closed container of propagating medium and trypsinisation the following day was then tried, as this had been successful with amnion. It also proved successful with thyroid. A number of large thyroids were processed in two parts, some of the tissue at once and the remainder on the following day. The results obtained with these show that yields were practically as good from stored tissue as from fresh. This is shown in Table 8. When it was more convenient, therefore, thyroid tissue was stored overnight in this way and trypsinised the following morning.

On one occasion trypsinised cells were stored as a very concentrated suspension in propagating medium and diluted and dispensed into tubes the next day. This gave successful cultures.

TABLE 8

Yields of tissue cultures from fresh and stored

thyroid tissue

| Reference number of thyroid | Yield of cultures in ml./g. of tissue | |
|---|---|---|
| | fresh | stored overnight |
| 20 | 17 | 15 |
| 22 | 13 | 10 |
| 24 | 15 | 10 |
| 27 | 20 | 20 |
| 33 | 7 | 5 |

## Analysis of results

### Yields of cultures

In assessing the success of the method in producing sufficient tissue cultures for routine needs from an unselected group of surgically removed thyroids, the first 5 have been disregarded because unsatisfactory trypsin solutions were used. Thyroids number 12 and 14 have also been excluded because little tissue was available for culture and they were grown not for virological purposes but at the request of colleagues studying the experimental pathology of thyroid disease.

The average yield from the remaining 27 was 300ml. or 345 tubes, as the average volume inoculated to a tube was 0.87ml. The yield from the most successful thyroid which was completely used for tissue cultures was 1100 ml. or 1280 tubes. There was a very great variation in the amount of tissue culture obtained per gram of different thyroids. The average was 9 ml./g. but as is shown in Table 6 there was a very wide variation from 0 to 300 ml./g. The first thyroid was quite exceptional in the very large yield per gram.

Of the 27 thyroids in the series which was analysed, 4 did not give any successful cultures. It was of interest that 2 of these were from patients treated with radioactive iodine before operation. In these 4 cases cultures were not obtained because of the failure of cells to be released during trypsinisation.

Contamination of tissue cultures was not encountered. This contrasted with the loss of some amnion cultures by contamination, and since both tissues were processed in the same laboratory it further

suggested that the contamination of amnion occurred during delivery and not in the laboratory.

## Time required for monolayer formation

Complete monolayers of thyroid cells formed with remarkable speed. The average time was 2 - 3 days with a range from 1 day to 6 days. One of the first 5 thyroids which was processed with unsatisfactory trypsin took as long as 10 days. When the inoculum of thyroid cells was heavy monolayers formed rapidly and with small inocula monolayer formation was much longer delayed.

## Microscopic appearances of tissue cultures

Fresh unstained cultures and stained preparations were made and examined by the same methods as those described in Chapter III for amnion tissue cultures.

## Monolayers in tubes

Clumps of thyroid cells stuck to the glass of the culture tubes very rapidly. Within a few hours of the cells being dispensed into the tubes very many of them had attached themselves to the glass. This is shown in Figure 2, a photomicrograph taken 2 hours after the culture tube was inoculated. At this stage most of the attached cells were in the form of aggregates of 10 - 50 cells, and individual cells were small and round. By 18 hours a complete monolayer had formed in some cases but in others less heavily seeded with thyroid cells, only patches of monolayer had formed and many unchanged cell aggregates were present between these patches. The cells forming areas of monolayer were most often triangular in shape with 2 long sides and a shorter base and had extremely clear translucent cytoplasm.
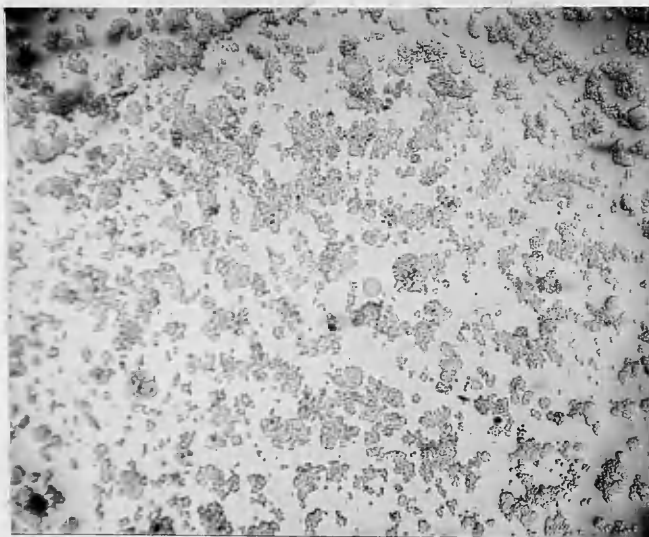
Figure 2    Human thyroid culture 2 hours after inoculation.
                    Unstained.
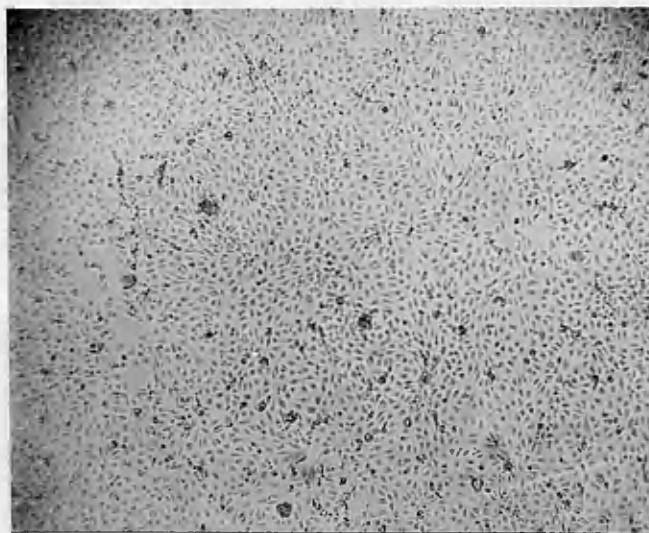


Figure 3    Human thyroid culture showing complete monolayer.
                    Unstained.

Figure 4    Peeling of lower end of monolayer of human thyroid
culture.   Unstained.
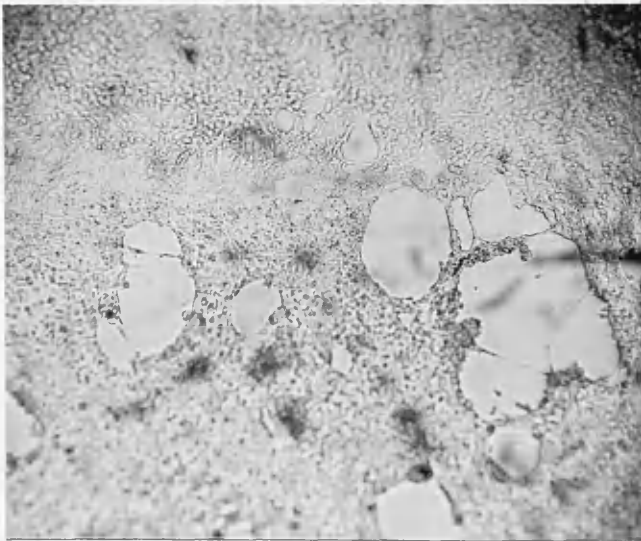


Figure 5    Degeneration of monolayer of human thyroid due to age.
Unstained.

As growth proceeded the irregular gaps in the monolayer became smaller until none remained. A mature monolayer consisted of small triangular and fusiform cells of great translucency which when compared with other types of tissue culture gave the impression of great uniformity of the component cells. This is illustrated in Figure 3. As described in the section on the size of the cell inoculum, over-seeding of cultures led to peeling of the lower part of the monolayer. After this happened the peeled monolayer usually became heaped up 1 to 2 cm. from the lower end of the tube and the peeling progressed no further. This effect is shown in Figure 4. Monolayers remained unchanged in morphology for at least 3 weeks and not infrequently for 4 or 5. When the layer eventually degenerated with age large gaps appeared giving a lacework effect, an early stage of which is shown in Figure 5. Finally all the cells separated from the surface of the tube.

Other than that seen in old cultures due to age, degeneration and cytopathic changes were not seen in uninoculated cultures from any of the thyroids. This suggests that human thyroid cells unlike monkey kidney cells do not commonly harbour latent viruses which can infect tissue cultures of the cells.

## Stained coverslip preparations

Coverslip preparations, stained with toluidine blue, of cultures at 2 hours after inoculation, at the stage of monolayer formation, and at the half-way stage, were examined. At 2 hours all the thyroid cells were small and round with an extremely thin halo of pale blue cytoplasm surrounding a darker blue reticulated nucleus. Some cells were single, some were in aggregates of 4 or 5, and some

in large aggregates of about 30 - 50 cells. At a later stage when patches of monolayer had formed over about half the area, the cells forming areas of monolayer were triangular or spindle-shaped. The nuclei of these cells were the same size as before but the cytoplasm filled a much larger area so that the diameter of the whole cell had become 3 times that of the nucleus. The cytoplasm was finely vacuolated throughout and some cells also contained a few larger vacuoles. Peripherally in the cell the cytoplasm was very thinly stained so that the margins of individual cells could not be defined. In areas between the patches of monolayer and to a lesser extent within these patches there were clumps of small round cells of the same appearance as those seen at 2 hours. When the monolayer covered the full area, all the cells were spindle-shaped, triangular, or trapezoid in shape and had the same morphology as the cells forming the incomplete monolayer at the previous stage. No clumps of round cells remained but an occasional solitary round cell was present. No mitotic figures were seen at any stage in any of the preparations examined.

## Growth of cells in culture

The complete failure of monolayer formation when the inoculum fell below a critical level, and the great rapidity with which monolayers formed from a suitable inoculum, pointed to monolayer formation being simply a rapid redistribution of cells without multiplication. Counts of thyroid cells in cultures made during the formation of monolayers substantiated this. The cells were counted in the same way as amnion and HeLa cells in the experiments described in Chapter III except that dilutions for counting were made in a

crystal violet, citric acid solution (Sandford et al., 1951) to
get rid of the many red blood cells which were present in the cul-
tures before the medium was changed. The results are shown in Table
9. Only about half the cells of the inoculum stuck to the glass and
there was little change in total cells from the time of setting up
the cultures until monolayer formation. This is very similar to the
results obtained with human amnion cultures, shown in Table 4, and
differs markedly from the findings with HeLa cells which are given
in Table 5.

## TABLE 9

### Numbers of cells in growing cultures of human thyroid

(Counts expressed as multiples of $10^5$)

| Day | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| Cell inoculum | 5.5 | | | |
| Cells in fluid | | $3.3^m$ | $0.03^m$ | 0.001 |
| Cells attached to glass | | 2.3 | | 2.0 |
| Total cells[c] | 5.5 | 5.6 | | 5.3 |
| Stage of growth | | Half monolayer | Complete monolayer | |

m - medium in all tubes changed just after this count.

c - total includes cells in fluid, cells attached to tubes, and
any cells removed at previous changes of medium

Thyroid cells appear therefore to form a monolayer in the same way as human amnion cells by redistribution of a proportion of the cells of the inoculum. The main differences between the two are that thyroid cells undergo this redistribution much more quickly and have greater powers of changing size as well as position in order to cover the glass surface, and that amnion cells multiply to a minor extent while thyroid cells showed no evidence at all of mitosis and cell division.

### Pathological condition of the thyroid glands

An attempt was made to correlate the gross pathological condition and the histological appearances of individual thyroid glands with the success or failure of tissue culture. Sections stained with H and E of 24 of the thyroids were examined. From 10 representative thyroids sections were stained with Van Giesen's stain to allow more accurate assessment of the amount of fibrous tissue present in the glands.

There was very wide variation in the pathological condition of different thyroids. In some, epithelial hyperplasia was prominent and in others colloid storage was the main feature. The picture of the underlying disease condition was often modified by pre-operative medication with thiouracil, iodine, potassium perchlorate, or car- bimazole. Most of the thyroids were from cases of thyrotoxicosis, a few were from cases of simple nodular goitre, and one was from a case of Hashimoto's thyroiditis.

In gross appearance the glands giving some of the best yields of tissue cultures were composed of soft friable tissue which when

chopped up for trypsinisation yielded fine mushy material and not the mass of very small firm cubes obtained from more fibrotic glands. Histologically this soft friable tissue usually consisted of numerous small acini containing little or no colloid - the picture of epithelial hyperplasia in unmodified thyrotoxicosis. Thyroids number 6, 17, and 32 had this histological appearance. Other thyroids, however, which also gave excellent yields of cultures were not of this type but showed the picture of colloid storage with large acini filled with deeply-stained colloid and lined by flattened epithelium. Thyroids number 1 and 20 were good examples of this type. Most of the thyroids giving good yields of tissue cultures of 15 ml. per g. or more, whether they showed the appearances of colloid storage or epithelial hyperplasia, contained much less fibrous tissue than glands giving poor yields. This was not invariable. Thyroid number 17, for example, gave an excellent yield but contained many fibrous septa enclosing small groups of acini. As a general rule, however, thyroids which had no fibrous septa separating acini gave better yields than glands with many septa. This was presumably because glands in which groups of acini were enclosed by fibrous tissue were less readily digested by trypsin than glands in which the acini were not thus protected from the action of the enzyme.

Among the thyroids which did not give any successful tissue cultures, number 19 showed the appearance of Hashimoto's thyroiditis and number 29 though basically presenting the features of thyrotoxicosis showed also a degree of thyroiditis unusual in a thyrotoxic gland. Thyroid 26 which yielded only 2 ml. of tissue culture per g.

contained well-filled acini which were almost all separated from each other by strands of fibrous tissue. The peri-acinar fibrosis in thyroid 26 is shown in Figure 6, and it contrasts markedly with the total absence of fibrous tissue in thyroid 1 which gave a large yield of cultures and which is shown in Figure 7.

There was thus no exact correlation between the histological appearances of the gland and the yield of tissue cultures. In general an excellent yield was obtained from glands with little fibrosis but many epithelial cells which might be in either a hyperplastic or a relatively inactive state. Where there was much fibrosis of the gland, yields were poor. Between these extremes no correlation between the pathological condition of the glands and minor differences in yields was apparent.
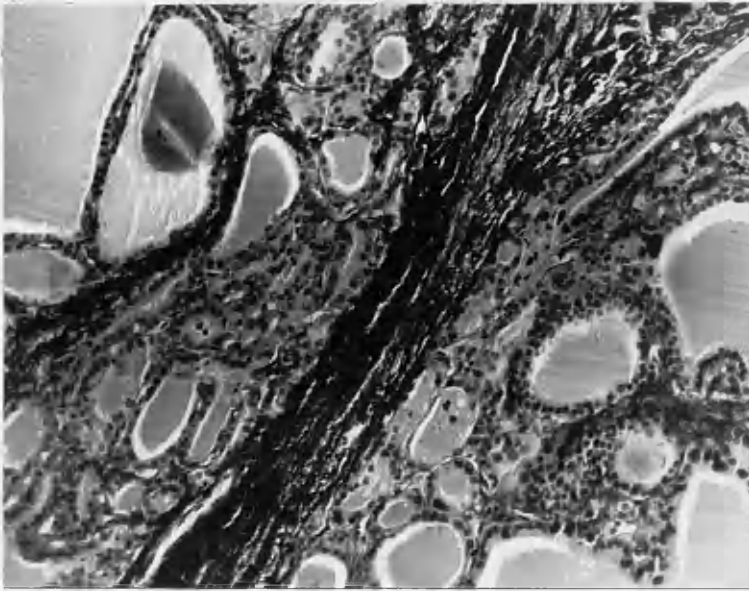
Figure 6    Histological section of thyroid 26 showing darkly
            stained thick fibrous septa between clumps of
            acini.   Van Giesen's stain.   x180.
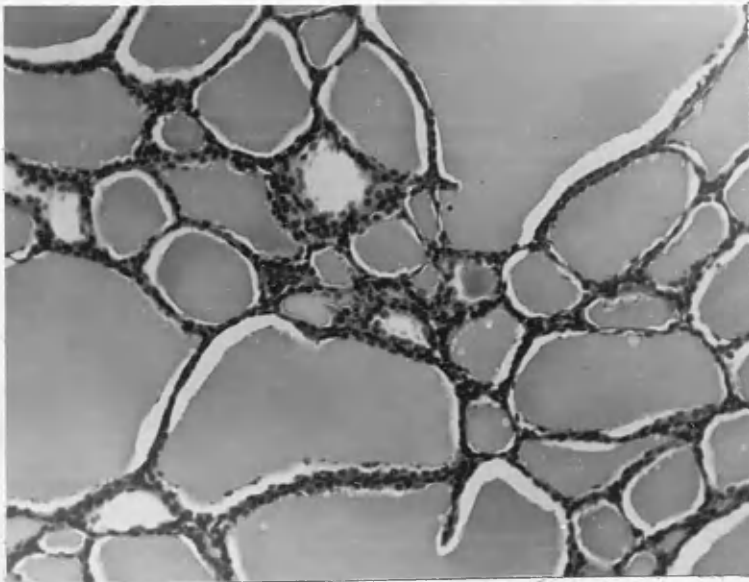


Figure 7    Histological section of thyroid 1 showing absence of
            fibrous septa.   Van Giesen's stain.   x180.

## CHAPTER V

## RANGE OF VIRUSES CYTOPATHOGENIC IN HUMAN THYROID

## TISSUE CULTURES

As a first step towards determining the probable value of thy-
roid tissue cultures for virus isolation, a variety of laboratory
strains of viruses were tested for cytopathogenicity in thyroid cul-
tures. The stock strains which were used are listed in Chapter II.
In addition, material from patients suffering from measles and chick-
enpox was tested in thyroid cultures because laboratory strains of
these viruses were not readily available. Viruses producing no cyto-
pathic effects when first tested were retested in thyroid cultures
derived from at least one other batch of thyroid tissue. Strains of
enteroviruses and adenoviruses which produced cytopathic changes in
2 or more passes in thyroid cultures were titrated in thyroid and at
the same time in the tissue cultures commonly used for the isolation
of the virus, in order to determine the sensitivity of thyroid cul-
tures as compared with the customary methods of isolation. Table 10
shows the range of viruses which were tested and which of them were
cytopathogenic. Each of the 4 groups of cytopathogenic viruses, the
enteroviruses, the adenoviruses, the pox viruses, and herpes virus,
produced a different type of cytopathic effect. These are described
in detail under the individual headings.

### Enteroviruses

The individual types of enteroviruses which were cytopathogenic
in thyroid cultures are detailed in Table 11. Thyroid showed cyto-
pathic changes with a much more restricted range of Coxsackie viruses

TABLE 10

Viruses cytopathogenic in thyroid tissue culture

| Poliovirus | + | Herpes | + |
|---|---|---|---|
| Coxsackie [x] | + | Influenza | - |
| ECHO [x] | + | Measles | - |
| Adenovirus | + | Varicella | - |
| Pox Group | + | Ovine abortion | - |

x   not all types examined were cytopathogenic

TABLE 11

Types of enteroviruses cytopathogenic in

human thyroid tissue culture

| Virus | Cytopathogenic types | Types not cytopathogenic |
|---|---|---|
| Poliovirus | 1  2  3 | - |
| Coxsackie | A9   B6 | B1 B2 B3 B4 B5 |
| ECHO | 1 2 3 5 6 7 8 9 11 12 13 17 18 19 20 21 22 23 24 25 | 4 10 14 15 16 26 27 |

TABLE 12

Titres (as $\log_{10}$ TCD$_{50}$) in thyroid amnion and kidney

cultures of prototype strains of enteroviruses

| Virus Type | Poliovirus | | | Coxsackie | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | A9 | B1 | B3 | B5 | B6 |
| Thyroid | 6.5 | 5.5 | 5.8 | 4.8 | – | – | – | 3.2 |
| Amnion | 6.2 | 3.5 | 5.5 | 5.5 | 0.8 | 6.5 | 1.5 | 2.5 |
| Kidney | 6.5 | 4.8 | 5.2 | 5.5 | 3.5 | 3.8 | 1.8 | 5.2 |

| Virus Type | ECHO | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 11 | 12 | 13 |
| Thyroid | 6.5 | 6.5 | 6.2 | 6.2 | 6.5 | 7.2 | 6.8 | 5.5 | 5.2 | 7.5 | 5.5 |
| Amnion | 6.8 | 6.8 | 6.5 | 6.8 | 7.2 | 6.5 | 6.5 | 6.5 | 5.8 | 6.2 | 6.5 |
| Kidney | 7.2 | 7.2 | 6.5 | 6.2 | 6.5 | 6.8 | 6.5 | 5.8 | 6.2 | 6.2 | 6.5 |

| Virus Type | ECHO | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 15 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
| Thyroid | – | 8.2 | 7.8 | 6.8 | 3.8 | 5.5 | 6.5 | >7.5 | 3.8 | 2.5 | – |
| Amnion | 4.8 | 8.2 | 7.2 | 6.2 | 6.5 | 5.8 | 2.5 | >7.5 | 5.8 | 5.5 | 4.5 |
| Kidney | 4.8 | 7.5 | 7.5 | 8.2 | 3.5 | 2.2 | 4.2 | 6.5 | 4.8 | 5.8 | 6.2 |

– No cytopathic effect produced

than monkey kidney, in which all 6 type B viruses and A9 produced typical effects. Human amnion also showed cytopathic effects with more Coxsackie viruses; A9, B1, B3, and B5 all produced typical changes in amnion. All the ECHO viruses are cytopathogenic for monkey kidney cultures but 7 types failed to produce cytopathic changes in human thyroid cultures. According to more recent classification however, only 6 of these are now considered to be true ECHO viruses since Sabin (1959) removed type 10 to the new group of reoviruses. Two of the 6, types 15 and 26, were cytopathogenic in human amnion cultures, and the other 4 did not produce any cytopathic changes in amnion cultures either. The results of titrations in thyroid, amnion, and monkey kidney cultures of all the enteroviruses cytopathogenic in thyroid are given in Table 12. All the strains of virus tested, except poliovirus type 1, gave clear-cut and constant end points in thyroid cultures. The Mahoney strain of poliovirus type 1 gave more variable results. In 2 titrations the titre in thyroid was similar to that in kidney and amnion while in a third titration it was $10^5$ times lower.

The cytopathic effects in thyroid cultures were the same with all the enteroviruses. In fresh unstained cultures in tubes the earliest signs of infection were groups of 5 - 10 rounded cells which were more opaque than normal. These were often surrounded by a pallisade of normally shaped cells which appeared to have rotated so that their long axes radiated out from the clump of round cells in the centre. When the virus inoculum was small, few of these little groups of infected cells were present and they were scattered at

wide intervals over the monolayer. With heavy inocula they were
so close as to be almost confluent. The groups of rounded cells
became larger and more cells throughout the monolayer rounded up
and became more opaque. Finally all the cells rounded up, and most
of them separated from the glass until the culture finally consisted
of scattered single round cells adhering to the glass. This sequence
of changes is illustrated in Figures 8 and 9. The changes produced
by enteroviruses at all stages of infection were characterised by the
rounding up of single cells which showed no tendency to coalesce with
other infected cells but remained discrete and solitary first within
a layer of healthy cells and eventually as the survivors of the mono-
layer adhering to the glass of the tube.

The detailed cytopathic changes produced by ECHO virus type 9
were studied in stained preparations. Coverslip cultures of thyroid
infected with this virus were fixed in Rhodes' fixative and stained
with May-Grunwald and Giemsa stain. Colophonium resin solution in
acetone and spirit was used to differentiate the preparations. The
earliest stage of infection was the appearance of scanty small eosi-
nophilic granules within the nuclei. At a later stage a small round
eosinophilic inclusion body was seen in the cytoplasm of an otherwise
normal cell. The cytoplasmic inclusion bodies grew larger and im-
pinged on the nucleus which became more deeply staining and lost its
reticulated appearance. In some cells the nucleus was distorted
into a semilunar shape by the large eosinophilic mass in the cyto-
plasm. Finally the cell rounded up and became smaller, the cytoplasm
was stained a pinkish-purple colour, and the nucleus became small

Figure 8    Thyroid culture infected with an enterovirus
            Earliest focus of infection.    Unstained.
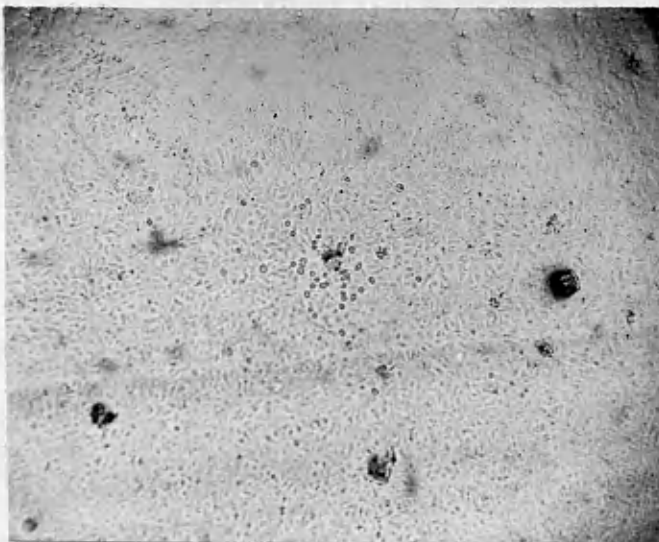


Figure 8a   Thyroid culture infected with an enterovirus
            Early stage.    Unstained.
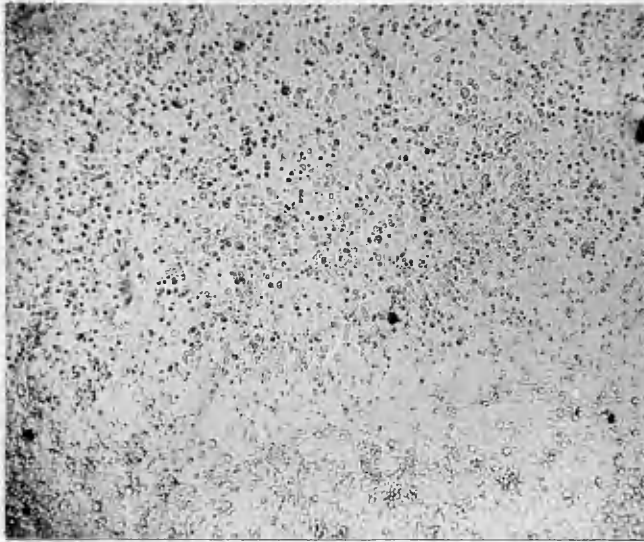
Figure 9    Thyroid culture infected with an enterovirus
            Infected cells scattered throughout monolayer.
            Unstained.
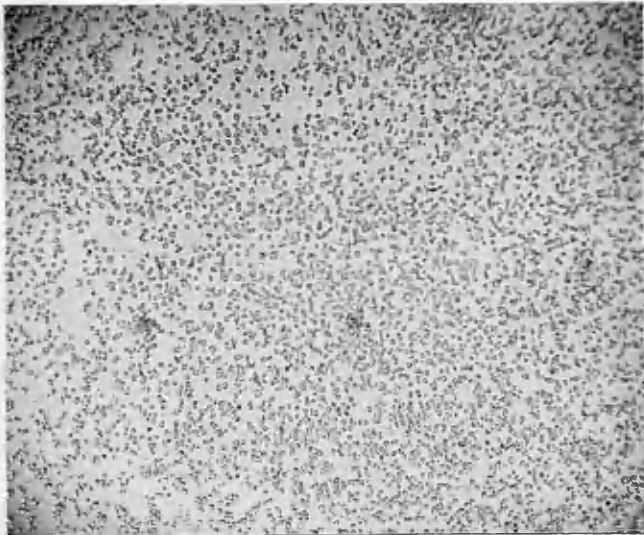


Figure 9a   Thyroid culture infected with an enterovirus
            Final stage of complete cell degeneration.
            Unstained.

and pyknotic and stained dark blue. These various appearances were very similar to those described by Shaver, Barron, and Karzon (1958) in monkey kidney cultures infected by enteroviruses.

## Adenoviruses

Adenoviruses of types 12 and 18 were not available for examination. The other 16 types which were tested were all cytopathogenic for thyroid tissue cultures and each was successfully passed at least twice. The type 8 seed was of extremely low potency and produced cytopathic changes very slowly in both passes. Its titre was not therefore estimated. The titre of each of the other 15 strains was determined simultaneously in thyroid and in HeLa cultures. The results of these titrations are shown in Table 13. These tests were read at 14 days. Even uninfected HeLa cell monolayers seldom survived much longer than this. Thyroid monolayers, on the other hand remained in perfect condition for a further 1 - 2 weeks, and higher end points were noted at that time than those recorded. Technically thyroid proved a very much easier tissue than HeLa cells for the growth of adenoviruses. The maintenance medium of Earle's solution plus 5% calf serum used for thyroid cultures did not have to be changed during the 2 - 3 weeks required for adenoviruses to produce cytopathic changes. It was not found possible to maintain the HeLa cells used in the laboratory in Earle's solution plus calf serum, and the less well buffered Hanks' solution plus calf serum had to be used. This maintenance medium became acid very quickly and had to be changed every 3 - 4 days. This was most laborious. Moreover cytopathic changes could be seen much more readily and at an earlier

## TABLE 13

### Titres (as log₁₀ TCD₅₀) in thyroid and HeLa cultures of prototype strains of adenoviruses

| Type of adenovirus | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| Thyroid | 3.5 | 2.8 | 4.5 | 2.5 | 3.2 | 4.5 | 4.5 | 4.5 | 2.5 |
| HeLa | 3.2 | 4.5 | 3.5 | 1.5 | 3.2 | 3.5 | 4.5 | 1.2 | 0.5 |

| Type of adenovirus | 11 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|
| Thyroid | 4.5 | 4.5 | 4.5 | 3.8 | 2.8 | 3.5 |
| HeLa | 3.5 | 4.5 | 3.5 | 2.2 | 0.5 | 3.5 |

stage in thyroid than in HeLa cultures because the thyroid monolayer consisted of much more uniform and translucent cells in which the earliest changes were easily observed.

The cytopathic effects seen in fresh unstained cultures were similar with all the adenoviruses examined. The various stages of infection of the monolayer are shown in Figures 10 - 13. At the earliest stage single more opaque cells were seen scattered through the monolayer. These were considerably larger than cells infected with enteroviruses and there was some slight variation in their size. These cells then began to clump together, thus occupying a smaller area and leaving gaps in the monolayer. The process eventually affected the entire monolayer and when all the cells were infected
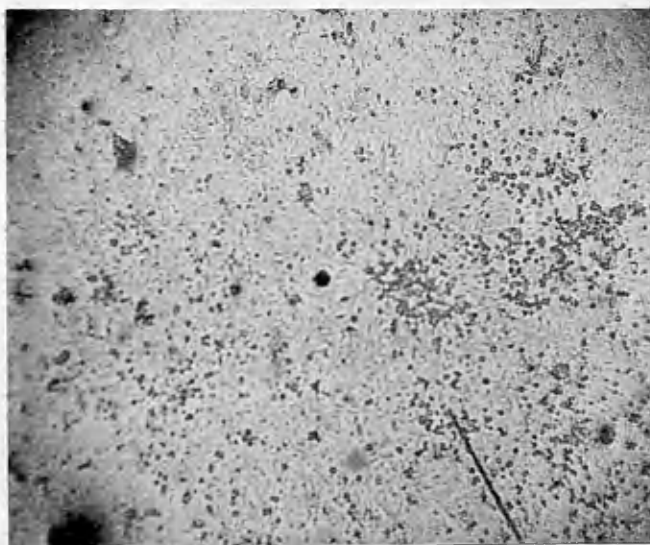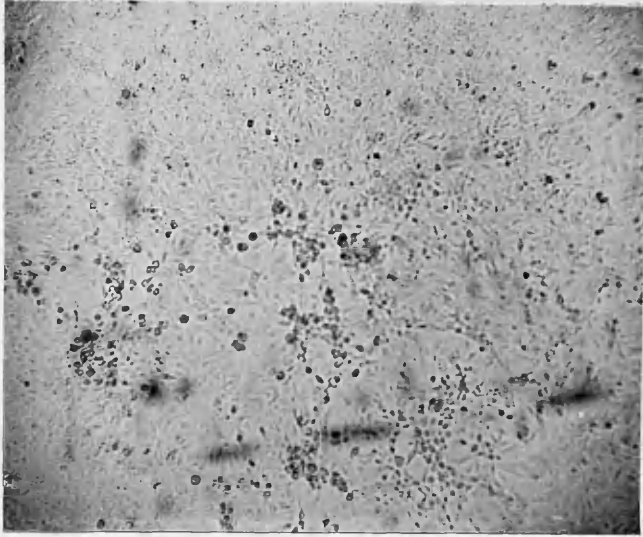
Figure 10    Thyroid culture infected with an adenovirus.
             Early stage.    Unstained.
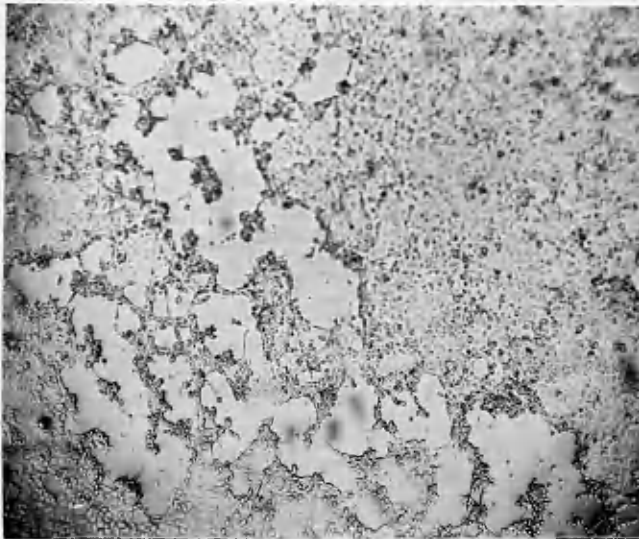


Figure 11    Thyroid culture infected with an adenovirus.
             Early clumping of infected cells. Unstained.
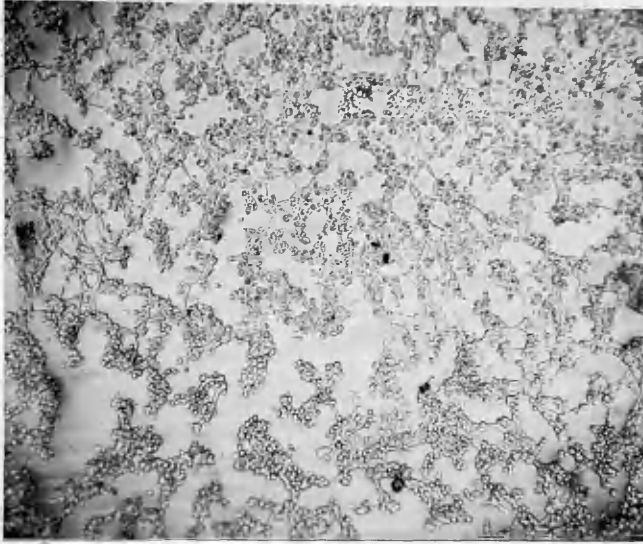
Figure 12    Thyroid culture infected with an adenovirus.
             Widespread clumping of cells.         Unstained.
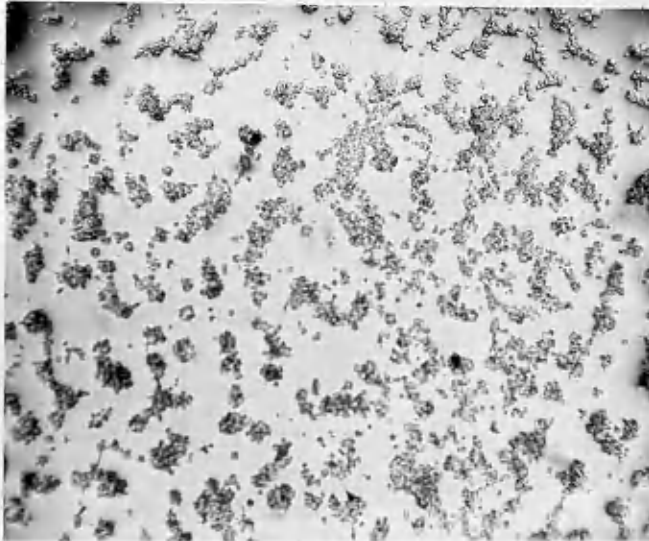


Figure 13    Thyroid culture infected with an adenovirus.
             Cell degeneration complete.         Unstained.

there remained on the glass rounded clumps of cells which had lost
their detailed individual structure as they fused together into the
rounded up masses.

Coverslip preparations were fixed and stained by the methods
described in the section on enteroviruses in this chapter. Type 7
adenovirus was used. The most obvious changes were in the nuclei
of infected cells. Early changes were the appearance of a very
finely granular eosinophilic area within an otherwise normal nucleus,
or in some cells the appearance of a densely purple staining reti-
culum in the centre of the nucleus with a narrow clear rim at the
periphery of the nucleus. At a later stage the nuclei consisted of
many fine granules or fewer coarse ones staining pink to reddish-
purple and surrounded by a wide clear zone. In some cells densely-
stained eosinophilic inclusions of moderate size were seen. Late in
the course of the infection the cells were smaller and contained a
round dense dark purple zone in the centre with a rim of paler blue.
A few showed a dense dark purple zone in the centre with scalloped
edges with a surrounding clear area subdivided into sections by divi-
sions radiating out from the central mass. This effect was identical
to the flower-like nuclear form described by Boyer, Leuchtenberger,
and Ginsberg (1957) in HeLa cells infected with adenovirus types 3
and 4. The changes seen at other stages of infection also resembled
those described by these workers in HeLa cells infected with adeno-
virus types 3 and 4. Pereira (1959) states that type 7 probably
belongs to the same group of adenoviruses as types 3 and 4 as regards
the type of cytopathic effects. Type 7 therefore appears to cause

exactly the same effects in thyroid as in HeLa cells. In thyroid cells the absence of mitotic changes in the cells makes the recognition of the viral effects somewhat easier than in HeLa cells.

## Pox group

Vaccinia and cowpox viruses were tested in thyroid cultures and both were cytopathogenic. Vaccinia was carried through 3 passes and cowpox through 13. Good yields of virus were obtained in these pass fluids.

Similar cytopathic effects were observed with both viruses. The earliest change was the appearance of groups of 10 - 30 small rounded opaque cells very similar individually to cells infected with enteroviruses, and all of the same size. The process of infection spread very slowly outward from these areas and irregular gaps appeared in the monolayer giving a lacework effect. Areas of monolayer between the centres of infection remained completely unaffected. This differed from enteroviral and adenoviral infections at a similar stage in that the infective process in these cases involved the whole monolayer sooner. With the pox viruses there was gradual centrifugal spread from the original foci, which continued until all the enlarging foci had joined together. The number of original foci bore a direct relationship to the concentration of virus in the inoculum. Eventually most of the cells separated from the glass and the final appearance resembled that of complete degeneration in cultures infected with an enterovirus — solitary small round cells adherent here and there to the wall of the tube.

Coverslip cultures infected with cowpox virus were prepared by

the methods already described. At the periphery of the foci of infection cells were seen containing 1 to 3 well-stained eosinophilic inclusions. In some cells with these inclusions the nucleus was distorted from its normal circular shape. At a later stage nuclei became dark purple and the nuclear membrane became indistinct. Finally infected cells rounded up and consisted of a dark purple centre with a very thin pale blue halo. Thin strands of cytoplasm connected some infected cells. A few eosinophilic masses exactly like the inclusions were seen lying free outside cells.

## Virus of herpes simplex

Only 1 laboratory strain of herpes virus, the HFEM strain, was tested in thyroid cultures. Virus seed grown in chorio-allantoic membranes was inoculated to thyroid cultures which showed cytopathic changes with vacuolation of cells and giant cell formation. Fluid from the infected tissue cultures was then passed again to eggs and characteristic pocks were produced on the chorio-allantoic membranes. The details of the cytopathic changes seen with herpes virus will be discussed in Chapter VI because a number of strains were isolated from clinical material and considerable differences in the types of cytopathic effect were seen with different strains.

## Other viruses

A classical A strain, an A1 strain, and an A2 strain of influenza virus, 2 type B strains, and 1 type C strain were inoculated to thyroid cultures. Cytopathic effects were not seen in the original culture tubes or in one further passage of tissue culture fluid. Attempts were made to demonstrate haemadsorption as described by Vogel and

Shelokov (1957) lest the influenza viruses had infected the cultures without producing cytopathic effects. All these attempts were unsuccessful.

A single strain of ovine abortion virus grown in the yolk sac of the hen's egg was inoculated to thyroid cultures. No effects were observed in either of two passes. This virus was selected for testing as a representative of the psittacosis group which was not a human pathogen and therefore presented no hazard in the laboratory.

Specimens of saliva were obtained from 5 patients with clinically typical measles and nasal mucus obtained from 3 of them. These specimens were inoculated to thyroid and no changes were observed in the cultures. In 3 of the patients the rash had just appeared and in 2 the rash had not yet appeared when the specimens were taken.

Fluid from fresh vesicles in 1 case of chickenpox was inoculated to thyroid cultures. No changes were observed.

## CHAPTER VI

## ISOLATION OF VIRUSES FROM CLINICAL SPECIMENS IN

## HUMAN THYROID TISSUE CULTURES

The previous chapter gave an account of the preliminary part
of the work of evaluating thyroid cultures in diagnostic virology.
This chapter describes their actual use in a routine diagnostic lab-
oratory. First their use in parallel with monkey kidney and human
amnion cultures for the isolation of viruses from all stool samples
examined in the laboratory over a one year period is described.
Since polioviruses were almost absent from the community during the
year of the trial, a number of stool extracts which had been stored
at $-20^{\circ}C$ since previous years were inoculated into thyroid cultures
in order to assess the value of thyroid for the isolation of polio-
viruses. Some evidence of the relative sensitivities of thyroid and
other cultures in detecting virus was provided by the number of iso-
lations in each. This information was supplemented however by titra-
ting the virus present in representative stool samples simultaneously
in thyroid and in the other tissue cultures. The work carried out
with prototype strains of adenoviruses and the few isolations of
adenoviruses made from stools during the year's trial suggested that
thyroid cultures were very suitable for adenovirus isolation. The
occurrence during the following year of a small epidemic of adenovirus
infection provided the opportunity of testing the value of thyroid
cultures more extensively for the isolation of adenoviruses. Finally
a limited comparison is reported of thyroid cultures, other types of
tissue culture, and the hen's egg for the isolation of herpes virus.

## Isolation of viruses from stools during 1959

During the year 1959 a total of 670 specimens of faeces was examined for viruses. An extract of each stool was made and the extract divided into 3 aliquots, 1 being inoculated into monkey kidney cultures, 1 into human thyroid cultures, and the third kept at -40°C as a spare. If a virus was isolated in either kidney or thyroid cultures the spare aliquot was later inoculated into human amnion cultures. Thus the plan of the trial allowed a complete comparison of thyroid with monkey kidney which was the tissue culture in standard use, but limited the trial of amnion largely to determining if amnion was at least as good as kidney. I adopted this compromise method of testing amnion only because I was unable to carry out the considerable task of testing every stool extract in amnion cultures as well as in the other two types of tissue culture.

Fulvertaft et al., in the original paper on the use of thyroid cultures in virology considered that this tissue culture was likely to be less satisfactory than monkey kidney because of its greater susceptibility to toxic factors present in stool extracts. I found that only a small proportion of stool extracts were more toxic for thyroid than for kidney monolayers. There appeared, however, to be some variation between different batches of thyroid cultures and I gradually formed the impression that cultures in which the monolayer had only recently formed were most affected. This impression was confirmed experimentally. Undiluted fluid, a 1:2 dilution, and a 1:4 dilution of a particularly toxic stool extract were each inoculated to 2 tubes of thyroid number 22 the day the monolayer formed,

1 day later, and 4 days later. This was also done with thyroid 23. The routine method of inoculation was used. As shown in Table 14 the 4 day old cultures were less susceptible to stool toxicity than newly formed monolayers. In future therefore I did not inoculate stool extracts to thyroid cultures until 4 days or more after the formation of the monolayer, and when this precaution was taken only rare stool extracts were more toxic to thyroid than to kidney tissue cultures.

TABLE 14

Toxicity of stools for thyroid cultures of various ages

| Thyroid number | Age of monolayer (days) | Toxic stool extract | | |
|---|---|---|---|---|
| | | Undiluted | 1:2 | 1:4 |
| 22 | newly formed | P | P | U |
| | 1 | P | P/U | U |
| | 4 | P/U | U | U |
| 23 | newly formed | P | U | U |
| | 1 | P | U | U |
| | 4 | U | U | U |

P - monolayer peeled off the glass in both tubes

P/U- monolayer peeled in 1 tube; undamaged in other

U - monolayer undamaged in both tubes

Apart from the minor problem of stool toxicity thyroid cultures were technically very satisfactory to use. The monolayers invariably survived in excellent condition for the 2 weeks during which the

cultures were observed. This was not always the case with monkey
kidney cultures and a second pass to fresh cultures was then re-
quired. Furthermore, kidney cultures and also more rarely amnion
cultures sometimes required a change of medium during the 2 weeks.
Thyroid cultures did not need any medium change.

The viruses isolated during 1959 from 670 stool specimens in
the 3 types of tissue culture are listed in Table 15. It is clear
that both thyroid and amnion cultures were considerably inferior to
monkey kidney cultures for the isolation of Coxsackie B viruses.
However it is interesting that 3 strains of Coxsackie B5 were cyto-
pathogenic for thyroid tissue culture, although the prototype strain
was not. In all, 31 viruses were isolated in kidney but not in thy-
roid cultures. Ten of these were Frater virus, 9 were Coxsackie B5,
4 were Coxsackie B2, and 4 were ECHO 14. On the other hand 74 viruses
were isolated in thyroid but not in kidney tissue cultures. Most of
these, 49 in all, were Frater virus, 10 were adenoviruses, and 10 have
not yet been typed. Two were ECHO 6, and 2 were ECHO 7. A remarkably
large number of the viruses isolated in thyroid but not in kidney cul-
tures could not be typed as enteroviruses at present recognised;
there were 10 such viruses as well as the 49 Frater viruses. Thyroid
was also better for the isolation of adenoviruses.

Amnion cultures were inferior to kidney cultures for the isola-
tion of Coxsackie B viruses. Of 24 viruses isolated in kidney but
not in amnion, 14 were Coxsackie B viruses. Amnion tissue cultures
lay between kidney and thyroid cultures in their efficiency in iso-
lating adenoviruses, Frater virus, and viruses which could not be typed.

## TABLE 15

## Viruses isolated from stools in different types of

## tissue culture during 1959

| Virus | Poliovirus | | Coxsackie | | | | ECHO | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 3 | A9 | A x | B2 | B5 | 5 | 6 | 7 | 11 | 14 | 15 |
| Thyroid | 1 | 2 | 1 | 1 | 1 | 3 | 0 | 4 | 2 | 6 | 0 | 0 |
| Amnion | 1 | 2 | 1 | 1 | 1 | 2 | 0 | 2 | 1 | 5 | 1 | 1 |
| Kidney | 1 | 2 | 1 | 0 | 6 | 10 | 2 | 2 | 1 | 6 | 4 | 1 |

| Virus | Adenovirus | | | | | | Frater Virus | Not yet typed |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 5 | 7 | 15 | | |
| Thyroid | 4 | 4 | 2 | 1 | 3 | 2 | 65 | 10 |
| Amnion | 2 | 2 | 1 | 0 | 2 | 1 | 58 | 5 |
| Kidney | 1 | 2 | 1 | 0 | 1 | 0 | 26 | 1 |

x  A other than 9

## Isolation of poliovirus from stored stool extracts

Only 3 isolations of poliovirus were made during 1959. It was obviously necessary to test the efficiency of thyroid tissue cultures in the isolation of poliovirus much more extensively than this, and therefore 48 stool extracts which had been stored at -20°C for 2 years were inoculated into thyroid and amnion tissue cultures. The original isolations from these specimens had been made in monkey kidney tissue cultures. One of the specimens contained poliovirus type 2 and the other 47 contained type 1 virus.

The virus was isolated in both types of tissue culture in every case. In 38 cases cytopathic changes occurred at the same time in both tissues. In the other 10, changes first appeared at about the same time in both, but in thyroid they remained stationary for from 2 to 6 days when about half the cells were visibly affected. After this delay the changes later progressed to complete degeneration. This temporary arrest of the progress of infection was seen only with poliovirus in thyroid tissue cultures. A second pass was made to thyroid cultures of each of the 48 isolates and 19 of them were also passed a third time in thyroid cultures. In every pass typical cytopathic effects developed. Poliovirus was thus isolated in thyroid tissue culture from each stool specimen of both the stored series and the small 1959 group which yielded virus in kidney or amnion cultures.

Neutralisation tests with poliovirus type 1 antiserum were done with 19 of the strains. All these tests gave clear-cut positive results.

## Titres in thyroid and other tissue cultures of

## viruses in stool extracts

In order to obtain further information on the relative sensi-
tivities of thyroid and the more commonly used types of tissue cul-
ture in detecting viruses, a number of stool extracts were titrated
at the same time in the various tissue cultures. The number of dif-
ferent types of viruses which were tested in this way was limited by
the strains which chanced to be isolated and the amount of stool ex-
tract left after all other tests had been done. The ECHO viruses
tested were particularly limited. The results of titrations of var-
ious enteroviruses are shown in Table 16. Thyroid and amnion cul-
tures gave very similar results. Both gave somewhat lower titres
than kidney tissue cultures with polioviruses and rather higher titres
with ECHO viruses. The results of titrations of 7 strains of adeno-
viruses in thyroid and HeLa cell cultures are given in Table 17. The
differences in titre between the two types of culture were not great.
Monkey kidney would appear to be the more sensitive tissue for the
isolation of poliovirus, but it is of interest that despite this,
thyroid was as successful as kidney under practical conditions of
isolation.

## Investigation of an outbreak of adenovirus infection

It was evident from the numbers of adenoviruses isolated in each
type of tissue culture from stool specimens during 1959, that thyroid
cultures were more suitable for the isolation of adenoviruses than
human amnion or monkey kidney cultures. The titrations shown in
Table 17 of some of these isolates in thyroid and HeLa cell cultures

TABLE 16

Titres (as $\log_{10}$ TCD$_{50}$) in thyroid, amnion and kidney cultures of

enteroviruses in stool extracts

| Virus | Poliomyelitis | | | | | ECHO | | | Coxsackie |
|---|---|---|---|---|---|---|---|---|---|
| Type | 1 | 1 | 1 | 2 | 3 | 6 | 11 | 11 | A9 |
| Thyroid | 1.5 | 2.0 | 2.5 | 1.5 | 0.5 | 5.0 | 3.5 | > 5.0 | 1.7 |
| Amnion | 1.5 | 0.5 | 2.5 | 0.5 | <0.5 | 4.5 | 3.5 | > 5.0 | 2.0 |
| Kidney | 3.0 | 1.5 | 3.5 | 2.5 | 3.5 | 2.2 | 1.7 | 2.7 | 2.0 |

## TABLE 17

### Titres (as log₁₀ TCD₅₀) in thyroid and HeLa cell cultures of adenoviruses in stool extracts

Titres (as $\log_{10}$ TCD$_{50}$) in thyroid and HeLa cell cultures of adenoviruses in stool extracts

| Type of adenovirus | 1 | 1 | 2 | 2 | 3 | 5 | 7 |
|---|---|---|---|---|---|---|---|
| Thyroid | 2.5 | 2.5 | 1.7 | 3.7 | 3.2 | 2.5 | >5.0 |
| HeLa | 1.5 | 3.0 | 2.0 | 3.7 | 1.7 | 1.5 | >5.0 |

showed further that thyroid did not differ greatly from HeLa cell cultures in its degree of sensitivity to infection by adenoviruses. The number of strains involved in these tests was very small and a more extensive test of the value of thyroid tissue cultures for adenovirus isolation seemed desirable. The occurrence of a small outbreak of adenovirus infection in the Spring of 1960 provided the opportunity of carrying out such a test.

The outbreak affected 5 families living on the same street, but only the members of two of the families were fully investigated virologically. The full details of this outbreak have been published (Duncan and Hutchison, 1961). In brief, the illness consisted in most of the patients of fever accompanied by diarrhoea, vomiting and abdominal discomfort, and in 3 only of the definite picture of pharyngo-conjunctival fever. The course of the illness, the specimens taken, and the viruses isolated are shown in Figure 14 for each member of the two families which were fully investigated. A total of 34 specimens was obtained for attempted virus isolation, 25 of which were stool samples, 2 were rectal swabs, 4 were conjunctival swabs, and 3 were throat washings. Paired sera were also taken from one patient, Mr. A., at the acute and convalescent phases of his illness in order to show if an antibody rise had occurred. Each of the specimens for virus isolation was inoculated to 2 tubes of thyroid tissue culture, which were observed for 21 days. Aliquots of those specimens which did not yield a virus in thyroid cultures were later inoculated into HeLa cell cultures.

Adenovirus type 3 was isolated from 13 of the 27 faecal speci-
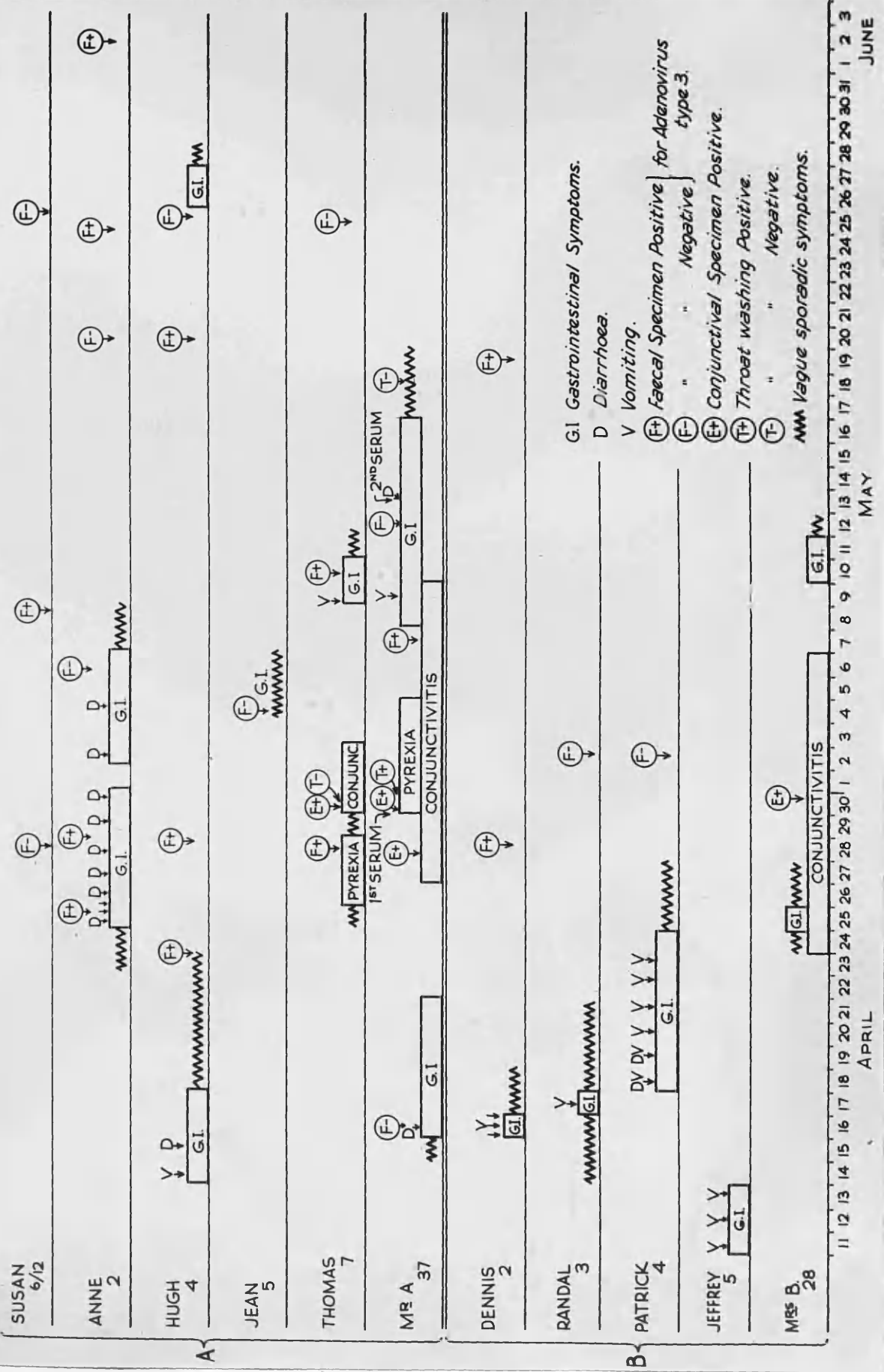
Figure 14. Details of outbreak of adenovirus infection.

mens, from all 4 conjunctival swabs, and from 1 of the 3 specimens
of throat washings. All of these isolations were made in thyroid
tissue cultures and no additional isolations were obtained in HeLa
cell cultures. Three of the stool specimens were inoculated into
both thyroid and HeLa cell cultures. The virus was in each case iso-
lated in both types of tissue culture, but cytopathic changes appeared
sooner in the thyroid cultures. A rise of neutralising antibodies
to adenovirus type 3 from less than 1:4 to 1:16 was found in the sera
of Mr. A. The concentration of adenovirus in 11 of the specimens
was titrated in thyroid tissue cultures. These titrations showed,
as might be expected, that the time between inoculation of the speci-
men and the appearance of cytopathic changes in thyroid cultures de-
pended entirely on the concentration of virus in the specimen. These
findings are given in Table 18.

The investigation of this outbreak thus provided evidence that
human thyroid cultures were most satisfactory for adenovirus isolation.
Thyroid proved no less sensitive than HeLa cultures in detecting virus.
Moreover the thyroid cultures required very much less technical atten-
tion than HeLa cell cultures. The thyroid monolayers remained in per-
fect condition over the 21 day period of observation and a change of
medium was very seldom required. HeLa cell layers, on the other hand,
required frequent changes of medium and also aged rapidly. On average,
6 changes of medium were required in addition to at least one pass to
fresh cultures during the 21 day period of observation of HeLa cell
cultures. Neutralisation tests for the identification of isolates
and the titration of serum antibodies gave much more easily readable

results when performed in thyroid tissue cultures, because there was
absolutely no confusion between cytopathic changes and cell degener-
ation due to age as there very often was when HeLa cell cultures
were used.

## TABLE 18

### Time required to isolate virus and concentration of

### adenovirus in the specimen

| Days until cytopathic changes | 3 | 3 | 4 | 4 | 5 | 9 | 10 | 10 | 12 | 14 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Concentration of virus in specimen ($TCD_{50}$/ml.) | 5.5 | 5.5 | 4.0 | 3.5 | 3.5 | 2.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |

### Isolation of herpes virus

Only a small scale comparison was attempted of thyroid tissue
cultures with other host systems for the isolation of herpes virus.
Specimens from 20 possible cases of herpes simplex of the skin or
buccal mucosa were inoculated into thyroid cultures, and to the
chorio-allantoic membranes of fertilised hens' eggs, and in some
cases also into human amnion or HeLa cell cultures.  Six specimens
did not yield herpes virus in any culture system.  Of the remaining
14, all except the 3 earliest specimens were inoculated into amnion
and HeLa cell cultures as well as into thyroid cultures and eggs.
Herpes virus was isolated from all of the 14 specimens in thyroid
cultures and from 10 of the 14 in eggs.  Tissue culture fluid from
3 of the 4 which were positive in thyroid cultures but not in eggs

produced herpes pocks when passed to the chorio-allantoic membranes
of eggs. Of the 11 specimens also inoculated into amnion and HeLa
cell cultures, all were positive in amnion cultures and only 5 were
positive in HeLa cell cultures. Typical cytopathic effects of herpes
virus in thyroid and amnion tissue cultures were often seen 18 hours
after inoculation and usually within 48 hours. Definite herpes pocks
on the chorio-allantoic membranes of eggs were not recognisable until
24 hours later with many of the specimens. This small series of iso-
lations suggests that thyroid and amnion tissue cultures are somewhat
more sensitive than the hen's egg for the isolation of herpes virus,
and that HeLa cells, or at least the strain in use in this laboratory,
are less sensitive than the egg.

The cytopathic changes produced by herpes virus in thyroid cul-
tures varied from strain to strain but there were certain features
in common. The first change to be observed in fresh unstained cul-
tures was the appearance of scattered groups of rounded cells, which
differed from those seen in enterovirus and pox virus infections in
their marked variation in size. Some infected cells were 2 or 3 times
the normal size. Clear areas like vacuoles which were of various
sizes were often seen in the cells. Very thin threads of cytoplasm
were sometimes present connecting cells. Giant cells were seen in
some cultures. Cytopathic changes began focally throughout the mono-
layer and spread outwards from the foci until they became confluent.
Strain differences in cytopathic effects corresponded closely to the
3 varieties described by Gray et al., (1958). Full records were kept
of the cytopathic effects of 11 of my isolates; 5 produced the non-

proliferative type of effect, 3 the proliferative, and 2 the giant cell. One of my stains could not be assigned to any of the 3 varieties of Gray et al. It caused less clumping of cells than usual and the outstanding feature was the presence of vacuoles of various sizes, sometimes several to a cell, in practically all the infected cells.

Coverslip cultures of human thyroid cells infected with herpes virus were prepared by the methods described in Chapter V. A strain which produced a non-proliferative type of effect was used. At the periphery of infective foci where the cells showed an early stage of infection, basophilic intra-nuclear inclusions were observed varying in size from little larger than the nucleolus to just less than the nucleus itself. In a few of the cells many small basophilic inclusions were seen, but most cells contained a single inclusion body. Near the centre of infective foci where the cells were at a later stage of infection, the inclusions were either densely eosinophilic or pale pink and of a foamy appearance. These inclusions closely resemble those described by Wolman and Behar (1952) in chick embryo cells infected with herpes virus.

## CHAPTER VII

## DISCUSSION

The primary object of the work reported in this section of the thesis was the assessment of human amnion and human thyroid cultures as substitutes for monkey kidney cultures for enterovirus isolation. In making such a comparison it is necessary to consider the preparation of the tissue cultures themselves as well as the virus susceptibilities of the various types of cells. Amnion is the most readily obtainable of the three and is in fact available in almost unlimited amount. It proved as easy to grow as monkey kidney, and its only disadvantages were the losses from contamination and failure to grow. The loss through contamination was 13% of the total number of tubes set up and the loss from failure of the cells to grow was only 3%. Even with these losses an average of 455 tubes of satisfactory cultures was obtained per amnion. This represents a fairly low rate of failure for the culture of a tissue so liable to trauma and infection before it reaches the laboratory. Other workers such as Ferguson and Tobin (1958) have also found human amnion reasonably easy to grow. Human thyroid is a less consistently satisfactory source of tissue cultures than human amnion for a routine virology laboratory. The supply of the tissue is much less certain since it depends on the number of patients with thyroid disease who happen to be treated surgically at the time the tissue cultures are required. Moreover, considerably more detailed care is necessary in growing thyroid than amnion cells. Once thyroid cells have been obtained, however, monolayer formation is rapid and the cultures remain intact for 1 or 2

weeks longer than amnion cultures.

Any tissue culture which is to be used instead of monkey kidney cells for virus isolation from stools must as a first essential be as sensitive in detecting poliovirus. Both amnion and **thyroid were** susceptible to infection by prototype strains of the 3 types of poliovirus but thyroid irregularly showed the phenomenon of viral growth in cells without an accompanying cytopathic effect. Pulvertaft, Davies, Weiss, and Wilkinson (1959) found this phenomenon much more constantly than ⊥ did when the inoculum was small, and they considered that it was essential to have a second type of tissue to which a blind pass could be made if thyroid was to be used for poliovirus isolation. This, of course, would make the use of thyroid cultures for poliovirus isolation quite unpractical. However, I obtained closely similar titres in comparative titrations of poliovirus prototype strains in thyroid and monkey kidney cultures, although with wild strains the titres were lower in both thyroid and amnion than in kidney. The practical test of attempting to isolate 50 strains of poliovirus from stool specimens showed that the amounts of virus usually present in the faeces could be detected equally well by all 3 types of tissue. Like Hovel and Schneider (1960) I was able to make further passes of polioviruses in thyroid cultures and still obtain cytopathic changes. My results showed that either amnion or thyroid could serve adequately for the isolation of poliovirus.

Neither amnion nor thyroid could be considered satisfactory for the isolation of the Coxsackie viruses. Thyroid had a very restricted range of susceptibility limited to types A9 and B6 and some wild

strains of type B5. Amnion failed to show cytopathic changes with types B2 and B4 and detected far fewer strains of type B5 in stool specimens than did kidney. This is a serious shortcoming of both amnion and thyroid since the Coxsackie B viruses cause a very considerable amount of enteroviral disease.

Both amnion and thyroid cultures proved much more suitable for the isolation of ECHO viruses than Coxsackie viruses. Of the first 27 types, only types 4, 10, 14, 16, and 27 were not cytopathogenic in amnion cultures. These same 5 types and in addition types 15 and 26 failed to produce cytopathic changes in thyroid cells. The other types of ECHO viruses gave similar titres in amnion, thyroid, and monkey kidney cultures. Amnion gave a higher titre than the other two with ECHO 20 and both amnion and thyroid gave higher titres than kidney with ECHO 21. Titrations of wild strains of ECHO 6 and ECHO 11 suggested that both amnion and thyroid were more sensitive than monkey kidney cultures for the isolation of these viruses. Both amnion and thyroid cultures would therefore be capable of detecting a wide range of the ECHO viruses. Of the types which are not cytopathogenic for amnion and thyroid cells, ECHO 10 is not a true ECHO virus (Sabin, 1959) and ECHO 15 (Macrae, 1959) and ECHO 26 and 27 (Hammon, Yohn, and Pavia, 1960) have not yet been incriminated as disease producers. Thus amnion and thyroid would fail to isolate only 3 ECHO viruses which are of clinical importance, types 4, 14, and 16. Type 14 has been associated only with sporadic cases of aseptic meningitis (Meyer et al., 1957). Failure to detect types 4 and 16 would be a more serious disadvantage because both these types

may cause epidemics of disease. Type 4 has caused considerable out-
breaks of aseptic meningitis (Lehan et al., 1957; Chin, Beran and
Wenner, 1957) and type 16 has caused outbreaks of both aseptic men-
ingitis and a febrile disease with a rash and ulceration of the
throat which has been called Boston exanthem (Kibrick, Melendez,
and Enders, 1957; Neva, Feemster and Gorbach, 1954).

It is of interest that almost the same range of ECHO viruses
was not cytopathogenic for either amnion or thyroid cells. More-
over these same types of ECHO viruses and a few others have been
shown by Guerin and Kitchen (1961) to be the types which fail to
produce cytopathic changes in a continous line of human kidney
cells. Types 4, 10, 14, 16, and 27 did not produce cytopathic
changes in any of these 3 types of human cells. This kind of dif-
ference in host range of different members of the ECHO virus group
might serve as a means of subdividing and classifying this rapidly
growing group of viruses. ECHO type 10 has already been removed
from the ECHO group (Sabin, 1959) partly on the basis of its very
different host cell range from that of other ECHO viruses (Hsiung,
1958). Possibly types 4, 14, 16, and 27 might form a subgroup with-
in the ECHO group of viruses. Lehmann-Grube (1961b) has made some-
what similar observations concerning the cytopathogenicity of ECHO
viruses for primary human amnion cultures. However, he found that
types 17, 18, 22, and 23 also tended to have variable effects in
amnion cultures, and he included these four with types 4, 14, and
16 as members of a group differing in their cytopathogenic properties
from the other ECHO viruses. However, the whole enterovirus family

show such a variety of common features that it is likely that any

small sub-groupings, within the ECHO group for example, would have

to be made in fairly general terms and with boundaries which were

not too rigid. Hsiung (1961) has reviewed the differences between

the enteroviruses in cytopathogenicity for various primary cell cul-

tures and in their ability to form plaques in monkey kidney cells.

All these characteristics of enteroviruses may be of value in their

fuller classification.

Probably the most interesting point to emerge from the use of

human amnion and thyroid cultures for the isolation of viruses from

stool samples was the isolation of far more untypeable viruses in

the human cells than in monkey kidney cells. Most of these were

found to be strains of the same virus which was called Frater virus

and which is discussed fully later in the thesis. A few others

could not be identified as Frater virus or any of the enteroviruses

to which sera were available, although they caused the same cyto-

pathic effects as known enteroviruses. Only 1 such virus was iso-

lated in monkey kidney cultures but 10 were isolated in thyroid

cultures and 5 in amnion. These results certainly suggest that

primary cultures of human cells are more sensitive than monkey kid-

ney cells for the isolation of some human viruses, probably of the

ECHO group. The designated types of ECHO viruses are types which

are cytopathogenic for monkey kidney cells, mainly because monkey

kidney cultures have been much more widely used than human cells

over the last decade. Probably more and more human strains of entero-

viruses will emerge with the greater use of human cells for virus

isolation.

Apart from the preparation of the cultures and the range of viral susceptibility of the cells, two minor points have to be considered in the assessment of amnion and thyroid cells as substitutes for monkey kidney cells. A substitute should be no more susceptible to the toxic action of stool extracts than monkey kidney cells. Amnion was similar to kidney in its susceptibility, and although young thyroid cultures were considerably more susceptible than monkey kidney cultures, thyroid cultures when used more than 4 days after monolayering were in practice damaged by stool toxicity little oftener than the other types of cultures. The other point of comparison is in the frequency of infection by naturally occurring viruses. This is common with monkey kidney cultures. It was not encountered at all with human amnion and thyroid cultures.

All things considered, human amnion cells are a more satisfactory alternative to monkey kidney cells than human thyroid cells. The supply of amnion tissue is much more constant, its culture requires less detailed care, and it is susceptible to a few more Coxsackie and ECHO viruses than human thyroid. For the laboratory without access to monkeys, human amnion cells offer a satisfactory alternative to monkey kidney cells, and particularly if suckling mice are available in addition to tissue culture, relatively few enteroviruses would fail to be isolated. Moreover even in virus laboratories in which monkey kidney cultures are regularly employed, the additional use of amnion cultures would greatly increase the chance of isolating viruses such as Frater virus which are less

readily detected by monkey kidney cells. Amnion cultures are also most useful in such a laboratory for performing titrations and neutralisation tests on viruses originally isolated in monkey kidney cells. Human amnion is probably therefore the most suitable type of primary cell culture to use instead of monkey kidney cells for isolating enteroviruses. The alternative would be to use a continuous cell line. The MK 1 line of monkey kidney cells of Westwood, Macpherson, and Titmuss (1957) is susceptible to almost all the ECHO viruses and to the Coxsackie B viruses and would appear suitable, but it is very troublesome to grow because the line is contaminated with pleuropneumonia-like organisms (Flewett, 1959). The more recently established HK/55 line of human kidney cells of Guerin and Kitchen (1961) is susceptible to fewer ECHO viruses than primary human amnion cultures and it has yet to be fully tested under routine conditions. At present, human amnion cells would seem to be generally preferable to the available continuous cell lines for the isolation of enteroviruses.

The place which thyroid most usefully fills in the diagnostic virus laboratory is in the isolation of adenoviruses. It is more sensitive than human amnion and monkey kidney cells in detecting adenoviruses, and it is at least as sensitive as HeLa cells. The long survival of thyroid monolayers for 3 weeks or more is ideal for adenovirus isolation. The very uniform monolayers of thyroid cells allow cytopathic effects of virus to be observed at a very early stage. I found thyroid cultures much more satisfactory for adenovirus isolation than HeLa cells because of the need for frequent

medium changes and periodic blind passes when HeLa cells were used.
My isolation of adenovirus type 15 (Duncan, 1960) in thyroid cells
was of some interest as it was the first reported isolation of this
type of adenovirus since the prototype (Murray et al., 1957; Rowe,
Hartley, and Huebner, 1958) and 4 other strains (Bell et al., 1959)
were discovered in Saudi Arabia. Later van der Veen and van der Ploeg
(1960) reported that 3 isolations had been made in Holland between
1957 and 1959. My isolations of adenovirus type 15 were from patients
suffering from diarrhoea. Thyroid cultures are particularly useful
for attempted virus isolation from diarrhoeal faeces as thyroid cells
are susceptible to both ECHO viruses and adenoviruses. Several ECHO
viruses have been associated with diarrhoea (Eichenwald et al., 1958;
Sommerville, 1958), and adenoviruses are being increasingly incrim-
inated as a cause of viral diarrhoea (Kjellen et al., 1957; van der
Veen and van der Ploeg, 1958; Gardner et al., 1960; Duncan and Hut-
chison, 1961).

Amnion and thyroid are both excellent for the isolation of her-
pes virus. My small series suggested that they were somewhat more
sensitive than the chick chorio-allantoic membrane and considerably
more sensitive than the strain of HeLa cells used in the laboratory.
Since amnion and thyroid cells were of equal sensitivity in detecting
herpes virus, amnion would be the more generally useful in routine
work because of the ease with which it may be obtained.

PART 2


ASEPTIC MENINGITIS AND CLINICAL PARALYTIC

POLIOMYELITIS IN SCOTLAND IN 1959

# CHAPTER VIII

## REVIEW OF THE LITERATURE

In the review of the literature of tissue culture in Part 1 of the thesis, the general comment was made that the study of viruses progressed steadily but rather slowly during the first half of this century largely because most of the work depended on the existence of suitable animal hosts. From 1949 onwards the new methods of tissue culture led to an unprecedented rate of advance. As the turning point was the work of Enders, Weller, and Robbins in growing poliovirus in tissue cultures, it is not surprising that the study of poliovirus provides an excellent example of the general progress which has been made.

The literature reporting investigations on poliomyelitis and the polioviruses by tissue culture methods is vast, and no more is intended in this brief review than to outline some of the main ways in which tissue culture methods have contributed to our knowledge of the disease and its prevention, and of the properties of the virus itself. The differentiation of aseptic meningitis caused by other viruses from true non-paralytic poliomyelitis, the evidence that other viruses than poliovirus may cause disease clinically indistinguishable from paralytic poliomyelitis, and the incidence of poliomyelitis and aseptic meningitis over recent years in Scotland, will be discussed more fully because of their particular relevance to the work of this thesis.

### Tissue culture methods in the study of

### poliomyelitis

All the laboratory investigations of poliomyelitis which were carried out before poliovirus was grown in tissue cultures, stemmed directly from two discoveries. First Landsteiner and Popper in 1909 showed that the disease was indeed due to a virus and that the rhesus monkey was a susceptible laboratory animal. A generation later Armstrong (1939) found that cotton rats and mice could be infected with at least some strains of poliovirus. With these rather cumbersome tools virologists had elucidated many of the major epidemiological problems of poliomyelitis before tissue culture methods were available to them. It was shown that poliovirus was present in the faeces as well as in the affected central nervous system (Paul and Trask, 1941). The concept that infection with poliovirus might produce in some people little or no clinical illness had become accepted on the basis of several investigations. Schabel et al., (1950) showed that symptomless contacts of poliomyelitis patients excreted poliovirus in their faeces, and poliovirus was demonstrated in the sewage of communities at times when the clinical disease was entirely absent (Mundel, Gear, and Wilson, 1946). Bodian, Morgan, and Howe (1949) clearly demonstrated the existance of the 3 serological types of poliovirus, and their work was confirmed on a larger scale by an investigation which was probably the last of the major poliomyelitis research projects which depended entirely on the use of great numbers of monkeys (Committee on Typing of the National Foundation for Infantile Paralysis, 1951). The first steps towards poliomyelitis vaccina-

tion were also taken in these last few years before tissue culture methods came into use. Morgan (1948) was able to protect monkeys against infection with the homologous strain of virus by vaccination with formalin-inactivated Lansing strain poliovirus. By 1950 Koprowski (1960) had succeeded in attenuating a type 2 strain of poliovirus by cotton rat passage and had already successfully immunised a human being with his attenuated virus.

These investigations employing animals laid down the lines along which future advances might be made. The discovery by Enders, Weller, and Robbins in 1949 that poliovirus would grow in cultures of tissues other than nerve tissue provided the means of making these advances infinitely more quickly than the older methods could possibly have permitted. Whereas formerly a single rhesus monkey was the minimum biological unit for the detection of poliovirus now one monkey may yield as many as 2,000 tubes of monkey kidney cultures, each one of which would serve to isolate a virus.

Using the new methods of tissue culture, various workers demonstrated that poliovirus spreads much more widely than had been thought among clinically unaffected members of the community. Bodian and Paffenbarger (1954) found that most household contacts of paralytic cases were infected with the virus. Practically every non-immune contact in a household with a case of poliomyelitis is infected (Bhatt, Brooks, and Fox, 1955). The wide spread of the virus in the school population as well as the family was shown by Nolan, Wilmer, and Melnick (1955) in an outbreak in a prosperous community near New York with excellent sanitary conditions. The infection rate with

poliovirus in a household containing an infected child has been

estimated as 92% in non-immune children and 20% in those possessing

antibodies to the strain (Gelfand et al., 1957). Many investigations

thus made it amply clear that a case of poliomyelitis leads to far

more symptomless infections than to clinical cases. The proportion

of symptomless infections to clinical cases has been variously esti-

mated. One large-scale investigation in which laboratories all over

England and Wales participated showed that the ratio for type 1

poliovirus in children under 5 years was 140 symptomless excreters

for each notified case of poliomyelitis (Spicer, 1961). Communities

have also been studied in which, despite the total absence of clini-

cal poliomyelitis over many years, children in the community were

shown to have acquired antibodies to polioviruses (Fallon, 1956;

Macrae, 1957). These findings explained the failure of quarantine

measures in the control of poliomyelitis. Goffe and Parfitt (1955)

in fact demonstrated the spread of type 1 virus through the community

in an outbreak in which quarantine was carefully applied.

Tissue culture methods made it possible to settle the question

of the spread of virus to the central nervous system much more readily

than the older methods of investigation would have allowed. Two

single isolations of poliovirus from the blood of patients were made

in 1946 and 1949 but it was not known whether or not these were chance

findings. Horstmann, McCollum, and Mascola (1954) were able to iso-

late the virus from 6 of 33 patients and to show that 18 of those

from whose blood the virus was not isolated, already had circulating

antibodies to the virus. Bodian and Paffenbarger (1954) demonstrated

a viraemia in 5 of 9 patients without homologous antibody. Blood-
borne spread of the virus from alimentary tract to central nervous
system has been fairly generally accepted as the likely route of
spread. The duration of carriage of poliovirus by those clinically
or sub-clinically infected has been determined. Goffe and Parfitt
(1955) found that 5 of a group of 8 infected people were still car-
rying the virus after 7 weeks and that 1 of the group was still
carrying it at 12 weeks. Probably the average period of carriage is
about 7 weeks as estimated by Gelfand et al., (1957).

Undoubtedly the greatest advance which has been brought about
by the use of tissue culture methods in poliomyelitis research has
been the extremely rapid perfection of poliovirus vaccines and their
utilization on a vast scale throughout the world. Experimental
animals have also played a part in this work. Monkeys were used
for potency and safety testing of formalin-killed vaccine. They
were essential in the work of attenuation of live vaccine. Even in
this work, however, tissue culture methods such as the plaque tech-
nique of Dulbecco and Vogt (1954) were invaluable in producing pure
cultures of the most attenuated strains in mixed populations of polio-
viruses.

Salk made use of tissue culture methods to produce an inactivated
vaccine which, unlike older vaccines such as the formalin treated
monkey spinal cord vaccine of Morgan (1948), could safely be given
to human beings. In 1953 Salk reported the successful vaccination
of human beings with his vaccine. This led to the great controlled
trial of Salk vaccine in the field in 1954, which conclusively proved

its efficacy in preventing poliomyelitis (Francis et al., 1957). A reduction in paralytic poliomyelitis of 70% was shown in the treated group as compared with a control group. It was concluded from the trial that the protection depended almost entirely upon the ability of the vaccine to produce antibodies in children previously lacking them. Further experience has confirmed the protective efficacy of Salk vaccine. Langmuir (1961), discussing the poliomyelitis experience of the United States up to the end of 1959, states that clinical poliomyelitis had almost disappeared in well-vaccinated groups of the population. Fairly severe outbreaks of poliomyelitis occurring in the U.S.A. in 1959 were limited to unvaccinated groups of the population, and Langmuir considers that the failure of Salk vaccine to control the disease as yet in the United States is due more to failure to make full use of the vaccine than to inadequacy of the vaccine itself. In 1959 Canada experienced one of the highest incidence rates of paralytic poliomyelitis ever recorded in the country, although 45% of the population up to the age of 40 years had been fully immunised with Salk vaccine (Kubryk, 1960). Almost all the cases were among the unvaccinated half of the population. In those who had had 3 or more injections of vaccine the protection rate was estimated to be 95%. It seems likely that highly purified and therefore more potent killed vaccine (Hilleman et al., 1961) will give an even better protection rate for fully vaccinated individuals.

Tissue culture methods have played an essential part in the work of producing live attenuated vaccines. Sabin (1955) by repeatedly picking virus from plaques for sub-culture succeeded in seg-

regating from mixed virus populations strains which were avirulent
for monkeys. Koprowski (1957) also used the plaque method to purify
virus attenuated by passage through mice and chick embryo tissue
culture. Although the virulence when injected into the central ner-
vous system of a monkey has been the criterion of attenuation for
virus strains used for vaccines, various tissue culture "marker"
properties of viruses show a correlation with virulence. These in-
clude the "d" marker, the plating efficiency under an acid agar over-
lay (Vogt, Dulbecco, and Wenner, 1957), the "MS" marker depending
on plaque size in a monkey stable cell line (Kanda and Melnick, 1959),
and the "T" marker depending on growth at $40^{\circ}C$ (Lwoff, 1959). The
existence of these marker properties provides a valuable preliminary
means of checking strains. Very wide-scale trials of live attenuated
vaccine have been made. The Sabin strains, which appear to be the
most attenuated, had by the end of 1960 been used to immunise over
70 million people in Russia and 13 million in other countries, in
campaigns in which all the members of a community were simultaneously
immunised (Chumakov et al., 1961). In these huge trials there was
no evidence of reversion of the vaccine strains to greater virulence.
Levels of antibodies were produced as high as those obtained with
Salk vaccine, and in addition some local intestinal immunity devel-
oped. Since interference was encountered from other wild entero-
viruses at other times, winter appeared to be the optimal season for
vaccination programmes. The vaccination of the community prevented
the normal increase in poliomyelitis the following season in the
areas in which it had been used. Finally, mass immunisation of the

whole community brought to an abrupt cessation a severe epidemic

of type 1 poliomyelitis in Tashkent in 1959. The production by

tissue culture methods of the live vaccine used in these Russian

trials has been described by Chumakov (1960). Sabin-type live vac-

cine has been tested on a small scale in England (Public Health

Laboratory Service, 1961). The report on this investigation puts

forward the conclusions that 3 doses of trivalent vaccine should be

given, that this scheme produces good levels of circulating antibody

and also some definite intestinal immunity, and that live vaccine

offers advantages over killed vaccine in providing immunity to polio-

myelitis not only in the individual but also in the herd.

As recently as 1953 the best that could be offered for the

prophylaxis of poliomyelitis was the transitory protection conferred

by an injection of pooled human gamma-globulin (Lancet, 1953). It

is a striking commentary on what has been achieved in the course of

a few years, largely through the availability of tissue culture

methods, that the poliomyelitis problems discussed in leading articles

in today's medical journals are not those of the proper use of the

limited supplies of gamma-globulin available but those of the choice

between two highly effective alternative methods of active immunisa-

tion (Lancet, 1961; Brit. med J., 1961).

Tissue culture methods have also helped to elucidate the physical

and biological properties of the poliovirus itself. Sabin, Hennessen,

and Warren (1954) measured the 3 types of polioviruses by ultrafil-

tration and electron microscopy, using seed grown in tissue cultures.

By gradocol membrane filtration the size was estimated at about 12

to 20 mμ and by electron microscopy at about 30 mμ. Benyesh et al.,
(1958) using a method which employed ionizing radiations made esti-
mates of just under 30 mμ. With a new negative staining technique
Horne and Nagington (1959) demonstrated the detailed subunit structure
of the virus and showed that the live virus measured 30 mμ in dia-
meter. The existence of the 3 serological types of poliovirus had
been determined before tissue culture methods were widely used in
poliomyelitis investigations. The more refined methods of tissue
culture have been employed in further investigations which revealed
that there are serological subdivisions within types (McBride, 1959).
It has been calculated from the results of experiments with tissue
cultures infected with polioviruses that a single particle of polio-
virus can initiate an infection (Dulbecco and Vogt, 1954). Alexander
et al., (1958) have shown that HeLa cells can be infected with a
preparation of poliovirus ribonucleic acid which is entirely free
from intact virus particles. Such investigations as these have been
greatly facilitated by tissue culture methods which have made avail-
able large quantities of relatively pure virus for study.

### The aetiology of aseptic meningitis

It has long been recognised clinically that during poliomyelitis
outbreaks patients are seen with all the general and meningeal signs
and symptoms of poliomyelitis except that paralysis is absent. This
disease has been referred to as non-paralytic poliomyelitis on the
assumption that all such cases are caused by polioviruses. In times
of epidemic prevalence of poliomyelitis this assumption may be
largely true. For example, during the severe epidemic due to type 1

poliovirus in Stockholm in 1953 poliovirus was isolated in tissue culture from 121 of 213 patients with non-paralytic poliomyelitis. In the age group under 16 the isolation rate was 82% (Svedmyr, Melen, and Kjellen, 1956). However as these workers also demonstrated in their investigations of the disease in Stockholm the following year, poliovirus may be isolated very rarely from such cases when paralytic poliomyelitis is not prevalent. It is now clear that the disease is not in fact a single disease entity caused by the poliovirus but a syndrome which may be due to any one of many viruses. For this reason it is now generally referred to as aseptic meningitis. This seems a particularly appropriate name because the term "méningite aseptique aiguë" was used as early as 1925 by Wallgren to describe the disease which on epidemiological grounds he considered to be a separate entity unconnected with poliomyelitis. The poliovirus, indeed, is causing fewer cases of the disease in some countries than it did in former years as a result of widespread poliomyelitis immunisation. In Detroit during the 1958 poliomyelitis outbreak (Brown, Lenz, and Agate, 1960) and in California in the years from 1955 to 1957 (Lennette et al., 1959) a smaller proportion of cases of aseptic meningitis was found to be due to poliovirus among those who had been immunised against poliomyelitis than among those who had not.

The technique of virus isolation by suckling mouse inoculation was in general use before modern tissue culture methods. As a result, the Coxsackie viruses were incriminated as causes of aseptic meningitis before the ECHO group. Curnen, Shaw and Melnick (1949) isolated

Coxsackie viruses from the faeces of patients with aseptic meningitis
in Connecticut in 1948, and demonstrated antibodies to the virus in
the convalescent sera of these and other patients.  Rhodes and Beale
(1957) found 18 cases due to Coxsackie B viruses among 96 cases of
aseptic meningitis studied in Toronto between 1950 and 1955.  As
well as isolating the viruses from the patients' stools, these work-
ers isolated virus from the cerebro-spinal fluid of 5 of the patients.
This group of workers has also shown that monkey kidney tissue cul-
tures are as sensitive as suckling mice for the isolation of Cox-
sackie B viruses (Beale et al., 1956).  Coxsackie viruses of types
B1, 2, 3, 4, and 5 have now all been found to cause aseptic mening-
itis.  Many of the earlier reports of aseptic meningitis associated
with Coxsackie B viruses recorded only sporadic cases, but outbreaks
have been recorded due to several of them including B2 (Marchessault
et al., 1961) and B5 (Syverton et al., 1957).  Group A Coxsackie
viruses have also been shown to cause aseptic meningitis, Gear, Meas-
roch, and Prinsloo (1956) isolated group A Coxsackie viruses from
the faeces of 5 cases occurring in Johannesburg in 1953 and 1954 and
from the cerebro-spinal fluid of 3.  Melnick (1957) reported 9 cases
due to type A9 in Connecticut in 1955 and 1956, and 5 cases due to
this virus in Washington in 1952 have been recorded (Habel, Silver-
berg, and Shelokov, 1957).  Single cases due to type A7 and A9 have
been recorded by Kilbourne and Goldfield (1956).  Johnsson and Lund-
mark (1957) isolated Coxsackie virus type A7 from 20 cases in Stock-
holm in 1954.  In assessing the relative frequency of group B and
group A Coxsackie viruses as causes of aseptic meningitis, it must

be taken into account that monkey kidney tissue cultures are employed

much more regularly than suckling mice by many laboratories and this

may artificially inflate the number of group B isolations as com-

pared with group A. When monkey testis and later monkey kidney cul-

tures came into use for the investigation of poliomyelitis and aseptic

meningitis, cytopathogenic viruses which were neither polioviruses

nor Coxsackie viruses were isolated in increasing numbers. Melnick

and Agren (1952) isolated one of the earliest of these strains from

a healthy Egyptian child. Other strains were isolated from healthy

children in Cincinnati (Ramos-Alvarez and Sabin, 1954) and the Phil-

lipines (Ludwig and Hammon, 1956), as well as from some patients with

aseptic meningitis (Melnick, 1955). These agents were named ECHO

viruses and were at first regarded as non-pathogens. It was ori-

ginally planned that any ECHO type would be removed from the ECHO

group if it were later shown to be the cause of a definite disease

(Committee on the ECHO Viruses, 1955). It soon became clear however,

that ECHO viruses did cause disease and when in May 1956 a conference

was held in New York on "Viruses in Search of Disease", there was

ample evidence to associate ECHO viruses with aseptic meningitis

(Ann. N.Y. Acad. Sci., 1957).

In 1959 Macrae in a review article on the enteroviruses stated

that ECHO viruses of types 2, 3, 4, 5, 6, 7, 9, 12, 14, 16, and 21

had all been associated with aseptic meningitis. Others continue to

be added to this list, such as types 18 and 19 which have each been

isolated from the cerebro-spinal fluid of a patient with the disease

(Eckert, Barron, and Karzon, 1960; Faulkner and Ozere, 1960). Only

a few of the ECHO viruses have caused more than sporadic cases of
aseptic meningitis. The type associated with the largest outbreaks
has been type 9 which has caused epidemics on a world-wide scale.
Sporadic cases due to this virus had been encountered in Sweden as
early as 1952 - 1954 (Svedmyr, 1957), and the first outbreak asso-
ciated with the characteristic rash was observed in the east end of
London in 1954 (Crawford, Macrae, and O'Reilly, 1956). In 1955 an
outbreak occurred in East Grinstead (Boissard et al., 1957; Garnett,
Burlingham, and van Zwannenberg, 1957). The following year many
outbreaks were reported from Britain and from European countries.
A full review of these reports is given in a paper by Lepow, Carver,
and Robbins (1960) who list 11 papers from Britain, 2 from Bel-
gium, 2 from Holland, 4 from Sweden, 2 from Denmark, 4 from Germany
and 3 from Switzerland, which describe the outbreaks which occurred
at that time. In 1956 the disease was also prevalent in Canada
(LaForest et al., 1957; Sultanian and Rhodes, 1958; MacLeod, Faulk-
ner, and van Rooyen, 1958). In 1957 it appeared in the United States.
Many parts of the country were involved and considerable outbreaks
occurred in Milwaukee (Sabin, Krumbiegel, and Wigand, 1958; Lepow,
Carver, and Robbins, 1960) and Minnesota (Prince, St. Geme, and
Scherer, 1958). Sporadic cases were also reported from Massachusetts
(Kibrick and Enders, 1958). Properly, these epidemics should not
be said to have been due to ECHO virus type 9 but to Coxsackie virus
type A23 since strains of this immunological type which are patho-
genic for suckling mice have been reclassified in this way (Sickles,
Mutterer, and Plager, 1959). However the virus is much more gener-

ally known as ECHO type 9 despite the new classification.

ECHO types 4 and 6 have also caused a number of outbreaks, though fewer than type 9. The first reported outbreak due to type 4 took place in Iowa in 1955 (Lehan et al., 1957; Chin, Beran, and Wenner, 1957). The following year there was a large outbreak in Buffalo, New York State (Karzon et al., 1961). These workers succeeded in isolating the virus from the cerebro-spinal fluid of 32% of the patients affected. An outbreak occurred in Australia in the winter of 1956 - 1957 (Forbes, 1958; Ferris and Lewis, 1958). In South Africa 2 large outbreaks have been reported among children living in institutions (Malherbe, Harwin, and Smith, 1957; Wilsen et al., 1961; Howarth et al., 1961). In each of these epidemics half the inmates at risk developed the disease. Small numbers of cases have also been reported in Switzerland (Buser, Krech, and Moeschlin, 1957) and Sweden (Johnsson, 1957).

Habel, Silverberg, and Shelokov (1957) found 21 cases due to type 6 virus among 125 cases of aseptic meningitis in Washington in 1952. In 1954 Svedmyr (1957) isolated type 6 virus from 34 cases in Stockholm, and 40 cases were encountered in Massachusetts (Kibrick, Melendez, and Enders, 1957). Davis and Melnick (1956) reported a small outbreak in Connecticut in 1955, and Meyer et al., (1957) reported cases that year in army personnel in various parts of the United States. In the same year the largest outbreak so far reported due to type 6 took place in western New York State (Winkelstein et al., 1957). The virus was isolated from 77 cases and 11 isolations were made from specimens of cerebro-spinal fluid.

Type 16 was associated with a small outbreak in Massachusetts

in 1951 (Kibrick, Melendez, and Enders, 1957).

Apart from the enteroviruses, mumps virus is one of the commonest causes of aseptic meningitis. When mumps is prevalent in a community a considerable proportion of the cases of aseptic meningitis are caused by mumps virus. McLean et al., (1961) found that in 46 of 104 children admitted to hospital with aseptic meningitis in Toronto in 1960 there was virological evidence of mumps. The virus was isolated from the cerebro-spinal fluid of 23 of the patients. This coincided with an unusually high reported incidence of mumps in Toronto in that year. Stern (1961) found mumps to be the cause of about 10% of cases in London in 1959 and 1960. Meyer et al., (1960) and Lennette et al., (1959) both conducted large-scale investigations of viral diseases of the central nervous system occurring over several years in large geographical areas, and found that mumps played a significant part in the aetiology of aseptic meningitis. These workers also showed that a small proportion of cases were caused by herpes virus. In some areas cases are also caused by the lymphocytic choriomeningitis virus (Meyer et al., 1960).

It is evident that many viruses can cause aseptic meningitis and that clinical examination cannot differentiate between cases due to different viruses. The virological diagnosis of the disease is itself complex, involving not only the inoculation of tissue cultures and suckling mice but also serological tests based on neutralisation and complement fixation methods.

## Clinical paralytic poliomyelitis caused by

## viruses other than poliovirus

During the last few years evidence has been gradually accumulating that a few cases of disease clinically typical of paralytic poliomyelitis may be caused by other viruses than poliovirus. At first most workers tended to discount this possibility and only a few, particularly Steigman in the United States, considered it an acceptable concept. Recently more attention has been paid to the matter because of its obvious importance in the accurate assessment of the efficacy of poliomyelitis vaccines, and because the decrease in paralytic poliomyelitis as a result of immunisation has thrown cases due to other viruses into greater prominence. A closely analogous epidemiological situation has recently occurred with pulmonary tuberculosis. As the typical disease caused by the tubercle bacillus has become less common due to chemotherapy, clinically similar cases caused by chromogenic mycobacteria have been recognised for the first time. In each case when the major disease was common the rarer cases due to other agents were entirely overlooked.

The great difficulty in assessing the part played by Coxsackie and ECHO viruses in causing paralytic disease, arises because these viruses are so often found in the stools of healthy people. Honig et al., (1956) isolated Coxsackie viruses from about 2% and ECHO viruses from a further 2% of a large group of healthy children under school age in Virginia in a survey lasting 29 months. In times of particular prevalence of these viruses in an area the percentages would be much higher. Double infections may occur. Lennette et al.,

(1959) in a very large investigation in California found that in patients excreting poliovirus in the stool there was evidence of coincident infection with another virus in 2% of cases. It is therefore important in assessing a case not only to isolate another virus but also to try to prove serologically that poliovirus infection was absent. Davis and Melnick (1956) were able to prove by demonstrating a rise in poliovirus antibodies that patients with paralytic disease from whose stools Coxsackie A9 and ECHO 6 viruses had been isolated were in fact cases of poliomyelitis. In general the isolation of an ECHO or Coxsackie virus from the cerobro-spinal fluid in life or from the central nervous system after death is fairly suggestive evidence that the virus caused the disease, but even in such a case there may be a double infection though this is probably very rare. Verlinde, Wilterdink, and Mouton (1960) described a fatal case in an infant in which ECHO 9 virus alone was isolated from the stool, and both ECHO 9 and poliovirus type 2 from the central nervous system. The poliovirus was isolated only with very considerable difficulty. Even when another virus is isolated from the cerebro-spinal fluid of a patient, therefore, it would be necessary to show that there had not been a rise in antibodies to poliovirus.

One of the earlier viruses, other than the 3 known types of poliovirus, to be isolated from cases of clinical poliomyelitis was at first thought to be a fourth type of poliovirus. This virus, which was referred to as AB IV virus, was isolated from 3 pooled specimens from 6 paralysed children in Karaganda, U.S.S.R. (Chumakov et al., 1956). The Russian workers sent the virus to other virolo-

gists for further examination and it was found to be a strain of Coxsackie A7 virus (Johnsson and Lundmark, 1957; Habel and Loomis, 1957). Habel and Loomis also found that the virus produced the same pathological picture in monkeys as polioviruses. Seven further isolations of Coxsackie A7 virus have since been made by the Russian workers from paralytic cases in another outbreak in Karaganda (Voroshilova and Chumokov, 1959). Ranzenhofer et al., (1958) reported a case in a three year old boy in Kentucky. They isolated Coxsackie A7 virus and showed a rise in level of antibodies to this virus in the absence of a rise in poliovirus antibodies. The cases encountered by Grist (1960, 1961) in Scotland will be discussed later. A single case has been reported associated with Coxsackie type A9 in which the virus was isolated and in which there was no rise in poliovirus antibodies (Hammon et al., 1958).

Several members of the Coxsackie B group have been associated with clinical paralytic poliomyelitis. In a few cases a Coxsackie B virus was isolated from the stools, a rise in antibody was demonstrated to the homologous virus, and the poliovirus antibody levels were shown to have remained unchanged during the course of the illness. Steigman (1958) reported one such case associated with Coxsackie B5 and Stern (1961) reported another two due to this same virus. Johnson, Shuey, and Buescher (1960) found Coxsackie B2 virus causing two such cases and they also succeeded in isolating the virus from the cerebro-spinal fluid. Hammon et al., (1958) reported two such cases, one due to Coxsackie B3 and the other to B4 virus. On serological grounds alone Meyer et al., (1960) found a case showing

a rise in antibodies to Coxsackie B4 but no rise in poliovirus anti-
bodies. These cases seem very likely to have been caused by the
Coxsackie viruses. Other workers have reported cases of paralytic
disease in which the Coxsackie virus was isolated from the stool but
in which no serological studies were done. In these cases the signi-
ficance of the isolation remains in doubt. They include 2 cases in
which type B2 was isolated (Stern, 1961), 4 involving type B3 (Drouhet,
1960), 3 involving type B5 (Drouhet, 1960; Brown, Lenz, and Agate,
1960), and 21 cases mentioned by Lennette et al., (1959) without
details of the types of Coxsackie B viruses involved.

The first suggestion that ECHO viruses might also produce para-
lytic disease was a report of the isolation of an untyped virus from
the spinal cord of a child who had apparently died of bulbo-spinal
poliomyelitis (Steigman, Kokko, and Silverberg, 1953). This virus
was later shown to be ECHO type 2 (Steigman, 1958). Poliovirus
could not be isolated in this case and no poliovirus antigen could
be detected in the cord but serological studies were not reported.
Steigman and Lipton (1960) reported a case of typical bulbo-spinal
paralysis in a 2 year old child. ECHO virus type 11 was isolated
from the faeces and a rise in antibodies to the virus demonstrated.
Poliovirus antibodies had not developed 6 weeks after the illness.
Buser, Krech, and Moeschlin (1957) isolated ECHO type 4 virus from
the faeces of a paralysed adult, who did not develop poliovirus
antibodies. Svedmyr (1957) isolated ECHO type 6 virus from one para-
lytic case and type 9 virus from another. In neither were poliovirus
complement-fixing antibodies detectable. Two cases due to ECHO type

4 and one due to ECHO type 16 were reported by Hammon et al., (1958).
In these cases the virus was isolated from the stool, a rise in
homologous antibody demonstrated, and no clear serological evidence
of poliovirus infection found. In these cases the assumption that
an ECHO virus caused the disease rests on fairly good evidence.
The other reports associating ECHO viruses with paralytic disease
are less definite either because the paresis was only slight or be-
cause the aetiological evidence was not fully conclusive. In an
outbreak of aseptic meningitis due to ECHO type 6 virus in Massa-
chusetts in 1951, Kibrick, Melendez, and Enders (1957) found evi-
dence of muscle weakness in 22 of 37 cases. Karzon, Barron, and
Winkelstein, (1956) also observed transient muscle weakness in cases
of aseptic meningitis due to ECHO 6 virus. Various workers includ-
ing Sabin, Krumbiegel, and Wigand (1958) have reported cases of
paralysis apparently due to ECHO type 9 virus during outbreaks of
aseptic meningitis due to this virus. Karzon et al., (1961) observed
8 cases with some muscle weakness among 82 cases of aseptic meningi-
tis due to ECHO type 4 virus in Buffalo, N.Y. in 1956. Hammon et al.,
(1961) have reported a single case with mild paralysis who was found
to have serological evidence of infection with ECHO type 13 virus.
By isolation of the virus unsupported by serological evidence, single
cases of paralytic disease have been associated with ECHO type 1
(Meyer et al., 1960), and ECHO type 12 (Drouhet, 1960). Lennette
et al., (1959) reported the isolation of ECHO viruses from 16 para-
lytic cases but the types were not specified.

## Aseptic meningitis and paralytic poliomyelitis in

## Scotland in recent years

Laboratory investigations of poliomyelitis in the West of Scotland were begun in 1955 and in addition to that year's specimens a number of specimens stored since 1953 and 1954 were then examined (MacGregor, Larminie, and Grist, 1956). Information on the probable prevalence of polioviruses in Scotland before this is available from surveys of poliovirus antibodies in children of various social groups in Glasgow, Dundee, Kilmarnock, and Stirlingshire in 1955 and 1956 (MacLeod et al., 1958). In general, antibodies appeared at a relatively early age, many children having acquired antibody to all 3 types before school age. This was observed to be in keeping with the age incidence of clinical poliomyelitis in Central Scotland; 60% of cases under 5 years and 75% under 10 years. Type 1 antibodies were acquired slightly sooner than other types but the differences were not great and all 3 types of virus seemed to have been fairly prevalent in the community. There were no age groups at which antibodies were present in a particularly high proportion of the children. This might have been expected if polioviruses had been very common in the community only in years of high incidence of paralytic disease as, for example, 1947, 1950, 1955. That this was not so suggests that polioviruses had also been widely prevalent in the community in non-epidemic years. Children from small towns and villages developed antibodies at a somewhat later age than those in the large towns. These findings contrast considerably with those of Macrae (1957) who carried out a survey in 1951 in the village of Torridon

in Wester Ross. This little village has never had a recorded case of paralytic poliomyelitis. The Torridon children were much later in acquiring antibodies. By 11 years 4 of a group of 15 had antibodies to only 1 type of poliovirus and the other 11 had no antibodies whatever. Older members of this community were shown to have antibodies to all 3 types of polioviruses.

In the Glasgow area the few isolations of poliovirus from patients with paralytic poliomyelitis in 1953 and 1954 were all of type 1 (MacGregor, Larminie, and Grist, 1956). Type 1 virus was isolated from 4 paralytic cases in 1955 and type 2 from another 2. In 1956 the main causes of paralytic poliomyelitis in Scotland were type 2 and type 3 polioviruses and type 1 played a much smaller part (MacGregor et al., 1958). Of particular interest in 1956 was the isolation of Coxsackie A7 virus from 1 paralytic case (Grist et al., 1960). Like other parts of Britain, Scotland experienced the epidemic of aseptic meningitis due to ECHO type 9 virus which was widespread all over Europe in 1956. ECHO type 9 virus was the main cause of aseptic meningitis that year in both the Glasgow and Dundee areas (Grist et al., 1960; Jamieson, Kerr, and Sommerville, 1958). In 1957 type 1 poliovirus was the predominant cause of paralytic poliomyelitis with type 3 as the next commonest. A number of viruses were isolated from individual cases of aseptic meningitis that year and there was a small outbreak caused by Coxsackie B3 virus. During the two years 1956 and 1957 when a moderate number of cases of paralytic poliomyelitis were seen, 16% of the cases of aseptic meningitis were due to polioviruses. In 1958 poliovirus type 1 completely overshadowed

all other viruses as the cause both of paralytic poliomyelitis and aseptic meningitis (Sommerville and Carson, 1958; Duncan and Sommerville, 1960). Type 1 virus was isolated from 117 paralytic cases and from 71 cases of aseptic meningitis, and type 2 virus from only 2 cases of aseptic meningitis. A poliovirus was isolated from 35% of the cases of aseptic meningitis.

The investigation of aseptic meningitis and poliomyelitis in Scotland in 1959 by tissue culture methods is reported in this thesis. By the inoculation of suckling mice Grist (1960, 1961) showed that Coxsackie A7 virus, which in its wild state is not cyto-pathogenic for tissue cultures at present in use, played an extremely important part in the aetiology of these diseases. This virus was isolated from 7 of the 13 cases of clinical paralytic poliomyelitis seen during the year, and from 29 cases of aseptic meningitis. In 9 of the 13 paralytic cases serological findings were against a diagnosis of poliomyelitis, and in 3 of those from whom Coxsackie A7 virus was isolated poliovirus antibodies were entirely absent from the convalescent sera. The virus was found in only 1 of 60 control patients examined when the outbreak was at its peak. The evidence that the virus was the cause of these paralytic cases and of the cases of aseptic meningitis was excellent. A number of cases of aseptic meningitis in 1959 were shown by serological means to have been caused by mumps, leptospirosis, and herpes (Combined Scottish Study, 1961).

In 1960 polioviruses were practically absent from the community (Grist, 1961) and considerable outbreaks of aseptic meningitis were

seen in both west and east Scotland due to ECHO virus type 9

(Landsman et al., 1960; Constable and Howitt, 1961).

## CHAPTER IX

## MATERIALS AND METHODS

Full details have already been given in Chapter II of most of the materials used for preparing tissue cultures and of the methods employed for the isolation of viruses. These same methods were used in Part 2 of the thesis, and the present chapter deals with the clinical specimens examined in this part of the work and the patients from whom they were obtained.

### Patients from whom specimens were obtained

Specimens were received for virological examination from three groups of patients; those suffering from clinical paralytic poliomyelitis, those with aseptic meningitis, and a mixed group of patients suffering from various other conditions. These were mainly non-infectious neurological conditions, non-specific febrile illnesses, diarrhoea, and respiratory diseases. Stool specimens from the third group served the useful purpose of providing an index of the incidence of enteroviruses in those not suffering from aseptic meningitis.

During 1959 specimens were received from 14 patients with clinical paralytic poliomyelitis, 227 with aseptic meningitis, and 443 other patients. Many more of the patients suffered from aseptic meningitis later in the year than earlier, 53 between January and June and 165 between July and December. Cases were considered as aseptic meningitis when signs of meningitis were present, when the cerebro-spinal fluid was abnormal, and when there was no evidence of a bacterial meningitis.

The patients were all under treatment in hospitals in the West of Scotland, Central Scotland, or the Dundee district. The patients with aseptic meningitis were in hospitals in the following areas:- Glasgow (112), Motherwell (51), Dundee (37), Greenock (13), Paisley (6), Falkirk (4), Ayrshire (2), Stirling (1), and Fife (1). Patients with paralysis were in hospitals in Glasgow (10), Dundee (2), Motherwell (1), and Ayrshire (1). The miscellaneous group of patients were similarly distributed amongst hospitals in these same areas.

### Types of clinical specimens

A specimen of faeces and two serum specimens were submitted from most of the patients with clinical paralytic poliomyelitis and aseptic meningitis. The current practice among the physicians in charge of these cases was to send only faecal and serum specimens and practically no specimens of cerebro-spinal fluid were received. The faecal specimens were taken as soon as possible after admission. The first serum specimen was taken at this time and the second 10 to 14 days later during convalescence.

Faecal specimens were obtained from 218 cases of aseptic meningitis. Specimens of serum from 81 of these cases were available to me for serological examinations in connection with the work of this thesis. Sera were submitted from a number of the other cases but were required for serological tests unconnected with my work. A further 9 sera were examined by me from cases of aseptic meningitis from whom no faecal samples were sent. Faecal specimens were obtained from all of the 14 patients with clinical paralytic poliomyelitis and sera were available from 4 of them. A sample of faeces was

obtained from each of the 443 patients who did not suffer from clini-
cal paralytic poliomyelitis or aseptic meningitis.

### Serum specimens

In addition to the sera from the patients mentioned in the
previous paragraph, serum samples were examined from a variety of
other people in order to determine the prevalence of antibodies to
Frater virus.

Sera were obtained from blood donors in the Glasgow and Lanark-
shire areas both before and after the 1959 outbreak of infection
with Frater virus. The earlier collection from 117 donors was orig-
inally taken for another purpose in the Autumn of 1958. The later
collection from 100 donors was obtained in the early months of 1960.
The donor groups included both males and females ranging in age from
20 to 60 years.

Paired acute and convalescent sera from 36 patients who suffered
from aseptic meningitis in 1958 and from 19 patients with clinical
paralytic poliomyelitis in 1958 were examined for antibodies to
Frater virus. These sera had been kept stored at $-20^{\circ}C$. All the
patients were from the West of Scotland.

Sera were obtained in 1960, 1 year after their illnesses, from
10 patients who developed antibodies to Frater virus at the time of
illness and from whom Frater virus was isolated at the time. All
these patients had been well during the year since their recovery
from the Frater virus infection.

### Gamma-globulin specimens

Samples of human gamma-globulin prepared for prophylactic and

therapeutic use from the blood of Scottish donors were examined for antibodies. These were prepared by the Edinburgh and South-East Scotland Blood Transfusion Service. Eight samples were obtained, each prepared from blood withdrawn from donors in a different year. The blood from which the different batches were prepared was withdrawn respectively in 1951, 1952, 1953, 1954, 1956, 1957, 1958, and 1959. All the donors were resident in the Edinburgh area, the Lothians, the Borders or Fife except that some of the blood used to prepare the 1956 and 1958 samples came from donors in the Forces, some of whom may have served abroad.

## Antibody titrations

All sera were heated for 30 minutes at 56°C before being tested. Dilutions of serum and seed virus were made in PBS. Screening tests for antibodies to Frater virus were performed by mixing a 1:4 dilution of the serum with an equal volume of Frater virus diluted in PBS to give 100 $TCD_{50}$ per 0.1 ml. of virus dilution. The mixture was left at room temperature for 1 hour after which 0.2 ml. of the mixture was inoculated into each of 2 tubes of human amnion tissue cultures. The tubes were examined for cytopathic effects the day after control tubes containing 100 $TCD_{50}$ doses of virus without serum showed complete degeneration. If neutralisation occurred with a serum in this screening test, two-fold dilutions of the serum were made and the neutralising titre of the serum determined in a similar way. All titres were expressed as the dilution of serum in the serum-virus mixture.

Antibody levels in gamma-globulin samples were similarly determ-

ined.  Amnion cultures were also used for poliovirus and ECHO 9

titrations.  Two-fold dilutions of the samples were employed.

## CHAPTER X

## CASES ASSOCIATED WITH KNOWN ENTEROVIRUSES

The cases of aseptic meningitis and clinical paralytic polio-
myelitis referred to in this chapter are a rather highly selected
group. They were all patients who were treated in hospitals in the
area served by the University Virus Laboratory at Ruchill Hospital
and from whom at least one specimen was taken for virological in-
vestigation. The geographical area concerned is the part of Scot-
land south of a line roughly drawn from Fort William to Montrose
but excluding Fife, the Lothians and the Border counties. Patients
who were not admitted to hospital and patients in hospital from whom
no specimen was taken are not included. The series does not there-
fore include all the cases in the area but because of its size is
probably fairly representative of the diseases as they occurred in
Scotland in 1959.

The aspect of the virological investigation of these cases
which is reported in this thesis is limited to virus isolation in
tissue cultures and the detection of neutralising antibodies by
tissue culture methods; work which I myself carried out. The isola-
tion of viruses by the inoculation of suckling mice and the detec-
tion of complement-fixing antibodies to a variety of viruses were
done by my colleagues in the laboratory as the other parts of the
full virological investigation of the cases. The results of these
tests are not presented in this thesis because they are the work of
others but reference will be made in the discussion in Chapter XIII
to those of them which have been published.

My isolations of viruses in tissue culture showed that one virus, Frater virus, caused most of the cases of aseptic meningitis and that a small number of cases were associated with other agents. For convenience, the present chapter will deal with all the other viruses, and the following chapter will be devoted entirely to the outbreak caused by Frater virus.

### Aseptic meningitis

A virus cytopathogenic for tissue culture was isolated from 82 of the 227 patients from whom specimens were obtained. All these viruses were isolated from stool specimens. The types of viruses isolated are shown in Table 19.

### TABLE 19

### Viruses isolated from 227 patients with aseptic

### meningitis in 1959

| Virus Type | Coxsackie | | | ECHO | | | | | Adeno-virus | Frater | Untyped |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A9 | B2 | B5 | 5 | 6 | 11 | 14 | 15 | 1 | | |
| Number isolated | 1 | 4 | 7 | 2 | 2 | 1 | 1 | 1 | 1 | 61 | 1 |

For comparison, the viruses isolated from 443 other patients who were treated during 1959 in the same hospitals as the patients with aseptic meningitis are shown in Table 20. These results give some indication of the general incidence of infection by these viruses in patients without aseptic meningitis and thus help in assessing the significance of the isolation of a virus from a patient with aseptic meningitis.

TABLE 20

Viruses isolated from 443 control patients in 1959

| Virus | Poliovirus | Coxsackie | | ECHO | | | | Adenovirus | Frater |
|---|---|---|---|---|---|---|---|---|---|
| Type | 3 | Ax | B5 | 6 | 7 | 11 | 14 | | |
| Number isolated | 2 | 1 | 5 | 2 | 3 | 5 | 3 | 15 | 12 |

x    not types A7 or A9

It is clear from both Tables 19 and 20 that polioviruses were
almost absent from the community in 1959. The two type 3 strains
were isolated from infants in Glasgow in August and October.  One
had gastro-enteritis and the other had croup.  Apart from Frater
virus, which will be considered in Chapter XI, only Coxsackie B2
and B5 viruses were associated with anything more than purely spor-
adic cases of aseptic meningitis.  The Coxsackie B2 infections all
occurred in May and June.  One was in Dundee and 3 were in Glasgow.
The 7 cases associated with Coxsackie B5 occurred in July, September,
and October.  Two were in Lanarkshire, one in Dundee, and the rest
in Glasgow.

The scanty isolations of similar types of ECHO viruses from the
control group make it very difficult to assess the significance of
the finding of the ECHO viruses in the faeces of some of the patients
with aseptic meningitis.  Some may have been the aetiological agents
and some simply coincidental carried strains.  Dr. N.R. Grist isolated
Coxsackie A7 virus from the same specimen as that from which I ob-

tained adenovirus type 1. The Coxsackie virus appears much the more likely cause of this patient's aseptic meningitis.

## Clinical paralytic poliomyelitis

Among the 14 patients with a disease which clinically appeared to be paralytic poliomyelitis, there were only 3 from whom a virus was isolated in tissue culture. Case 1 was that of a 29 year old man who in August developed paralysis of one leg of sufficient severity to require orthopaedic treatment. Poliovirus type 1 was isolated from the patient's faeces, so that this case both clinically and virologically would seem to have been a true case of paralytic poliomyelitis. In the other cases polioviruses were not isolated but other enteroviruses were. These two cases are therefore described in detail.

Case 2: This 9 month old male infant was admitted to hospital on 14 October, 1959 with a history of restlessness for 2 days and failure to use the left arm for 1 day. At the time of admission the left arm was weak and flaccid but not completely paralysed. The grip in the left hand was much weaker than that in the right. No other muscles were observed to be weak and neck rigidity was absent. Lumbar puncture, performed on admission, yielded cerebro-spinal fluid at normal pressure and containing 21 lymphocytes per cubic mm. The protein in the fluid was 15 mgm.% and the sugar 64 mgm.%. The day after admission the left deltoid muscle was still weak and by the day after that no muscle weakness was detectable. Coxsackie virus type B2 was isolated from the infant's stool on the second day of illness. Serum specimens were obtained on the second day and ten

days later. The results of titrations of antibodies in these sera are given in Table 21.

Case 3: This patient was a 14 month old boy who developed stiffness of the neck and left arm on 28 July 1959. On admission to hospital the following day there was obvious paralysis of the left arm and forearm but not of the hand. The cerebro-spinal fluid on admission contained 61 cells per cub.mm., 60% lymphocytes and 40% polymorphs. The protein level was 30 mgm.%, the sugar 68 mgm.%, and the chloride 760 mgm.%. On the day after admission the left hand was also paralysed. Three weeks after the onset of illness there was recovery of hand and wrist movements but the elbow and shoulder muscles remained paralysed and there was definite deltoid wasting. One year after the illness orthopaedic treatment had produced good function of the elbow muscles but complete deltoid paralysis remained. This patient had received 2 doses of killed poliomyelitis vaccine in infancy. Frater virus was isolated from a single stool specimen submitted on the 10th day of illness and the antibody levels in the only two serum specimens, obtained on the 16th and 29th days of illness, are given in Table 21.

In addition to these patients with diseases which clinically appeared to be paralytic poliomyelitis, one child with aseptic meningitis apparently due to Frater virus, developed a transient paresis. This case lies in an intermediate position between uncom-plicated aseptic meningitis and paralytic disease. It was classi-fied as aseptic meningitis but the clinical details are given here for the sake of completeness.

## TABLE 21

### Levels of antibodies in acute-and convalescent-phase

### sera of patients with paralysis

| Patient Number | Virus Isolated | Acute/convalescent antibody titres to | | | | | |
|---|---|---|---|---|---|---|---|
| | | Poliovirus | | | Coxsackie | | Frater Virus |
| | | 1 | 2 | 3 | A7 | B2 | |
| 2 | Cox.B2 | 0/0 | 0/0 | 0/0 | 16/16 | 8/64 | ... |
| 3 | Frater | 0/0 | 32/32 | 32/32 | 0/0 | ... | 256/256 |
| 4 | Frater | 512/512 | 512/256 | 128/128 | 0/0 | ... | 128/256 |

... not done

Case 4: This 13 year old boy developed a sudden illness on 16 August, 1961 with headache, anorexia, difficulty in swallowing, and pain on moving his legs. On admission to hospital the next day he appeared acutely ill. There was nuchal rigidity and weakness of the muscles of the arms and right leg. This weakness lasted for only one day. Complete general recovery had occurred by 10 days after the onset of illness. The cerebro-spinal fluid at the time of admission contained 25 lymphocytes per cub. mm. and the protein was 12 mgm.%. This boy had received a full course of killed poliomyelitis vaccine. Frater virus was isolated from a stool sample on the 2nd day of illness. The antibody levels in sera on the 2nd and 14th days of illness are shown in Table 21.

## CHAPTER XI

## EPIDEMIC OF ASEPTIC MENINGITIS ASSOCIATED WITH

## FRATER VIRUS

Only a few of the viruses isolated in tissue culture from the stools of patients with aseptic meningitis could be identified by neutralisation tests with the antisera available in the laboratory. I therefore prepared antisera by immunising guinea pigs with several of these untyped viruses. Cross-neutralisation tests, which are discussed in detail in Chapter XVI, showed that the isolates were serologically the same, and all the untyped viruses were therefore tested with one of the first sera to be prepared which had a sufficiently high titre of neutralising antibodies. The virus used to prepare this serum was designated as the prototype strain, and was named Frater after the surname of the patient from whom it was isolated. As the new virus appeared to belong to the ECHO group, this method of naming the prototype strain followed the precedent set for the prototypes of the accepted types of ECHO viruses. The patient Frater did not in fact have typical aseptic meningitis, but was the child with paralytic disease referred to as case 3 in Chapter X.

The viruses isolated from 61 of the patients with aseptic meningitis in 1959 were identified with the antiserum to the prototype Frater virus as strains of the same virus. All the available specimens of sera from aseptic meningitis patients in 1959 were tested for antibodies to Frater virus, and another 8 cases were found on serological grounds to be apparently due to Frater virus. There were thus 69 cases of clinically typical aseptic meningitis with

evidence of infection by Frater virus.

## Clinical features

Aseptic meningitis associated with Frater virus could not be distinguished clinically in any way from aseptic meningitis due to other viruses. The patients had signs and symptoms of meningitis unaccompanied by paralysis and without a bacterial cause, and had some abnormality of the cerebro-spinal fluid. Apart from these features, which were the criteria necessary for a case to be classified as aseptic meningitis, there was a wide range of clinical features.

In most cases the onset of illness was sudden with headache and vomiting. As well as headache a considerable number of the patients complained of pain in the eyes and of photophobia. A number of patients were markedly irritable and some experienced muscle pains in the limbs, in the trunk, or in both. Most of the patients were moderately ill when admitted to hospital. A few appeared severely ill as, for example, case 4 referred to in Chapter X. Not all patients were fevered and in those who were, the pyrexia seldom reached high levels. In most cases nuchal rigidity and Kernigism were readily detected. The cerebro-spinal fluid in the acute phase of the illness contained from 18 to 500 cells per cub. mm., mostly lymphocytes. In some cases the protein level was raised and in others it was normal.

The acute stage of the illness usually lasted for about 3 to 5 days and the patients were usually discharged from hospital fully recovered after a hospital stay of about 2 weeks. No sequelae of

the disease were noted.

The typical features of the disease are illustrated by the following three case histories.

Case 5:   A 5 year old boy was admitted to hospital on 17 September, 1959 having suddenly developed a severe headache and vomiting 2 days before admission.  He had also been listless and drowsy.  On admission the temperature was 101°F and the pulse 98 per minute. There was nuchal rigidity but Kernig's sign could not be elicited. He appeared moderately ill.  The findings in the C.S.F. the day after admission were:- Cells - 25 lymphocytes per cub.mm.; protein - 40 mgm.%; sugar - 65 mgm.%.  The temperature fell to normal on the 4th day of illness and there was obvious general improvement.  The patient was discharged from hospital 11 days after admission completely recovered. Frater virus was isolated from a stool obtained on the day after admission, and a rise in titre of antibodies to Frater virus was demonstrated from 1:8 on the 3rd day of illness to 1:512 on the 13th day.

Case 6:   A 5 year old boy was admitted to hospital on 16 July 1961 with a history of headache and vomiting of 1 day's duration. On admission he was moderately ill, with a temperature of 100.4°F and a pulse rate of 116 per minute.  There was nuchal rigidity and Kernig's sign was readily elicited.  Lumbar puncture was done on admission and the C.S.F. findings were:- Cells - 435 per cub.mm.; protein - 15 mgm.%; sugar - 60 mgm.%.  The child improved rapidly and was dismissed after 20 days in hospital.  Frater virus was present in the faeces on the day after admission, and a rise in antibodies to Frater

virus from less than 1:8 to 1:32 was demonstrated between the 3rd and 13th days of illness.

Case 7: A 28 year old man developed vomiting and diarrhoea accompanied by abdominal pain, headache, and general febrile symptoms. The symptoms became worse and he was admitted to hospital on 24 August, 1959, 2 days after their onset. Pulse rate and temperature were normal on admission. There was rigidity of the neck muscles. C.S.F. findings on the day of admission were:- Cells - 280 per cub.mm., mostly lymphocytes; protein - 75 mgm.%; sugar 52 mgm.%. The day after admission the temperature rose to 99°F. Signs of meningeal irritation lasted for 4 days and then recovery was rapid and uneventful. The patient was discharged from hospital after 15 days. Frater virus was found in a stool specimen obtained on the 11th day of illness and the levels of antibodies to Frater virus in the patient's sera on the 11th and 22nd days of illness were 1:32 and 1:128.

## Epidemiology

The ages of the patients varied over a wide range from 4 months to 39 years. Details of the age distribution are given in Table 22. Approximately 60% of the patients were children of school age, an age-group at which there are ample opportunities for the spread of an infectious disease. Thirteen patients were 20 years and over. This suggests that even the adults in the community did not have protective antibodies to the virus, which would indicate that the virus may have been new to the community. Table 22 also shows the marked preponderance of male patients over female. For all age-groups taken together, 70% were males and 30% females.

There were two instances of pairs of siblings developing
aseptic meningitis due to Frater virus within a few days of each
other.  One pair were aged 3 years and 5 years, and the other pair
7 years and 11 years.  In addition the sister of one of the patients
with aseptic meningitis developed an encephalitis-like disease and
Frater virus was isolated from her stools.

The epidemic began in July 1959 and reached a peak in August
and September.  This is shown in Table 23.  It gradually subsided
during the last 3 months of the year.

The geographical location of the cases is illustrated in
Figure 15.

Most of the cases occurred in Glasgow and in the Clyde valley,
west and south-east of Glasgow.  There were 5 cases in Greenock and
isolated cases in Clydeside towns such as Yoker, Dumbarton, Port
Glasgow, and Paisley.  Single cases also occurred in Uddingston,
Hamilton, Motherwell, Airdrie and other Lanarkshire towns.  Together,
the Glasgow, Clydeside, and Lanarkshire cases accounted for 55 of
the total of 69 cases.  There were 2 cases in Ayrshire and 1 each
in Falkirk, Bridge of Allan, and Perth.  A group of 10 cases occurred
in Dundee.  It might appear that there were two areas affected by
epidemics, one around Glasgow and one in Dundee.  However, the con-
centration of cases in these areas is probably only a reflection of
the main centres of population in the region served by the labora-
tory.  The outbreak probably affected the whole region, possibly
even the whole of Scotland, and the number of cases in each area in
this series depended partly on the size of the population in each

TABLE 22

Age and sex distribution

| Age in years | 0-2 | 3-4 | 5-9 | 10-14 | 15-19 | 20-24 | 25-29 | over 30 | Total |
|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 8 | 14 | 15 | 6 | 3 | 2 | 0 | 48 |
| Female | 1 | 1 | 7 | 3 | 1 | 2 | 3 | 3 | 21 |
| Total | 1 | 9 | 21 | 18 | 7 | 5 | 5 | 3 | 69 |

TABLE 23

Monthly incidence of cases

| Month | March | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|
| Number | 1 | 1 | 9 | 23 | 25 | 6 | 3 | 1 |

area and partly on the readiness with which physicians in different hospitals sent specimens for virological examination. The staffs of the larger fever hospitals were particularly helpful in this respect, and consequently most of the cases were from a limited number of such hospitals.
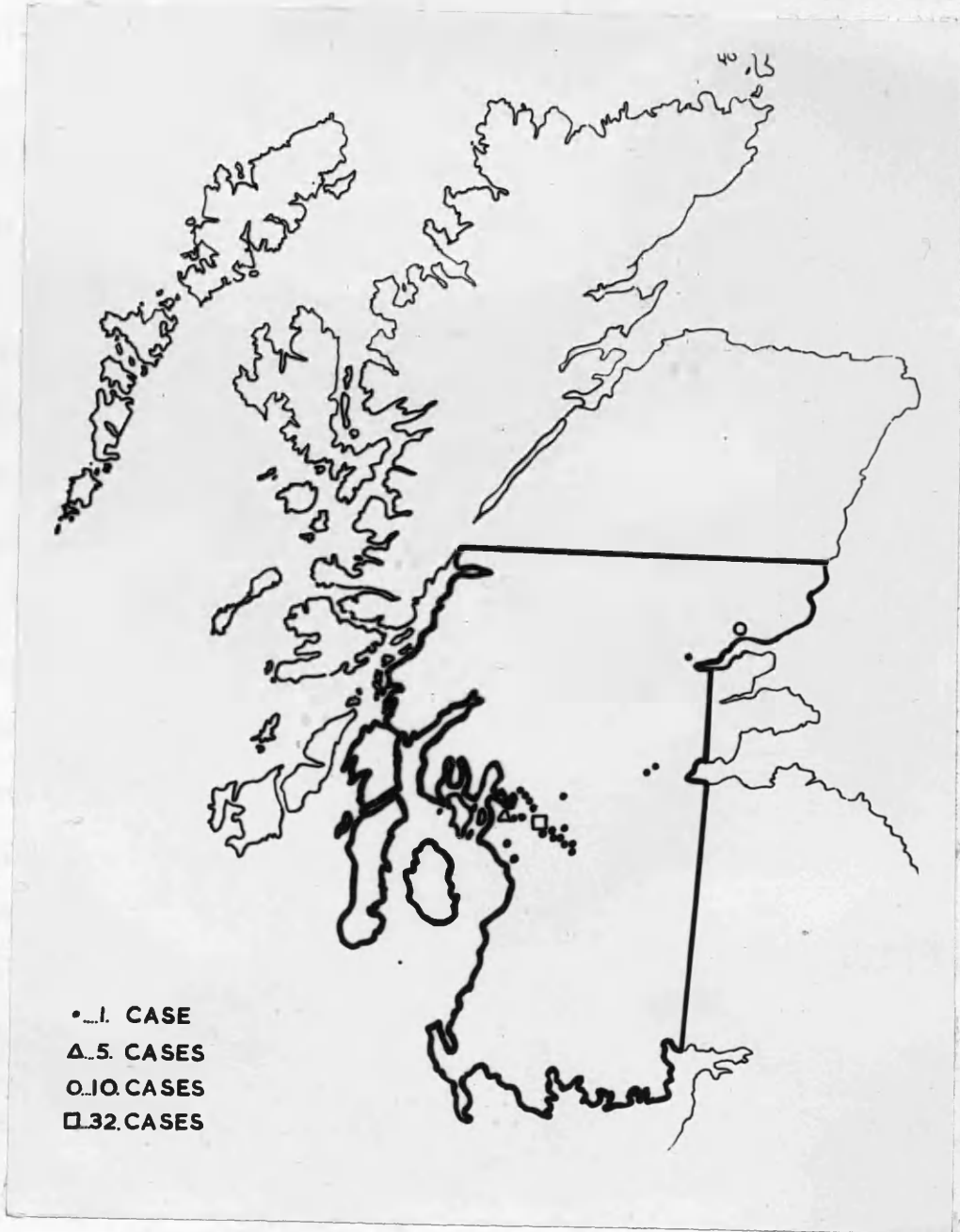
<u>Figure 15</u>    Map of Scotland showing geographical location of cases.
Area served by the laboratory is enclosed by heavy lines.

## Isolations of Frater virus

All the isolations of Frater virus were made in the second
half of 1959. Although there were two cases of aseptic meningitis
associated with Frater virus in March and June, they were diagnosed
by serological means only. The half year from July to December 1959
was therefore chosen as a suitable period for making a comparison
of the relative incidences of isolation of Frater virus from patients
with aseptic meningitis and from control patients. This was done
to help to assess the aetiological significance of the virus in
aseptic meningitis. During the period, Frater virus was isolated
from the stools of 61 of the 165 patients with aseptic meningitis,
an isolation rate of 37%. There were only 12 isolations in the con-
trol group of 215 patients with other illnesses, a rate of 6%. This
considerable difference between the isolation rates in the two
groups was also observed when the results were broken down further
by age groups as shown in Table 24.

## TABLE 24

### Isolations of Frater virus from aseptic meningitis and from control patients (percentage figures)

| Age groups in years | 0-4 | 5-9 | 10-19 | over 20 |
|---|---|---|---|---|
| Aseptic menin-gitis patients | 22 | 42 | 48 | 33 |
| Control patients | 6 | 7 | 9 | 0 |

In all, 75 isolations of Frater virus were made. The virus

was isolated on 2 occasions from one patient with aseptic menin-

gitis. There were therefore 62 isolations among the group of

patients with aseptic meningitis, 12 in the control group, and 1

from the patient, previously mentioned, who had a paralytic disease.

### Serological investigations

Paired acute-and convalescent-phase sera were available from

37 of the patients with aseptic meningitis from whom Frater virus

was isolated. The antibody titres of Frater virus in these sera

were measured in order to make certain that acute infection with

the virus in these patients coincided with their clinical symptoms,

thus establishing more fully the aetiological role of Frater virus

in aseptic meningitis. The results of these titrations are shown

in Table 25. A four-fold or greater rise in titre between the two

specimens was demonstrated in 23 cases. Titres of 64 or more in

both sera but no four-fold rise were found in 11 pairs, and in 3

pairs titres of 16´or less were found in both. Dr. N.R. Grist also

tested 31 of these 37 pairs of sera for antibodies to Coxsackie

type A7 virus. The other 6 pairs could not be tested because in-

sufficient serum was left over from thé previous tests. These tests

were done because Dr. Grist had shown (Grist, 1960 and 1961) that

Coxsackie A7 virus was the cause of many cases of aseptic meningitis

in the area at the same time, and it was considered that the aetio-

logical association of Frater virus with aseptic meningitis would

be further strengthened if it could be shown that the other virus

prevalent at the time was definitely not associated with the Frater

## TABLE 25

Antibody titres to Frater virus in acute-and convalescent-phase sera

of 37 aseptic meningitis patients from whom Frater virus was isolated

| Case | Day of illness acute/con-valescent | Frater antibody titre acute/con-valescent | Case | Day of illness acute/con-valescent | Frater antibody titre acute/con-valescent |
|------|------|------|------|------|------|
| 1 | 2/13 | <16/128 | 20 | 4/13 | <16/256 |
| 2 | 3/12 | 64/256 | 21 | 3/14 | < 8/16 |
| 3 | 6/16 | 256/256 | 22 | 7/20 | 128/128 |
| 4 | 5/18 | 64/512 | 23 | 3/13 | <16/64 |
| 5 | 2/13 | <16/256 | 24 | 5/28 | 64/128 |
| 6 | 4/15 | 64/128 | 25 | 8/19 | 16/128 |
| 7 | 2/13 | <16/32 | 26 | 10/20 | 256/1024 |
| 8 | 2/13 | <16/32 | 27 | 13/24 | 16/1024 |
| 9 | 6/19 | 64/64 | 28 | 3/15 | 32/128 |
| 10 | 3/17 | 256/64 | 29 | 2/14 | 128/256 |
| 11 | 3/10 | 512/2048 | 30 | 1/12 | <16/128 |
| 12 | 4/11 | <8/<8 | 31 | 2/14 | <16/128 |
| 13 | 5/11 | 256/4096 | 32 | 11/22 | 64/256 |
| 14 | 3/11 | 128/512 | 33 | 3/14 | 1024/1024 |
| 15 | 3/13 | 8/512 | 34 | 4/14 | 64/64 |
| 16 | 3/13 | 16/1024 | 35 | 4/16 | <16/64 |
| 17 | ... | 32/256 | 36 | 3/18 | 128/128 |
| 18 | 3/14 | 128/128 | 37 | 6/15 | <8/<8 |
| 19 | 1/10 | <16/<16 | | | |

... Not accurately known

virus cases. The titre of antibodies to Coxsackie A7 virus was less than 1:8 in both specimens of 29 of the 31 pairs. In the sera from patient number 15 in Table 25 the titres were 16/32 and in those from patient number 18 they were 8/16.

Single serum samples only were available from 16 of the patients with aseptic meningitis who had Frater virus in the stool. The stage of illness at which the serum was taken and the titre of antibodies to Frater virus in each of these 16 samples is given in Table 26.

Paired acute-and convalescent-phase sera were available from 57 patients who had aseptic meningitis during 1959 and from whom Frater virus was not isolated. A four-fold rise in antibodies between the 2 specimens was found in 4 cases. In 4 other pairs the same high titre was found in both samples of the pair, 1:256 in 2 pairs, 1:128 in 1, and 1:64 in 1. The first of the 2 serum specimens of each of the 4 pairs with the same titre in both samples was not taken until the patient had been ill for a week. These 8 cases were included in the series on serological grounds alone. No stool specimen was submitted from 1 of the 8 cases. Stools from each of the other 7 were tested and failed to yield any viruses. Included among the patients with aseptic meningitis whose sera were tested were 23 from whose stools Dr. Grist had isolated Coxsackie A7 virus. One of them had antibodies to Frater virus to a titre of 1:8 and the other 22 had no detectable antibodies to Frater virus.

## TABLE 26

### Antibody titres to Frater virus in single serum specimens

### of 16 aseptic meningitis patients excreting the virus

| Case | Day of illness | Antibody titre | Case | Day of illness | Antibody titre |
|------|----------------|----------------|------|----------------|----------------|
| 38 | 2 | < 8 | 46 | 4 | 256 |
| 39 | 3 | < 8 | 47 | 5 | 256 |
| 40 | 5 | 8 | 48 | 10 | 256 |
| 41 | 4 | 32 | 49 | 12 | 256 |
| 42 | 5 | 32 | 50 | 27 | 256 |
| 43 | 2 | 64 | 51 | 24 | 4096 |
| 44 | 4 | 64 | 52 | 12 | 8 |
| 45 | 4 | 64 | 53 | 21 | 32 |

CHAPTER XII

GENERAL EPIDEMIOLOGY OF FRATER VIRUS

The investigations discussed in the previous chapter on the epidemiology of aseptic meningitis associated with Frater virus suggested that the virus may have been a newcomer to the Scottish community. The number of adults affected and the fact that the virus did not appear to have been recognised previously, both favoured this hypothesis. It was possible to obtain further information about this by an antibody survey of blood donors who were bled in 1958. The results of this are shown in Table 27. Only 9% of the donors had detectable antibodies to Frater virus and even in those who had the levels were low. It was possible that immunity to the virus might have been short-lived in which case lack of antibodies in the donors would not necessarily have implied that the virus had not been present in the community. To obtain some idea of the probable persistence of antibodies after infection with Frater virus, serum specimens were taken a year after their illness from 10 of the patients who had aseptic meningitis associated with Frater virus. The titres of these patients' sera at the time of convalescence were all within the range 1:64 to 1:512. After a year the titre was unchanged in 3 and showed only a two-fold difference in 7, twice the original titre in 2 and half the original in 5. Taking into consideration the margin of error of the titration methods the antibody levels could be considered to have remained unchanged over the year. It seems probable therefore that the lack of antibodies in the 1958 donors is evidence that the virus had not been prevalent to any

great extent in the West of Scotland before 1959.

## TABLE 27

### Levels of antibody to Frater virus in the

### sera of blood donors in 1958 and 1960

| Antibody titre | Under 1:8 | 1:8 | 1:16 | 1:32 | 1:64 |
|---|---|---|---|---|---|
| Percent of 1958 donors | 91 | 7 | 2 | 0 | 0 |
| Percent of 1960 donors | 75 | 5 | 4 | 9 | 7 |

It seemed probable from its behaviour in 1959 that if Frater virus had been widely prevalent in previous years it would have been responsible for cases of aseptic meningitis in these years. The results of testing paired sera from patients who had virologically undiagnosed aseptic meningitis or clinical poliomyelitis in 1958 did not suggest that there had been wide-spread infection with Frater virus that year. Of a total of 55 cases there were no detectable antibodies to Frater virus in the convalescent sera of 43. In 10 the titres were 1:8. A 12 year old boy with aseptic meningitis had a titre of 1:64 which was unchanged at the 3rd and 16th days of illness. An 8 year old boy with paralytic disease had a titre of 1:32 on the 4th day of his illness and a titre of 1:64 on the 12th day. It is impossible to assess the possible significance of the antibody levels in these two children. Frater virus may have been

responsible. However, the lack of antibodies in the remaining 53
patients strongly suggests that Frater virus was not epidemic in
1958.

The evidence of antibody surveys suggests that the Scottish
community experienced considerable sub-clinical or mild infection
with Frater virus in 1959 as well as the epidemic of aseptic menin-
gitis. This may be seen from the antibody levels in the sera of
blood donors in 1960 which are shown in Table 27. The same percent-
age of 1960 donors as of 1958 donors had low levels of antibodies of
1:16 or less. In 1960, however, 16% had levels higher than any ob-
served in the 1958 donors. Some additional information was obtained
from the titration of antibodies in samples of gamma-globulin pre-
pared from the sera of blood donors in Edinburgh and the east of
Scotland. Antibodies to poliovirus type 1 and ECHO virus type 9
were also titrated. This was done as a rough check that variations
in the details of preparation of the gamma-globulin were not respons-
ible for changes in levels of antibodies. It seemed unlikely that
the level of poliovirus antibodies in adults would have changed mark-
edly from year to year. The results of these titrations are shown
in Table 28. Within the limits of error of the titrations poliovirus
antibodies remained fairly constant around 1:2000 and ECHO type 9
antibodies at 1:64 to 1:128. It was of interest that antibodies to
Frater virus were unchanged from 1951 until 1958 and that in 1959
they rose to 4 times their former level. Considering that levels of
antibodies to the other two viruses were unchanged in 1959 from
previous years it is most likely that the higher level of antibodies

to Frater virus in 1959 was not an artefact but was the result of infection with Frater virus in the general community.

TABLE 28

Antibodies to Frater virus, ECHO virus type 9, and poliovirus type 1

in gamma-globulin prepared from Scottish blood donors

| Years in which donors were bled | Titre of antibodies to | | |
|---|---|---|---|
| | Frater virus | ECHO 9 | Poliovirus type 1 |
| 1951 | 32 | 64 | 4000 |
| 1952 | 32 | 64 | 1000 |
| 1953 | 32 | 64 | 1000 |
| 1954 | 16 | 128 | 4000 |
| 1956 | 16 | 64 | 2000 |
| 1957 | 32 | 128 | 2000 |
| 1958 | 32 | 128 | 1000 |
| 1959 | 128 | 128 | 2000 |

The 1956 and 1958 samples contained gamma-globulin from members of the armed forces temporarily stationed in the area as well as from permanent residents of the east of Scotland.

## CHAPTER XIII

## DISCUSSION

The epidemiology of clinical paralytic poliomyelitis and aseptic meningitis in Scotland in 1959 presented three features of unusual interest. The first was the almost complete absence of polioviruses, the second was the major part played by Coxsackie A7 virus in the aetiology of clinical paralytic poliomyelitis, and the third was the appearance of a new virus causing a large epidemic of aseptic meningitis.

Wide variations in the number of cases of paralytic poliomyelitis from year to year have been long recognised to occur. However, the finding of only one case of true poliomyelitis in 1959 was remarkable when compared with 117 paralytic cases and 73 cases of aseptic meningitis in 1958 virologically proved to be caused by polioviruses. Yearly variations have seldom been quite so marked in extent. The disappearance of poliomyelitis might be attributed to the effect of immunisation of the community with Salk vaccine. However, other countries in which Salk vaccine has been widely employed have not reported anything approaching such complete prevention of the disease. Indeed in 1959 such well-immunised communities as Canada and the United States experienced severe outbreaks of poliomyelitis (Kubryk, 1960; Langmuir, 1961). Moreover in England, where Salk vaccine has been used to a similar extent as in Scotland, there were many isolations of type 1 virus from cases of poliomyelitis in 1959 (Stern, 1961). The total number of cases of paralytic poliomyelitis in England and Wales for 1959 was 739 as compared with 1717

in 1956, 3177 in 1957, and 1419 in 1958 (Bradley, 1961). Immunisation may have played a part in causing the great change in incidence of poliomyelitis in Scotland but it seems unlikely to have been the only factor involved.

It has been demonstrated (Dalldorf, 1955) that in mice and in tissue cultures Coxsackie viruses may interfere with infection of the host by polioviruses. This may occur under natural epidemiological conditions as well. However, Coxsackie B viruses rather than A viruses show this interference effect with poliovirus, and in Scotland in 1959 the prevailing Coxsackie viruses belonged to group A. Although there were a number of isolations of B2 and B5 there did not appear to have been sufficiently wide-spread infection of the community by group B viruses to have had a significant interfering effect with polioviruses. Less is known about interference between ECHO viruses and poliovirus, but there is epidemiological evidence that most of the enteroviruses may interfere with each other. It is not impossible that the spread of Frater virus in the community may have interfered with the spread of poliovirus.

Whatever the underlying causes may be, similar changes in poliomyelitis incidence have been seen in other countries, even before the introduction of poliomyelitis immunisation. In Finland which is similar to Scotland in the size of its population, there were 619 paralytic cases in 1956 and only 81 in 1957 (Penttinen, 1961). An even more striking change was seen in Stockholm between 1953 and 1954. In 1953, 248 isolations of type 1 poliovirus and 1 of type 3 were made from paralytic cases, and 121 of type 1 from cases of

aseptic meningitis. The following year 2 isolations of type 1,
4 of type 2, and 9 of type 3 were made from paralytic cases and 2
each of types 1 and 3 from cases of aseptic meningitis (Svedmyr,
Melen, and Kjellen, 1956). In both countries these changes were
observed in the days before poliomyelitis immunisation.

The virtual absence of polioviruses in Scotland in 1959 pro-
vided an unusually suitable epidemiological background against which
to assess the aetiological significance of other viruses isolated
from cases clinically resembling paralytic poliomyelitis. The three
such cases which I investigated myself were described in Chapter X
as Cases 2, 3, and 4. Case 2 is the easiest to assess aetiologically.
Only Coxsackie B2 virus was isolated from the stool, poliovirus anti-
bodies were absent even in convalescence, Coxsackie A7 antibodies
remained at the same low level throughout the illness, and an eight-
fold rise of antibodies to Coxsackie B2 virus was demonstrated.
This was almost certainly a true case of paralysis of short duration
and with full recovery caused by Coxsackie B2 virus. Case 3 was
clinically a typical case of paralytic disease with residual paralysis.
As the first specimen of serum was not submitted until the 10th day
of illness it is impossible to exclude the possibility that a double
infection with a poliovirus and Frater virus may have taken place.
Only Coxsackie A7 infection can be excluded on serological grounds.
This boy had received two injections of Salk vaccine so that an in-
fection with a wild poliovirus would have acted as a secondary anti-
genic stimulus and a higher level of antibodies than 1:32 might have
been expected to be present to one of the types of polioviruses.

However, this is only hypothesis and formal proof is impossible from
the information available. The third case, case 4, showed only trans-
ient paralysis and is probably best described as aseptic meningitis
with minimal paralysis. Because of previous immunisation, the levels
of antibodies to all 3 types of poliovirus in this patient were
high but they did not change during the illness. Coxsackie A7 infec-
tion was excluded serologically. Frater virus was isolated from the
stool but only a two-fold rise of antibodies to Frater virus was
demonstrated. However the case seems most probably to have been
due to the Frater virus infection. This sort of transient paralysis
has also been reported in infections due to ECHO type 6 virus (Ki-
brick, Melendez, and Enders, 1957; Karzon, Barron, and Winkelstein,
1956).

The 7 cases of paralytic disease from whom my colleague, Dr.
Grist, isolated Coxsackie A7 virus by suckling mouse isolation were
clinically definite paralytic cases (Grist, 1961). In most of these
patients serological evidence did not suggest coincident poliomye-
litis infection. In all, there were 14 cases during 1959 which
clinically could have been taken for paralytic poliomyelitis. Cox-
sackie A7 virus was the probable cause of 7 of them, Coxsackie B2
of 1, Frater virus may have caused 1, and poliovirus caused only a
single case. The rarity of polioviruses in the community adds con-
siderable additional weight to the probability that 9 of the 14
paralytic cases were due to other viruses than poliovirus. This
unusual aetiology of so many paralytic cases in one area during one
year emphasises the necessity of full virological investigation of

cases thought to be poliomyelitis. Without virological information on aetiology it is impossible to obtain accurate poliomyelitis statistics which are essential for the assessment of the results of poliomyelitis vaccination programmes.

The third unusual feature of the epidemiology of virus infections of the central nervous system during 1959 was the appearance of infections due to Frater virus. This virus seemed to be definitely associated with aseptic meningitis but as it was an unknown agent it was necessary to try to prove that it was indeed the aetiological agent of the disease. Two main criteria have been laid down by the Committee on the Enteroviruses (1957) for establishing an aetiological association between an enterovirus and a particular disease. First the virus must be isolated from a considerably higher proportion of patients with the disease than of normal people of the same age, and of the same social and economic status in the same area at the same time. Secondly, antibodies to the virus should develop in patients during the course of their disease. Frater virus satisfied both these conditions in its association with aseptic meningitis in Scotland in 1959.

Although the group of people forming the controls for assessing the incidence of Frater virus were hospital patients rather than healthy members of the community they probably reflected reasonably accurately the population at large in their carriage of Frater virus. Stool samples were obtained from them as soon as possible after admission to hospital so that any viruses isolated were unlikely to have been acquired in hospital. The patients in this

group were admitted to hospital mainly with respiratory diseases,
non-infectious diseases of the central nervous system such as
vascular conditions, and diarrhoeal disease. If anything, such a
control group might tend to err on the side of having a higher in-
cidence of infection with enteroviruses than the normal community
in view of the association of enteroviruses with diarrhoea (Sommer-
ville, 1958; Ramos-Alvarez and Sabin, 1958). The control patients
were drawn from the same areas as the patients with aseptic men-
ingitis. The comparison between the groups was made over the same
six month period, and separate comparisons were made for the various
age groups. Every case of aseptic meningitis was included in the
totals, even those known to be associated with other viruses. At
all age groups there was a much higher incidence of infection with
Frater virus in the aseptic meningitis group than in the control
group. The rate was particularly high in those between 5 and 19
years. This was the age-group at which most of the aseptic meningi-
tis cases associated with Frater virus occurred. The rate was low-
est in children under 4, probably because many cases of aseptic
meningitis in this age group were caused not by Frater virus but by
Coxsackie A7 virus (Grist, 1961). However, even although the per-
centages of aseptic meningitis patients excreting Frater virus were
reduced by the inclusion in the totals of cases clearly due to other
viruses, there was at all age-groups a markedly higher isolation
rate of Frater virus from aseptic meningitis patients than from con-
trol patients.

It was possible to demonstrate the development of antibodies to

Frater virus in the sera of a considerable number of patients with
the virus in the stool. Paired acute-phase and convalescent-phase
sera were obtained from 37 of these patients. A clear four-fold or
greater rise in antibody titre between the two specimens was ob-
tained in 23 cases, thus proving that the acute stages of infection
with the virus coincided with the clinical features of aseptic
meningitis. When account is taken of the consistently low levels
of antibodies to Frater virus observed in the community before the
epidemic, as shown by the titrations of antibody in blood donors'
sera, levels of antibody of 1:64 or higher in a single convalescent
phase serum or in both sera of a pair in which a full four-fold rise
was not found, are probably indicative of recent infection with the
virus.

Thus the aetiological connection of the virus with aseptic
meningitis appears to be reasonably well proved. It is unfortunate
that virtually no samples of cerebro-spinal fluid were submitted for
virological examination. Isolation of the virus from the C.S.F.
would have completed the proof of its connection with aseptic men-
ingitis.

The general features of the aseptic meningitis epidemic due to
Frater virus were very similar to those of other outbreaks due to
ECHO viruses. The marked preponderance of male patients has been
observed in other outbreaks such as that in New York State in 1955
due to ECHO type 6 virus (Winkelstein et al., 1957). The proportion
of older patients correlated well with the general lack of antibodies
to the virus in the community before the outbreak. In all probability

the epidemic in 1959 affected the whole of Scotland. The cases which
I have described came from a wide area of the western and central
parts of the country and from the Dundee region. In August and
September of 1959, Bell isolated Frater virus from 6 cases of
aseptic meningitis in Aberdeen. The viruses which he isolated were
antigenically the same as my Frater prototype strain. No isolations
of Frater virus were reported in the Edinburgh area but it seems
likely from the results of my antibody survey of gamma-globulin
from Edinburgh blood donors that this part of Scotland was also
affected by the 1959 epidemic. In 1960 Frater virus was isolated
from 5 cases of aseptic meningitis in the west of Scotland (Grist,
1961). There were also sporadic infections with Frater virus in
England in 1960. Mair (1961) isolated the virus from 4 patients
with aseptic meningitis in Leicester, and from another 4 symptom-
less children who were contacts of patients with aseptic meningitis.
Single isolations of Frater virus were also made in 1960 in Leeds,
Sheffield, Liverpool, London, and Portsmouth (Macrae, 1961). It is
significant that Mair in Leicester, who made 8 isolations of the
virus, used human amnion cells for routine virus isolation as well
as monkey kidney cells. The absence of isolations of Frater virus
in the Edinburgh area in 1959 and in most of England in 1960 does
not necessarily imply the absence of the virus from these areas.
It may simply mean that the types of tissue cultures, such as mon-
key kidney cells, in use in virus laboratories in these areas were
not highly sensitive to the virus.

The available evidence suggests that Frater virus may have

caused sporadic infections in previous years in Scotland but had probably not been at all widely prevalent until 1959. The finding of raised levels of antibody to the virus in the sera of two patients in 1958 and the low levels of antibody in gamma-globulin samples prepared from 1951 to 1958 suggest that the virus may have occurred sporadically in earlier years. However, this can be no more than a suggestion because these antibodies could have been produced in response to another virus. Antigenic crossing is known among the ECHO viruses. In human infections with ECHO viruses antibodies reacting with several ECHO types in addition to the infecting virus have been observed (Halonen, Rosen, and Huebner, 1959). Moreover cross-reactions have been described between ECHO types 1, 8, and 12 in hyperimmune monkey sera (Committee on the Enteroviruses, 1957). Both the number of older patients affected in 1959 and the lack of antibodies in most blood donors in 1958 strongly suggest that 1959 was the first time the community had experienced widespread infection with Frater virus.

This was in marked contrast to Dr. Grist's findings with respect to the outbreak of aseptic meningitis and paralytic disease due to Coxsackie A7 virus. He found that 70% of these cases occurred in children under school age, and that 46% of blood donors in 1958 had antibodies to Coxsackie A7 virus (Combined Scottish Study, 1961). It appeared that this virus was by no means new to the Scottish community.

The occurrence of two most unusual outbreaks of infection in Scotland in the same season emphasise the necessity of employing

many different complex virological methods if the epidemiology of viral diseases of the central nervous system is to be adequately unravelled. Without modern laboratory investigations it would have appeared that there were a few typical cases of paralytic poliomyelitis and a single large outbreak of non-paralytic poliomyelitis or aseptic meningitis. By using suckling mouse inoculation Dr. Grist uncovered the largest outbreak of paralytic disease due to Coxsackie A7 virus so far reported, and showed that there was an epidemic of aseptic meningitis in the earlier part of the summer also due to this virus (Grist, 1961). By employing human thyroid and amnion cells as well as the more usual monkey kidney cell cultures I found that there had been a second and larger epidemic of aseptic meningitis rather later in the summer but overlapping with the first, and that it had been caused by an entirely new virus.

As the investigations described in Part 3 of the thesis show, Frater virus has the characteristics of an ECHO virus. It is therefore of particular interest that it was associated with such a considerable outbreak of aseptic meningitis. Although there have also been large outbreaks due to ECHO types 4 and 6 in the United States, only type 9 had previously caused major outbreaks in Britain. Frater virus can now be added to this small group of ECHO viruses which can cause aseptic meningitis in epidemic proportions.

# PART 3

## PROPERTIES AND CLASSIFICATION OF FRATER VIRUS

## CHAPTER XIV

## REVIEW OF THE LITERATURE

The discovery of the ECHO viruses followed as a result of the widespread use of cultures of monkey kidney cells for the isolation of polioviruses. Many viruses were isolated from patients suffering from aseptic meningitis (Melnick, 1955) and from healthy children (Ramos-Alvarez and Sabin, 1954) which were neither polioviruses nor Coxsackie viruses although they resembled these viruses in the type of cytopathic effects they produced in tissue cultures. So many different strains of these viruses were isolated that it soon became urgently necessary to make a working classification of them, and the National Foundation formed a committee for the purpose. This Committee on the ECHO viruses in 1955 published the criteria for acceptance of a new virus as one of a new group called ECHO viruses and designated the first 13 types. The name orphan, forming part of the full title of enteric cytopathogenic human orphan (or ECHO) viruses, had been given light-heartedly to the group at the time when they had not yet been associated with any parent diseases. Their properties as stated by the committee were:- 1) cytopathogenicity for monkey and human cells in culture, 2) failure to be neutralised by pools of antisera to the 3 types of polioviruses, 3) failure to be neutralised by antisera to those Coxsackie viruses known to be cytopathogenic in tissue cultures and failure to infect suckling mice, 4) lack of relationship to other viruses which may be recovered from the alimentary tract by the inoculation of cultures of primate tissues, such as herpes virus, influenza viruses, mumps virus, measles virus,

varicella virus, ARD and APC viruses, 5) neutralisation by human
gamma-globulin and by individual human sera.

In 1957 the committee issued a further report in which the
polioviruses, the Coxsackie viruses, and the ECHO viruses were
grouped together under the name, enteroviruses (Committee on the
Enteroviruses, 1957). Six additional ECHO types were designated.
The criteria for inclusion in the group were not changed, but it was
stated that the reference serum for each type would in future be
prepared with virus seed which had been purified by the plaque method
or the method of terminal dilutions. The need for purified seed of
prototype viruses had been made clear by the discovery that the
original seed virus issued as type 13 was in fact a mixture of types
1 and 13 (Hammon et al., 1959). At present there are 30 accepted
types of ECHO viruses and reference antisera have been prepared for
the first 25 (Kamitsuka, Soergel, and Wenner, 1961). A full acoount
of types 26 and 27 was given by Hammon, Yohn, and Pavia (1960) and
of type 28 by Pelon (1961). One type, type 10, has been removed to
a new group of viruses called reoviruses (Sabin, 1959) because it
differed markedly in size, pathogenicity for tissue cultures, and
other properties from the remaining members of the ECHO group.

### Pathogenicity for animals and cytopathogenicity

The ECHO viruses are very limited in their pathogenicity for
animals. In monkeys no obvious clinical disease is produced but the
virus may multiply and may produce histological lesions in the
spinal cord. Itoh and Melnick (1957) showed that chimpanzees infected
with ECHO type 4 and with ECHO type 6 viruses excreted virus in the

throat and stools and developed antibodies in their sera. Kamitsuka,
Soergel, and Wenner (1961) observed lesions resembling those of mild
poliomyelitis in the spinal cords of monkeys which had been immunised
with live ECHO virus of types 1, 2, 3, 4, 6, 10, 19, and 24. No
such lesions were seen in monkeys immunised with the others of the
first 25 types of ECHO viruses. Only 2 of the ECHO virus types are
pathogenic for suckling mice. Type 10, now known as reovirus type 1,
produces lesions similar to those caused by type B Coxsackie viruses
(Sabin, 1959). Many strains of ECHO type 9 virus produce paralysis
in suckling mice if a large enough inoculum is used (Eggers and Sabin,
1959). Other strains of type 9 such as the prototype strain, Hill,
cannot multiply in suckling mice. Most virologists still refer to
strains pathogenic for suckling mice as well as to non-pathogenic
strains as ECHO type 9 virus. However the pathogenic strains have
been classified as Coxsackie type A23 (Sickles, Mutterer, and Plager,
1959) and the non-pathogenic strains remain as ECHO type 9 virus.
As both are immunologically very similar this separation is somewhat
arbitrary.

By definition, all the ECHO viruses must be cytopathogenic for
monkey and human cells in culture. Almost all the prototype strains
were isolated in rhesus monkey kidney cultures. Rhesus monkey kidney
cells are susceptible to all the ECHO viruses. Cultures of kidney
cells from other species of monkey are susceptible only to certain
ECHO types. Patas (Erythrocebus patas) monkey kidney cells are highly
susceptible to types 7, 10, and 12 but not to other types (Hsiung,
1961). Hsiung has also shown that cultures of kidney cells from

premature babies show cytopathic effects with all types of ECHO viruses. Primary human amnion cultures, on the other hand, show clear cytopathic effects with many ECHO types but not with all (Lehmann-Grube, 1961). Continuous cell lines derived from human cancer cells are not suitable for the primary isolation of ECHO viruses, but laboratory strains of virus can be adapted to grow in them and produce cytopathic changes. Archetti, Weston, and Wenner (1957) succeeded in adapting all of the first 13 types of ECHO viruses to produce cytopathic changes in HeLa cells. Ormsbee and Melnick (1957) found that some ECHO viruses would produce cytopathic effects in the Maben cell line, and some ECHO viruses have been adapted to grow in KB cells (Maisel, Moscovici, and La Placa, 1961). Few ECHO viruses are cytopathogenic for non-primate cells. Lenahan and Wenner (1960) tested 23 types in kidney cell cultures from 8 different animals. ECHO types 4 and 9 were cytopathogenic for calf cells, and type 4 was also cytopathogenic for pig cells. Type 10 gave cytopathic changes in all except rabbit cells.

The cytopathic changes produced by ECHO viruses resemble very closely those of polioviruses and Coxsackie viruses. Shaver, Barron, and Karzon (1958) found that all of the first 14 types of ECHO viruses except type 10 produced the same cytopathic changes as polioviruses in cultures of monkey kidney cells. The main changes were rounding of the cells, and the appearance of a round eosinophilic mass in the cytoplasm and of eosinophilic granules in the nucleus. The cytoplasmic mass later appeared to indent the nucleus and at an even later stage basophilic granules appeared in the cytoplasm.

These workers also examined ECHO viruses types 15 to 24 and found
similar appearances with all except types 22 and 23 (Shaver, Barron,
and Karzon, 1961). These 2 types showed changes similar to other
types in the cytoplasm. There was however, a characteristic disap-
pearance of nuclear chromatin and of the nucleolus and a thickening
of the nuclear membrane, not seen with other types. Wigand, and
Sabin (1961) observed that the cytopathic effects of these 2 types were
usually limited to the periphery of the monolayer and varied in deg-
ree from culture to culture. Types 26 and 27 produce effects similar
to polioviruses and to the majority of the ECHO types (Hammon, Yohn,
and Pavia, 1960), and type 28 produces a cytopathic effect which
characteristically progresses extremely slowly (Pelon, 1961).

ECHO viruses have been shown by Hsiung and Melnick (1957) to
produce plaques in rhesus monkey kidney cultures under an agar over-
lay. The ECHO viruses were subdivided into 3 groups by these workers
on the basis of plaque formation. Types 1, 3, 4, 6, 9, 11, 13, and
14 formed small irregular plaques in rhesus kidney cultures only
(group A). Types 7, 8 and 12 gave large circular plaques in both
rhesus and patas kidney cultures (Group B). Types 2, 3, 5, and 6
failed to form plaques. Maisel and Moscovici (1961) tested the more
recent ECHO prototypes and found that types 15 to 24, except type
21 which was not tested, gave plaques characteristic of group A in
rhesus kidney cells. Plaques were produced by type 26 virus but
types 27 (Hammon, Yohn, and Pavia, 1960) and 28 (Pelon, 1961) failed
to give plaques in rhesus kidney cells. It has been shown that the
failure of Hsiung and Melnick to obtain plaques with ECHO type 2

virus was not because this virus is incapable of forming plaques but because of a technical detail in their method which suppressed the production of plaques (Sommerville, 1960a). By using a lower concentration of neutral red to stain the culture cells Sommerville succeeded in producing plaques with this virus.

Sommerville has also made quantitative studies of various aspects of the growth of ECHO viruses in monolayers of monkey kidney cells. (Types 1, 2, 7, and 11 were used in all his experiments. The rates of adsorption of the 4 viruses to the cells of the monolayer varied considerably and the rate with type 2 was much slower than with the others (Sommerville, 1960b). The average maturation time of virus growing in monkey kidney cells was about 7 hours for types 2, 7, and 11, but only 3 hours for type 1 (Sommerville, McIntosh, and Carson, 1958). At the time of maturation the proportion of virus which was free in the culture fluid varied among the types from 0.08 to 2.6% of the total in the medium plus the cells. These findings are within the same range as those obtained by Howes and Melnick, (1957) for type 1 poliovirus. It appears that different types of ECHO viruses vary considerably in their growth characteristics but are broadly similar to other enteroviruses. Sommerville (1960c) working with the same 4 types of ECHO viruses has demonstrated interference between pairs of them growing in monkey kidney cultures.

## Haemagglutination

The different types of ECHO viruses vary in their ability to cause agglutination of human group O red cells. Goldfield, Srihongse, and Fox (1957) found that types 3, 6, 7, 10, 11, and 12 agglutinated

red cells even when the virus was well diluted but that only con-
centrated suspensions of type 4 virus caused haemagglutination, and
that types 1, 2, 5, 8, 9, 13 and 14 failed to produce haemagglutina-
tion at any concentration. The haemagglutination was caused by the
virus particle itself. They found, as also did Lahelle (1958), that
some types failed to produce haemagglutination at 37°C while others
gave haemagglutination both at 37°C and at lower temperatures. Gold-
field and his colleagues (1958) later reported that ECHO viruses
will elute off agglutinated red cells leaving them inagglutinable
by the same ECHO virus. They found a receptor gradient for different
ECHO viruses, similar to the well-known receptor gradient found with
the myxoviruses, and they showed that red cells from which ECHO
virus had eluted and which could no longer be agglutinated by the
ECHO viruses could still be agglutinated by myxoviruses. The re-
verse was also true. Philipson (1959) tested 6 different enzymes
for their ability to destroy the red cell receptors on which the
ECHO viruses act. Only chymotrypsin was successful. Red cells
treated with chymotrypsin, though no longer capable of agglutination
by ECHO viruses, could still be agglutinated by myxoviruses. Dif-
ferences in agglutinating ability have been demonstrated within ECHO
types. McIntosh and Sommerville (1959) examined 24 strains of ECHO
type 7 virus and found that only 8 of the strains caused haemag-
glutination. The haemagglutinating abilities of ECHO viruses can be
changed by adaptation in the laboratory. Maisel, Moscovici, and
La Placa (1961) found that strains of ECHO types 3, 11, and 19 lost
their powers of haemagglutination after adaptation from growth in

monkey kidney cells to growth in continuous lines of human cancer cells.  It seems probable that haemagglutination is a property not so much peculiar to a whole ECHO virus serotype as to the individual virus strain within the serotype.

## Antigenic properties

Each of the ECHO viruses possesses its own distinct antigen. Although minor sharing of antigens has been detected amongst a few types there is no common group antigen as there is, for example, among strains of influenza type A virus or among the adenoviruses. All the work of classifying the ECHO group and establishing prototype strains for each type has been based on neutralisation tests in monkey kidney cultures with hyperimmune monkey sera.  This major task has been performed by the members of the Committee on the Enteroviruses of the National Foundation.  Reference sera are being prepared for each type of ECHO virus and at present such sera have already been made for the first 25 types (Kamitsuka, Soergel, and Wenner, 1961).  Most of the reference sera have very high titres but it has not been found possible to prepare high-titred serum for types 4 and 21 even with the help of adjuvants.  Detailed recommendations on methods of using these sera for the identification of virus isolates have been made by the Committee on the Enteroviruses (1957) and by Kamitsuka and his colleagues (1961).  Most of these sera are specific for a single type of ECHO virus.  Types 1 and 8, however, share an antigen and there is reciprocal cross-neutralisation between the two.  Types 11 and 19 show minor antigenic relationships with types 6, 23, 24, and 25.  Type 23 serum shows a one-way cross-

neutralisation with type 22 virus. Routinely, neutralisation tests
are performed by inoculating replicate tubes of tissue culture with
mixtures containing 100 $TCD_{50}$ of virus and suitable dilutions of
antiserum. With some viruses this method is unsatisfactory. Most
strains of type 4 virus, including the prototype, are not neutralised
by specific serum in a tube test. Itoh and Melnick (1957) have
devised a neutralisation test for ECHO type 4 virus which depends
on a reduction in the number of plaques produced by the virus when
it is mixed with the specific antiserum. Because of the number of
ECHO types and the lack of group antigens, Lim and Benyesh-Melnick
(1960) and Shaw et al., (1961) have devised methods of identifying
isolates by neutralisation tests with a series of pools of sera in
order to reduce the labour involved in the use of large numbers of
monovalent antisera.

There is now abundant evidence of marked antigenic variation
within types of ECHO viruses. In 1957 the Committee on the Entero-
viruses recognised the existence of prime strains of ECHO viruses.
These possessed a broader antigenicity than the prototype strains
so that an antiserum to a prime strain would neutralise all strains
within the type while an antiserum to the prototype would neutralise
the prime strain only poorly. Karzon, Pollock, and Barron (1959)
made a study of the antigenic properties of a large series of strains
of ECHO type 6 viruses. They found that with this virus the range
of antigenic variation was much wider than simply strains resembling
the prototype and strains resembling the prime variant. Between
different isolates there might be reciprocal cross-neutralisation

to high titre, high-titred neutralisation in only one direction, or poor cross-neutralisation in both directions. These workers referred to strains which were well neutralised by all heterologous antisera as being in the broad or B phase. Strains poorly neutralised by heterologous antisera were said to be in the specific or S phase. It was found that passage in monkey kidney tissue culture would convert a virus in the S phase to the B phase. Barron and Karzon (1961) have also found S and B phase variation among strains of ECHO type 4 virus. Most strains were in the S phase but one strain "Du Toit" was found to be in the B phase. In the past it has been generally thought that ECHO type 4 virus is a very poor antigen since it has seldom been possible to demonstrate rises in antibodies to the virus in patients from whom it has been isolated. Barron and Karzon found that antibodies could be readily detected if the B phase Du Toit strain were used as the indicator antigen. They also found another type of variation within ECHO type 4. Some variants are not neutralised by hyperimmune rabbit sera but are neutralised by monkey or human sera, whereas other strains are neutralised by all three.

Variation has also been reported among strains of ECHO type 9 virus. Flugsrud, Abrahamsen, and Lahelle (1958) found two antigenic groups among strains isolated from the same epidemic of aseptic meningitis. Archetti, Dubes, and Wenner (1959) made a very thorough comparison of the prototype Hill strain and a number of strains pathogenic for suckling mice. Antigenically the suckling-mouse-pathogenic strains behaved like prime variants in their antigenic relationship

to the prototype strain. They also differed from the prototype in the type of plaque produced in monkey kidney cells. Now that strains which are pathogenic for suckling mice are designated as Coxsackie viruses (Sickles, Mutterer, and Plager, 1959) it might be considered that this is not a valid example of variation within an ECHO virus type. The U virus, which was shown to be a variant of ECHO type 11, (Philipson and Rosen, 1959) and the Boston exanthem varient of ECHO type 16 (Neva, Malone, and Lewis, 1959) are two other good examples of viruses showing marked antigenic variation from the prototype strain of their type.

Since Melnick (1955) showed that ECHO viruses growing in monkey kidney cultures released an antigen which would react in a complement fixation test, several groups of workers have used this method to study the antigens of the ECHO virus group. Archetti, Weston, and Wenner (1957) found that live ECHO viruses which had been grown in HeLa cells were satisfactory antigens for complement fixation tests, and generally reacted specifically with the homologous sera. There was crossing between types 1, 8, and 13, but the relationships with 13 were probably not genuine because the original type 13 seed is now known to have been contaminated with type 1 virus. Halonen, Rosen, and Huebner (1958) obtained closely similar results with viruses grown in monkey kidney cells as antigens. They used the complement fixation method successfully to type unknown isolates. Yohn and Hammon (1960) advocate the use of complement fixation tests for the identification of isolates as ECHO type 4 because of the great difficulty of typing strains of ECHO 4 virus by neutralisation methods.

Godtfredsen (1960) has also employed the complement fixation method
in typing isolates. In these investigations hyperimmune monkey sera
were employed and they proved almost as specific as when used in
neutralisation tests. Tests for ECHO virus antibodies in the sera
of human beings infected with ECHO viruses give much more evidence
of antigenic crossing between different types. Halonen, Rosen, and
Huebner (1959) found significant heterologous antibody responses in
human beings, linking ECHO virus types 2, 4, 5, 6, 7, 8, 11, 14, 16,
and 20. The complement fixation test therefore, though it appears in
general to detect much the same antigens as those reacting in the
neutralisation test, provides rather more evidence of common antigens
among different ECHO types.

The specific antigens of those ECHO types which agglutinate
human group O red cells react with homologous antisera by the
haemagglutination inhibition method (Goldfield, Srihongse, and Fox,
1958). Lahelle (1958) has shown that the haemagglutination inhibi-
tion method with ECHO viruses is considerably less specific than
neutralisation tests. The types of ECHO virus which Lahelle found
by haemagglutination inhibition methods to have antigens in common
were not the same as those which Halonen, Rosen, and Huebner showed
by complement fixation methods to share antigens.

### Physical properties

The ECHO viruses like the other enteroviruses are stable viruses.
They survive treatment with ether (Committee on the ECHO Viruses,
1955) and with sodium desoxycholate (Hammon, Yohn, and Pavia, 1960).
Philipson (1958) showed that the U virus strain of ECHO type 11 was

slightly more resistant to formalin than poliovirus, and also that some virus survived storage in balanced salt solutions for 10 hours at all ranges of pH between 3 and 10. ECHO type 28 has been shown to survive for 24 hours in the presence of 95% and 70% ethanol, 20% ether, and 5% phenol (Mogabgab and Pelon, 1957).

Different ECHO types vary in their heat stability. Lehmann-Grube and Syverton (1959) studied the stability at 37°C of the prototypes of types 1 to 20. After 24 hours the titre of the most stable type, ECHO 6, was unchanged and that of the least stable, ECHO 20, had fallen from $10^{-5.7}$ to $10^{-2.3}$. ECHO type 6 virus closely resembled poliovirus type 1 in its thermal stability. Tyrrell et al., (1958) compared the stability of a suckling-mouse-pathogenic strain of ECHO type 9 and a poliovirus at several different temperatures and found their survival almost identical.

In size, the ECHO viruses are similar to polioviruses. Melnick (1955) reported investigations carried out in his laboratory by Macrae who showed by filtration through gradocol membranes that ECHO types 1, 2, 3, and 4 were the same size as type 1 poliovirus and were therefore about 30 mμ in diameter. Benyesh et al., (1958) determined the size of ECHO types 1 and 7 and poliovirus type 1 by a method employing ionizing radiations. All these viruses appeared to be in the region of 30 mμ in diameter. By the sucrose density gradient method Tyrrell et al., (1958) estimated the size of a suckling-mouse-pathogenic strain of ECHO type 9 virus as 33 mμ. Different methods are thus in agreement that the ECHO viruses are about 30 mμ in diameter, the same size as poliovirus. Only ECHO

type 10 appears to be different from the others in size (Sabin, 1957)
and this was one of the anomalous properties of this virus which led
to its removal from the ECHO group.

### Relationship of the ECHO group to other
### enteroviruses

In size, in resistance to chemicals and changes of temperature,
and in the type of cytopathic effect produced, ECHO viruses are very
similar to polioviruses and Coxsackie viruses. However in 1955 when
the Committee on the ECHO Viruses issued its first report, the 3
groups of viruses each appeared quite distinct from one another.
The ECHO viruses could be distinguished from polioviruses on sero-
logical grounds and from Coxsackie viruses by their lack of patho-
genicity for suckling mice. By 1957 the committee had changed its
name to the Committee on the Enteroviruses as it was recognised that
the 3 groups belonged to the same family of viruses. More recently
it has become increasingly clear that there is considerable over-
lapping in the properties of some ECHO and Coxsackie viruses, which
makes it very difficult to place them unequivocally in one or other
group.

The prototype strain of ECHO type 9 virus cannot multiply in
suckling mice and is non-pathogenic for mice (Eggers and Sabin, 1959).
However, the great majority of strains possessing the specific anti-
gen of this virus, which were later isolated throughout the world,
proved to be pathogenic for suckling mice. Accordingly Sickles,
Mutterer, and Plager (1959) gave these strains the designation of
Coxsackie type A23. This leads to the strange situation of a Cox-

sackie virus and also an ECHO virus possessing the same antigen and reacting with the same specific antiserum. Schmidt, Fox, and Lennette (1961) reported a similar situation with two other viruses. Lennette's Coe virus, which is not pathogenic for suckling mice and which has not yet been placed in any particular virus group, was shown to be immunologically the same as Coxsackie type A21 virus. On the other hand, strains of Coxsackie type A9 and of the Coxsackie B viruses have been encountered which resemble ECHO viruses in their ready isolation in tissue culture and their lack of pathogenicity for suckling mice (Kalter and Hall, 1959; Godtfredsen, 1960; Lycke, Lund, and Hultgardh, 1959). Hsiung (1960) was able to make Coxsackie A9 virus avirulent for suckling mice by adapting it to grow in patas monkey kidney cells, and subsequently to restore the virulence by passage in rhesus monkey kidney cells.

There are considerable differences between different ECHO viruses in their effects on the central nervous system of monkeys (Kamitsuka and his colleagues, 1961). The same is true of different Coxsackie viruses. Some ECHO viruses agglutinate red cells and others do not, and again this is true of Coxsackie viruses (Goldfield, Srihongse, and Fox, 1958).

There may even be some minor antigenic factors shared between ECHO viruses and polioviruses, because Lennette, Schmidt, and Magoffin (1961) have observed rises in poliovirus complement-fixing antibodies in patients who were infected with ECHO viruses and were very unlikely to have a coincident poliovirus infection.

The present method of classification of an enterovirus as either

a poliovirus, a Coxsackie virus, or an ECHO virus is clearly far

from perfect. The alternative would be to abolish the 3 categories

and to redesignate the members of each group simply as "entero-

viruses" with a type number. This could be done on purely immuno-

logical grounds and would allow of variation in such characters as

suckling mouse pathogenicity within a single type. Some virologists

would favour such a change (Schmidt, Fox, and Lennette, 1961) but

others might consider that the existing names are already so well

established that to change them would cause unnecessary confusion.

The members of the Committee on the Enteroviruses themselves con-

sider that a new classification is necessary and their proposals

for this classification will shortly be published (Wenner, 1962).

## CHAPTER XV

## PATHOGENICITY FOR TISSUE CULTURES AND FOR ANIMALS

### Cytopathogenicity for different types of cells

In the course of the comparison of human amnion, human thyroid,
and monkey kidney cultures for virus isolation, Frater virus was
isolated from 75 stool specimens. Each of these specimens was inocu-
lated to all 3 types of culture. The virus was isolated from 65 of
the specimens in thyroid cultures, from 58 in amnion cultures, and
from only 26 in monkey kidney cultures. All 75 strains were later
passed without difficulty to amnion cultures, in which all neutral-
isation tests were performed. Amnion cultures were used because
they were available in the largest quantities. Four strains not
originally isolated in kidney cultures were later passed to kidney
cultures without difficulty, and 1 strain not originally isolated
in thyroid cultures was similarly passed to thyroid. This was done
in an attempt to find if the very different isolation rates in the
different types of cells were due to differences in cytopathogenicity
between strains of Frater virus or simply to differences in the sus-
ceptibility of the different types of cell for all strains. Every
isolate readily produced typical cytopathic changes in each type of
cell to which it was passed. The differences seemed to be due there-
fore to basic differences in the susceptibilities of the 3 cell
types to infection by the virus.

In order to obtain further support for this hypothesis, 12
stool extracts known to contain Frater virus were titrated in all 3
types of cultures. The titres obtained are shown in Table 29.

## TABLE 29

## Titres[x] in thyroid, amnion, and kidney cultures of

## 12 wild strains of Frater virus

| Thyroid | 3.0 | 3.0 | 5.0 | 4.2 | 2.5 | 1.2 | 0.5 | 0.2 | 0.7 | 0 | 0 | 0 |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|-----|-----|
| Amnion | 1.7 | 1.5 | 5.0 | 4.5 | 0.7 | 0 | 0 | 0 | 0.7 | 1.2 | 0 | 0.5 |
| Kidney | 1.7 | 0.2 | 3.5 | 4.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0 |

x  Titre expressed as $\log_{10}$ $TCD_{50}$ per 0.1 ml.,

the inoculum per tube being 0.1 ml.

In general, higher titres were obtained in thyroid than in amnion, and higher titres in amnion than in kidney. However, in those speci-mens which contained very little virus the chance distribution of virus particles among the various aliquots of inoculum appeared to have determined the result more than the relative susceptibility of the cultures. Seed of 5 strains of tissue-culture-adapted virus known to be of considerably higher titre than the wild viruses in the stool extracts was also titrated in the 3 types of cultures. As shown in Table 30, the titre was highest in amnion and lowest in kidney in every case. These results clearly showed that human thyroid, human amnion, and monkey kidney cells varied markedly in their susceptibility to infection by Frater virus.

A single experiment was carried out to find if Frater virus would produce plaques in human amnion or thyroid cells under an agar overlay. Monkey kidney cells were not investigated because of their lesser susceptibility to the virus. Monolayers of each

## TABLE 30

### Titres[x] in thyroid, amnion, and kidney cultures of

### 5 tissue-culture-adapted strains of Frater virus

| Thyroid | 5.8 | 6.5 | 6.5 | 6.5 | 5.5 |
|---------|-----|-----|-----|-----|-----|
| Amnion  | 4.2 | 5.8 | 5.2 | 5.2 | 4.5 |
| Kidney  | 1.8 | 3.8 | 2.8 | 3.5 | 2.5 |

x    Titre expressed as $\log_{10}$ $TCD_{50}$ per 0.1 ml.,

the inoculum per tube being 0.1 ml.

type of cell were grown in Petri plates 6 cm. in diameter. The
layers were washed with PBS, and 1 ml. of each of a series of ten-
fold dilutions of prototype Frater virus with an original titre of
$10^{5.5}$ $TCD_{50}$ was added to each of 2 plates. After 1 hour the layers
were again washed and an overlay of agar containing double strength
Earle's saline and 5% calf serum was added. Ten days later, when
cytopathic changes were observed, 1:1000 neutral red solution was
added. In the amnion monolayers nothing was seen in those which
received the $10^{-6}$ inoculum, a small number of apparent plaques were
observed in those which received the $10^{-5}$ dilution and many were
seen in those which received the $10^{-4}$ dilution. The plaque-like
areas were irregular in shape and 1-2 mm. in diameter. No evidence
of plaque formation was seen in the thyroid cultures. It would have
been essential to repeat these experiments to have been sure that
the appearances observed represented true plaque formation. I did
not have time to do this before leaving the laboratory but my col-

league Dr. M.C. Timbury carried out further experiments after I
left. These will be referred to more fully in the course of the
discussion in Chapter XVIII. Dr. Timbury did in fact succeed in
producing plaques in amnion cells with Frater virus and this adds
weight to the probability that the appearances I observed were
indeed true plaques. Moreover the last dilution of virus with
which apparent plaques were seen in my experiment was the same as
the last dilution possessing demonstrable infectivity for amnion
cultures in tubes.

Tissue-culture-adapted strains of Frater virus were also tested
in HeLa cell cultures and in guinea pig kidney cultures. The pro-
totype strain and 14 other strains were inoculated into HeLa cell
cultures which were observed for 2 weeks. In almost all cases a
blind pass had to be made during this time to fresh cultures because
of aging of the first cultures. Cytopathic effects were not seen
with any of the strains. No attempt was made to adapt Frater virus
to grow in HeLa cells by making a series of blind passes. The
virus was tested for cytopathogenicity for HeLa cells only for the
purpose of aiding its identification. As will be discussed in
Chapter XVI no antiserum to ECHO type 10 (Reovirus type 1) was
available in the laboratory for testing Frater virus, and at an
early stage of the work before the size of Frater virus was known
to differ from that of ECHO type 10 it seemed advisable to find by
some other means if Frater virus could be a strain of this virus.
Hsiung (1958) showed that ECHO type 10, unlike the true ECHO viruses,
is cytopathogenic for guinea pig kidney cultures, and I therefore

tested Frater virus in this type of culture. The cells were pro-
pagated in Hanks' saline plus 10% calf serum and maintained in the
maintenance medium routinely used. The prototype strain and 10
other strains of Frater virus, and the Lang strain of ECHO type 10
virus which served as a control, were inoculated into tubes of
guinea pig kidney cultures. The Frater virus strains caused no
changes, but the Lang virus produced complete degeneration of the
cultures.

### Growth characteristics in amnion and kidney cultures

As thyroid and human amnion cultures were found to be more
sensitive than monkey kidney cultures to infection by Frater virus,
I carried out a number of experiments in an attempt to discover the
basis for these differences. The comparison was made between kidney
and amnion rather than between kidney and thyroid, where the dif-
ference was even greater, because much larger numbers of amnion
than thyroid cultures were available for use in the experiments.

The seed of the tissue-culture-adapted strains of Frater virus
employed for the titrations reported in Table 30 was grown in human
amnion culture. It was possible that adaptation to amnion might
have had the effect of producing seed which would give a higher
titre in amnion than in kidney cultures. Accordingly the prototype
strain of Frater virus was passed twice in monkey kidney cultures
and this seed was titrated in human amnion and monkey kidney cultures.
The titre in amnion was $10^{-5.5}$ and that in kidney was $10^{-3.8}$. These
results are very similar to those shown in Table 30 for virus grown
in amnion cultures. Thus the type of tissue in which the seed virus

was grown did not appear to affect the relative titres in the two tissues.

The differences in titre of the same seed titrated in monkey kidney and human amnion cultures might have been due to the virus growing in both types of cell to the same dilution but producing obvious cytopathic changes in kidney cells only with the higher concentrations of virus. Such a phenomenon occurs with poliovirus growing in thyroid cultures. To determine if this was also taking place when Frater virus was growing in monkey kidney cultures, two experiments were performed. First, on the analogy of Hsiung's finding (1959a) that some wild strains of enteroviruses produced cytopathic changes in monkey kidney cell monolayers only under an agar overlay, an experiment was carried out to find if there was any evidence to suggest that Frater virus in the small doses which are present in the higher dilutions of tube titrations might produce cytopathic changes only under an agar overlay. Ten-fold dilutions of prototype strain Frater virus were inoculated in 0.1 ml. volumes to sloped tubes of monkey kidney cultures and human amnion cultures. After 1 hour at $37^{O}C$ the monolayers were washed with buffered saline and molten agar at $43^{O}C$ containing Earle's saline and 5% calf serum was added to each tube. The tubes were stoppered and incubated at $37^{O}C$. The virus titre in the monkey kidney cultures was $10^{-2.5}$ and in the human amnion cultures $10^{-5.5}$, results very similar to those obtained when Frater virus was titrated in tubes containing the usual fluid medium.

In the second experiment, seed of the prototype Frater strain

with a titre of $10^{-5.5}$ in human amnion was titrated in monkey kidney

cultures. Ten-fold dilutions were employed and 6 tubes were inoculated

with each dilution. All 6 tubes of the $10^{-3}$ dilution, 1 tube of the

$10^{-4}$ dilution, and none of the $10^{-5}$ or $10^{-6}$ dilutions showed cyto-

pathic effects. The contents of the $10^{-5}$ tubes, the $10^{-6}$ tubes, and

the 5 of the $10^{-4}$ tubes not showing cytopathic changes were frozen

and thawed twice to free into the culture fluid any virus present

inside the cells. The fluids from the tubes of each of the dilutions

were separately pooled, and each pool was titrated for Frater virus

in thyroid cultures. No virus was detected in any of them. Growth

of virus in the cells thus corresponded exactly with the visible

cytopathic effects.

There was therefore no evidence that the difference in titre

of Frater virus in monkey kidney and human amnion cultures was due

to growth without visible cytopathic changes of small virus inocula

in kidney cells. The most probable alternative explanation was that

a smaller proportion of the virus particles in a suspension of Frater

virus could successfully infect kidney than could infect amnion cells

under the conditions of the titrations. Marked differences in the

rates of adsorption of the virus to the two types of cell might ac-

count for this if the adsorption rate to kidney was so prolonged that

some virus was inactivated by heat before it could adsorb. An

experiment was carried out to determine the relative adsorption

rates of Frater virus to monkey kidney and human amnion cells. Mono-

layers of monkey kidney and human amnion were grown in 6 oz. bottles.

The mature monolayers were washed twice with maintenance medium and

the medium was removed from the bottles. An inoculum of 100 $TCD_{50}$ of prototype strain Frater virus in 10 ml. maintenance medium was added to each of 3 bottles, one containing an amnion monolayer, one containing a kidney monolayer, and one an empty bottle to serve as a control. The bottles were incubated at $37^{O}C$ for 8 hours and 0.35 ml. samples were taken from the supernatant fluid medium in the tissue culture bottles at 30 minutes, 1 hour and then hourly until 8 hours, and from the control bottle at 1, 2, 6, and 8 hours. The samples were immediately frozen at $-40^{O}C$ and all were later titrated in human amnion cultures. The results of the experiment are shown in Figure 16. There was a steady fall in titre of virus in the medium in the amnion bottle from the original of $10^2$ $TCD_{50}$ to $10^{0.6}$ $TCD_{50}$ at 7 hours. By 8 hours virus began to be freed from infected amnion cells into the culture fluid. The fall in virus level in the medium in the kidney bottle was considerably slower and at 7 hours was still $10^{1.5}$ $TCD_{50}$. Frater virus appears therefore to adsorb to amnion cells faster than to kidney cells. This one experiment does not explain why the adsorption process is slower. It is possible that only a proportion of the virus particles are capable of adsorption to monkey kidney cells. The results shown in Figure 16 suggest that virus adsorbs to kidney cells only for the first 5 hours, and that after this time no further adsorption occurs. Such an adsorption pattern would be expected if only a proportion of the virus particles in the suspension were capable of adsorption to the cell layer.

On many occasions I observed that cytopathic changes progressed much more rapidly in amnion than in kidney cells inoculated with the
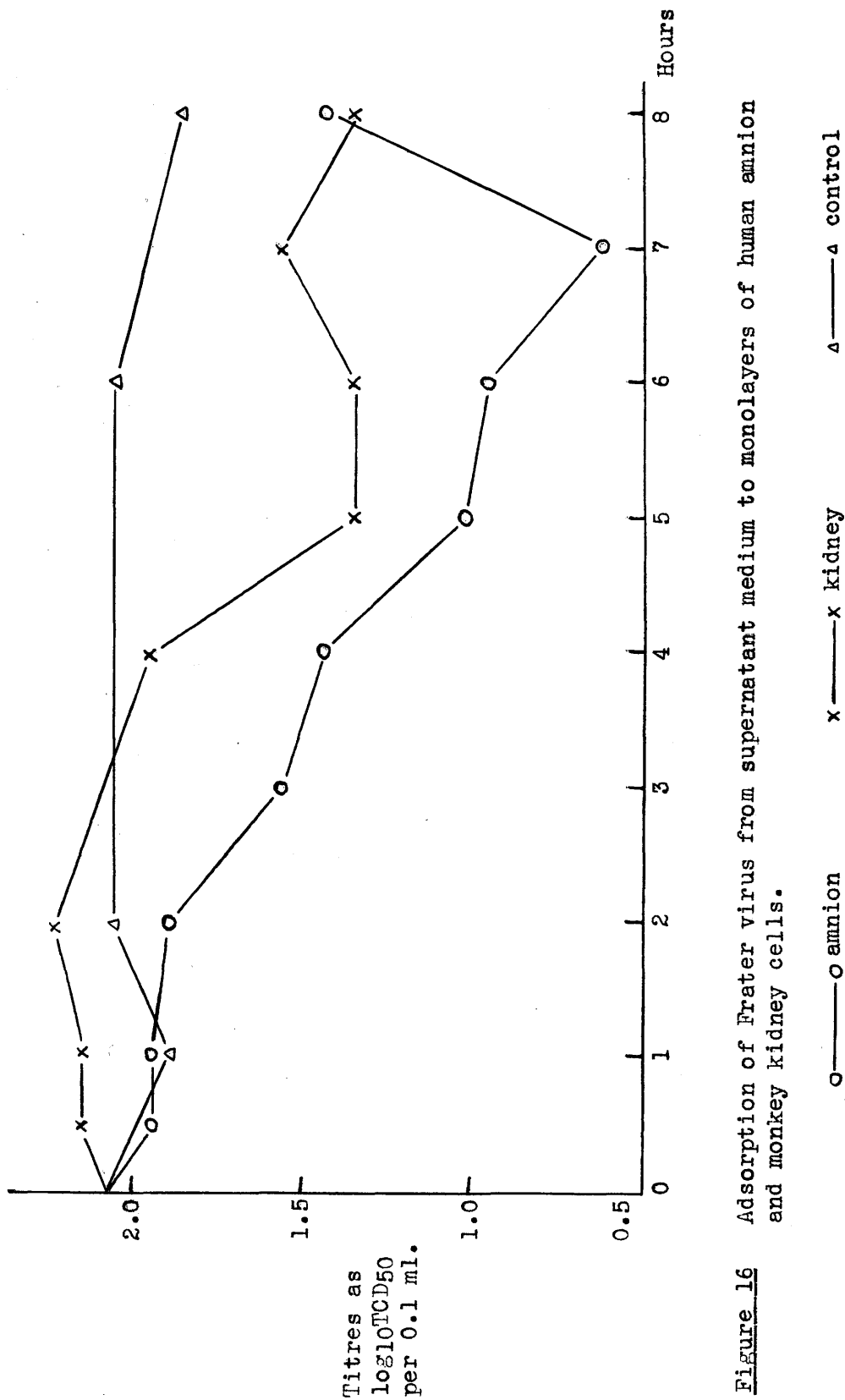
Figure 16    Adsorption of Frater virus from supernatant medium to monolayers of human amnion and monkey kidney cells.

o———o amnion     x———x kidney     △———△ control

same amount of Frater virus. A single experiment was done to show
if the release of virus into the medium occurred correspondingly
sooner with amnion cultures than with kidney. The same inoculum
of 100 $TCD_{50}$ of prototype strain Frater virus was added to 10 ml.
maintenance medium in each of two 6 oz. bottles, one containing a
monolayer of amnion cells and the other a monolayer of kidney cells.
The bottles were incubated at $37^{\circ}C$ and 0.3 ml. samples of medium
were removed from each bottle at 4 hours, 8 hours, 24 hours, and
then daily until 1 day after complete degeneration of the culture.
Each sample was immediately stored at $-40^{\circ}C$ and all the samples were
titrated at the same time in amnion cultures. Figure 17 shows
graphically the liberation of virus into the media during growth
of each type of cell, and Table 31 correlates the amount of virus
in the medium with the progress of the cytopathic effects. The
maximum yield of virus in the medium was at 2 days with amnion and
at 5 days with kidney while complete degeneration of the monolayer
was seen at 4 days with amnion and at 5 days with kidney.

## Cytopathic effects

All strains of Frater virus examined showed exactly the same
type of cytopathic effects as known types of enteroviruses in fresh
unstained preparations of human thyroid, human amnion, and monkey
kidney cultures. In thyroid cultures, for example, the cytopathic
effects of Frater virus were the same as those described in Chapter V
for strains of enteroviruses and illustrated in Figures 8 and 9.

The detailed descriptions which have been published of the cyto-
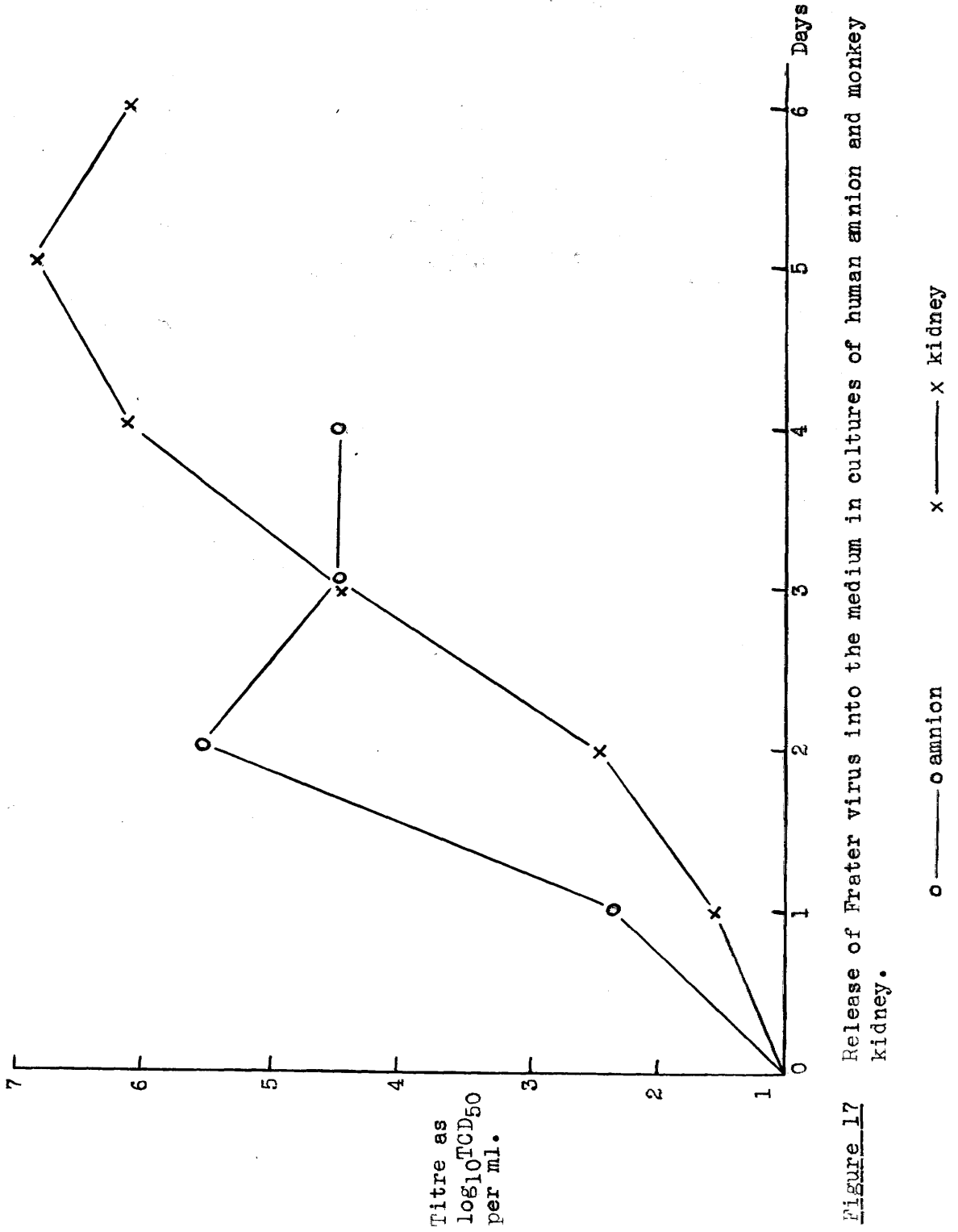pathic effects of ECHO viruses, refer to their growth in monkey

<u>Figure 17</u>    Release of Frater virus into the medium in cultures of human amnion and monkey kidney.

o ———— o amnion        x ———— x kidney

## TABLE 31

### Release of Frater virus into the medium and

### stage of cytopathic effect in tissue cultures

| | Day | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Kidney | CPE | 0 | 0 | Tr | 1 | 4 | 4 |
| | TCD$_{50}$/ml. in medium | $10^{1.5}$ | $10^{2.5}$ | $10^{4.5}$ | $10^{6.1}$ | $10^{6.9}$ | $10^{6.1}$ |
| Amnion | CPE | 0 | Tr | 3 | 4 | 4 | 4 |
| | TCD$_{50}$/ml. in medium | $10^{2.3}$ | $10^{5.5}$ | $10^{4.5}$ | $10^{4.5}$ | NT | NT |

0   No change                      3   75% of cells affected

Tr   Trace                          4   Complete degeneration

1   25% of cells affected          NT   Not tested

kidney cultures. Accordingly a series of coverslip preparations were made of monkey kidney monolayers infected with Frater virus. Kidney cell cultures were grown on coverslips in Petri dishes incubated in an atmosphere containing 5% carbon dioxide. When mature monolayers had formed a heavy inoculum of Frater virus was added to the medium in the Petri dish. Coverslips were removed at 1, 2, 4, 6, 8, 12, 24, 32, and 48 hours after inoculation of the virus. The monolayers were fixed in Rhodes' fixative and stained with Giemsa's stain. Uninfected monolayers were also stained in the same way to act as controls.

No changes were seen in the cultures until 6 hours after infec-

tion. At 6 hours and 8 hours, very scanty cells were observed to contain small eosinophilic inclusions in the cytoplasm. At 12 hours the cytoplasmic inclusions were much larger but were limited to a small proportion of the cells. Small granular eosinophilic inclusions were first observed in the nuclei of a few cells at 6 and 8 hours. At 12 hours larger intranuclear granules were visible. These were more densely staining and were seen in a larger proportion of the cells. At later stages cells were seen in which the cytoplasmic inclusion appeared to indent the nucleus giving the periphery of the nucleus the appearance of a membrane folded over upon itself. The nucleus then became contracted and pyknotic. At the final stage of infection the cells were small and rounded, and were composed of eosinophilic and sometimes rather more basophilic cytoplasm occupying most of the cells but with a semilunar rim of densely staining nuclear remains at one side of the periphery. Even at 48 hours, although cells at all stages of infection could be seen, only a small proportion of the cells of the monolayer showed cytopathic changes.

These changes seen with Frater virus were the same as those produced by most of the enteroviruses. No changes were seen resembling those produced in the nuclei by ECHO viruses types 22 and 23 (Shaver, Barron, and Karzon, 1961).

## Pathogenicity for animals

Suckling mice. Mice in the first 24-36 hours of life were inoculated with many strains of Frater virus. An entire family of mice was inoculated with each specimen. Each mouse received 0.03 ml. intra-peritoneally, and in addition those injected with 3 of the

tissue culture fluids received 0.01 ml. intra-cerebrally. All the
mice were observed for at least 2 weeks for signs of paralysis and
in any doubtful case a second pass was made to another family of
mice. Tissue culture fluid of 12 different strains of Frater virus
containing from $10^3$ to $10^{4 \cdot 5}$ $TCD_{50}$ per dose was injected into baby
mice. Two of the strains including the prototype strain were in-
oculated into two families of mice. In each case one family received
first tissue-culture-passage fluid and the other family eighth or
ninth passage fluid. None of these viruses caused disease in the
mice. Mice injected with 6 of the 12 strains were examined histo-
logically and no abnormalities were seen.

Aliquots of 23 stool extracts known to contain Frater virus were
also injected into suckling mice. Six of these stool extracts con-
tained strains of virus which were also injected into mice in the
form of tissue-culture-passage material. This included the prototype
strain. None of the mice injected with these 23 stool extracts be-
came sick.

In one or other form, as wild or as tissue-culture-adapted virus,
29 strains of Frater virus were inoculated to suckling mice. None
produced any effect on the mice.

Adult mice. Tenth tissue-culture-passage seed of the prototype
strain was injected intracerebrally into 6 adult mice in a dosage of
0.03 ml. The mice were observed for 4 weeks and they all remained
healthy.

Guinea pigs. Six guinea pigs received a course of 4 or 5 intra-
muscular injections of 1 ml. of one or other of 6 strains of Frater

virus for the purpose of preparing antisera to the viruses. One of

the viruses was the prototype strain. The animals showed no signs of

illness and appeared normal at autopsy.

Chick embryos. Prototype strain virus was inoculated on to the

chorio-allantoic membranes of 3 14-day-old hen's eggs, intra-amnio-

tically to 3 12-day-old eggs, intra-allantoically to 3 12-day-old eggs,

and into the yolk sac of 3 7-day-old eggs. The seed was tenth tissue-

culture-passage fluid and the dose in every case was 0.1 ml. No

lesions were seen on the chorio-allantoic membranes of eggs examined

3 days and 4 days after inoculation. Allantoic and amniotic fluids

removed on the 3rd and 4th days from eggs inoculated by the allantoic

route and from those inoculated by the amniotic route failed to agglu-

tinate human and fowl red cells. The chick embryos survived in all

the eggs, including those which were inoculated into the yolk sac and

observed until just short of hatching.

## Haemagglutination

Prototype strain and 6 other strains of Frater virus were grown

in the same batch of human amnion cultures. A control preparation

was made by incubating uninoculated amnion cultures of the same batch

and harvesting them in the same way as the virus-containing fluids.

The virus suspensions and the control fluid were tested for haemag-

glutination by mixing 0.3 ml. volumes of the fluids and of red cell

suspensions in plastic haemagglutination plates. Each was tested

with a 1% suspension of human group O cells and with a 0.5% suspension

of fowl cells of a type which gave strong agglutination with vaccinia

virus. All the tests were done in replicate at 4°C, 23°C, and 37°C.

Haemagglutination was not observed in any of the tests.

## CHAPTER XVI

## SEROLOGICAL INVESTIGATIONS

Most of the serological work of attempting to identify Frater

virus was done with two strains. Strain 1 was the prototype strain

isolated from the patient referred to as Case 3 in Chapter X. This

patient came from Darvel, Ayrshire. Strain 2 was isolated from a

Rutherglen patient with aseptic meningitis. These two viruses were

tested as completely as possible, and all other strains of Frater

virus were identified by means of antisera to one of these two. Most

of the strains were identified with strain 1 antiserum which was a

more potent serum than the other. Antisera were also prepared to 4

other strains so that cross-neutralisation tests might be carried out.

Strain 3 was from a Glasgow patient and strain 4 from a Greenock pa-

tient; both suffering from aseptic meningitis. Strain 5 was from a

Glasgow infant who was a carrier of the virus, and strain 6 was from

a Dundee child with aseptic meningitis. The strains selected were

thus representative of several different geographical areas affected

by the Frater virus epidemic.

### Neutralisation tests performed on isolates

At an early stage of the investigations before it became apparent

that most of the viruses isolated in 1959 were strains of a new virus,

19 isolates later found to be Frater virus were tested by the standard

neutralisation test described in Chapter II with the full range of

enterovirus antisera available. Strains 1, 2 and 3 were among the 19

tested. The antisera employed were sera against the 3 types of polio-

virus, Coxsackie viruses A9 and B1 to 5, and ECHO viruses types 1 to 20

but excluding types 10 and 16. Sera to types 10 and 16 could not be readily obtained. Clear neutralisation did not occur in any of the tests, but the Coxsackie B5 antiserum caused 1 to 2 days' delay in the appearance of cytopathic changes with a few of the isolates. This serum was of low titre and had to be used at a dilution of 1:8. It therefore seemed unlikely that this delaying of cytopathic changes represented specific neutralisation, particularly as the serum at this dilution gave quite clear neutralisation of known strains of Coxsackie B5 virus.

When antisera had been prepared against strain 1 and 2 Frater virus all unknown isolates were tested with these sera and thus identified as Frater virus, the more potent strain 1 serum being used in most cases.

### Neutralisation tests with enterovirus prototypes
### and antisera to two Frater viruses

Antiserum to strain 1 Frater virus was prepared by injecting a guinea pig intra-muscularly with a series of 1 ml. doses of virus suspension. A course of 8 injections was given, with 4 day intervals between injections. Antisera to strains 2, 3, 4, 5, and 6 virus were similarly prepared, except that only 4 injections were given of the strain 2 virus and 7 in the case of each of the others.

Neutralisation tests were carried out with strain 1 and strain 2 antisera against seed of the 3 types of polioviruses, Coxsackie viruses types A9 and B1 to B6, and ECHO viruses types 1 to 9 and 11 to 27. None of these viruses was neutralised by either antiserum.

Additional tests were carried out in order to determine if the

minor effect of Coxsackie B5 antiserum on some of the isolates was
of any significance. Neutralisation tests were performed with strain
1 Frater antiserum at 1:8 dilution against Coxsackie B5 virus in
doses of 1 $TCD_{50}$, 10 $TCD_{50}$, and 100 $TCD_{50}$, and also with Coxsackie
B5 antiserum at 1:8 dilutions against Frater virus in doses of 1 $TCD_{50}$,
10 $TCD_{50}$, and 100 $TCD_{50}$. In no case was neutralisation observed. At
the same time as the antibody titres to Frater virus were determined
in samples of gamma-globulin, as recorded in Table 28 in Chapter XII,
the titres of antibody to Coxsackie B5 were measured in 3 of the
samples. The Coxsackie B5 antibody levels in the samples were 1:64,
1:64, and 1:32 and the corresponding Frater virus antibody levels in
the same samples were 1:16, 1:32, and 1:128. This lack of correla-
tion in the levels provides additional evidence that Frater virus is
not related to Coxsackie B5 virus.

## Complement fixation tests

Seed of strain 1 Frater virus grown in human amnion cultures was
used as antigen in complement fixation tests with antisera to herpes
virus and to adenovirus. Controls of known strains of herpes virus
and adenovirus grown in tissue culture were also set up. The tests
with Frater virus and each of these sera were negative, while the con-
trol tests with homologous virus and antiserum were positive even
with diluted virus antigen.

The same Frater virus seed was also tested by the complement
fixation method against 3 human sera and 2 guinea pig sera. All 5 of
these sera had been shown previously to contain neutralising antibodies
to Frater virus. A serum taken on the 23rd day after the onset of

aseptic meningitis in a patient contained no detectable complement
fixing antibodies to Frater virus although the titre of neutralising
antibodies was 1:2048. Paired sera taken on the 3rd and 13th days
after onset of illness from another patient with aseptic meningitis
contained complement fixing antibodies to a titre of 1:8 in the first
specimen and to a titre of 1:64 in the second. The corresponding
levels of neutralising antibodies in the pair were 1:8 and 1:512.
Two sera from different guinea pigs both had levels of complement
fixing antibodies of 1:128, the levels of neutralising antibodies
being 1:256 in one and 1:512 in the other.

A single experiment was carried out to find at what stage in
the growth of Frater virus in tissue culture, complement fixing anti-
gen was released. Strain 1 virus was grown in human amnion cultures
in tubes, and at 18 hours, 2 days, and daily to 5 days tubes were
frozen, thawed, and centrifuged to remove cell debris. The super-
natant fluids were tested in complement fixation tests with one of
the guinea pig antisera known to contain complement fixing anti-
bodies to Frater virus. Two-fold dilutions of each fluid were tested.
Antigen was not detectable at 18 hours. By the second and third days
antigen was detectable in a 1:2 dilution of the fluid but not in a
1:4 dilution. On the fourth day when the cytopathic changes had pro-
gressed to complete degeneration of the monolayer, antigen was found
at 1:4 but not at 1:8 dilution of the fluid. Antigen was detected
at 1:2 but not at 1:4 dilutions of the fluids taken on the fifth and
sixth days.

## Cross-neutralisation tests with different strains

## of Frater virus

Each of strains 1 to 6 of Frater virus was tested in neutralisation tests against its homologous antiserum and each of the antisera to the other 5 viruses. The titre of each antiserum for its homologous virus was 1:1240 except for strain 2 antiserum which had a homologous titre of only 256. The results of these cross-neutralisation tests are shown in Table 32, adjusted to allow of better comparison in such a way that the titre of each serum for its homologous virus is represented as 1 unit. Thus with serum 4 the actual titre with virus 4 was found to be 1:1024 and with 2 to be 1:64. The titre of homologous virus with serum 4 being 1 unit the titre of virus 2 would be 16 units since 1024 divided by 64 is 16. Strains 3, 4, 5, and 6 show almost complete cross-neutralisation both with strain 2 and with the 3, 4, 5, and 6 group. Strain 2 behaves antigenically like the "specific phase" strains of ECHO type 6 described by Karzon, Pollock, and Barron (1959). These workers found that "specific phase" variants of ECHO type 6 could be changed to the "broad phase" by repeated passage through tissue cultures. In the tests reported in Table 32, 8th tissue-culture-passage seed of strain 2 virus was used, and an experiment was carried out to find if further passage in tissue cultures would yield seed which resembled the "broad phase" variants of Karzon and his colleagues. Eighth pass seed and 30th pass seed of strain 2 were tested against antisera to strains 1, 2 and 5. The titres in each case were the same with both batches of virus seed.

## TABLE 32

### Serological relationships of Frater virus strains 1 to 6

| Virus strain number | Antiserum to strain number | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 1 | 2 | 4 | 4 | 4 | 1 |
| 2 | 1 | 1 | 16 | 16 | 8 | 8 |
| 3 | 1 | 2 | 1 | 4 | 1 | 1 |
| 4 | 1 | 2 | 2 | 1 | 1 | 2 |
| 5 | 1 | 2 | 4 | 1 | 1 | 2 |
| 6 | 1 | 2 | 2 | 2 | 1 | 1 |

Titres expressed as number of units of antiserum, 1 unit
being titre of antiserum with homologous virus.

## CHAPTER XVII

## PHYSICAL PROPERTIES

The work reported in earlier chapters showed that Frater virus had the type of cytopathic properties of an enterovirus, but could not be identified as any known member of this family. I therefore carried out a number of experiments which are reported in this chapter, to find if Frater virus possessed the same degree of resistance to changes of temperature and to chemicals as known enteroviruses and to find if its size was similar to that of enteroviruses. All the experiments were performed with seed of the Frater prototype strain which had been purified previously by the method of terminal dilutions.

### Stability at different temperatures

Repeated freezing. 2 ml. of virus seed was frozen by immersing the tube in a mixture of solid carbon dioxide and alcohol and thawed by immersion in cold water. In this and also in the later temperature experiments the virus was suspended in Earle's saline plus 5% calf serum. The cycle of freezing and thawing was repeated 6 times. Samples removed after 2 cycles and after 6 cycles were titrated at the same time as the original seed. The titre of each of the 3 was the same, $10^{6.2}$ $TCD_{50}$ /ml.

Stability at $4^{o}C$. A stoppered $3 \times \frac{1}{2}$ in. tube containing 1.5 ml. virus seed was maintained at $4^{o}C$ for 12 weeks, and 0.1 ml. samples were removed after 3 days and after 1, 2, 3, 6, 7, and 12 weeks. The samples were frozen at $-40^{o}C$ and titrated in tubes of human amnion cells at the end of the experiment. The results of the titrations are shown in Table 33. It is clear that Frater virus retains its

activity for a long period at 4°C.

## TABLE 33

### Residual virus activity after storage at 4°C

| Period of storage | Days | | Weeks | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 3 | 1 | 2 | 3 | 6 | 7 | 12 |
| Titre as $\log_{10}$ TCD$_{50}$ | 5.5 | 5.5 | 5.5 | 5.2 | 5.2 | 4.8 | 4.8 | 3.5 |

Stability at **room temperature**. A stoppered 3 x $\frac{1}{2}$ in. tube containing 1.5 ml. virus was left at room temperature for 3 weeks, and 0.1 ml. samples were removed after 1, 2, 4, 7, 10, 14, and 21 days. The temperature varied between 20°C and 25°C during the period. The samples were stored and titrated in the same way as before. The results are presented in Table 34.

## TABLE 34

### Residual virus activity after storage at 20-25°C

| Days of Storage | 0 | 1 | 2 | 4 | 7 | 10 | 14 | 21 | 49 |
|---|---|---|---|---|---|---|---|---|---|
| Titre as $\log_{10}$ TCD$_{50}$ | 5.5 | 5.8 | 4.8 | 4.8 | 3.8 | 3.8 | 3.5 | 2.5 | nil |

Inactivation at room temperature was considerably more rapid than at 4°C.

Stability at 37°C. Two samples of virus were maintained at 37°C, each for a different period. A 1 ml. sample in a stoppered 3 x ½ in. test tube was held in a water bath at 37°C for 24 hours, and 0.1 ml. amounts were removed at 2, 4, 8, and 24 hours. A second 5 ml. sample was kept in a bijou bottle in a 37°C incubator for 3 weeks, and 0.5 ml. amounts were removed after 1, 3, 5, 7, 10, 15, and 20 days. The virus was suspended in Hanks' saline plus 2.5% calf serum, 0.5% lactalbumin hydrolysate, and 0.14% sodium bicarbonate. This method was chosen to be the same as that used by Lehmann-Grube and Syverton (1959) to test known ECHO virus prototypes, in order that valid comparisons might be made with Frater virus. The results are shown in Table 35.

TABLE 35

Residual virus activity after storage at 37°C

| Period of storage | Hours | | | | Days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 8 | 1 | 3 | 5 | 7 | 10 | 14 |
| Titre as $\log_{10}$ TCD$_{50}$ | 5.5 | 5.8 | 5.2 | 5.2 | 4.8 | 2.8 | 2.5 | 2.2 | nil | nil |

Stability at 56°C. A mixture of 2 ml. seed virus and 2 ml. PBS in a stoppered 3 x ½ in. tube was kept in a water bath at 56°C for 30 minutes. The phosphate buffer was used because at 56°C the carbon dioxide in the usual bicarbonate buffer was lost the first time the tube was unstoppered. Samples, each of 0.3 ml. volume, were taken

after 2, 5, 10, 20, and 30 minutes. The samples were titrated in amnion cultures at the end of the experiment. The results are shown in Table 36. Frater virus was clearly inactivated rapidly at 56°C.

TABLE 36

Residual virus activity after inactivation at 56°C

| Time in minutes | 0 | 2 | 5 | 10 | 20 | 30 |
|---|---|---|---|---|---|---|
| Titre as $\log_{10}$ TCD$_{50}$ | 4.5 | 0.5 | 0.5 | nil | nil | nil |

Stability at different pH levels

The pH of each of 9 aliquots of seed virus suspended in 1 ml. of Earle's saline was adjusted with 0.5 N hydrochloric acid or 0.5 N sodium hydroxide to give a range of pH levels each differing by 1 unit from 2 to 10. The 9 aliquots and an untreated aliquot which served as a heat control, were held at 37°C for 16 hours. The pH of each sample was then restored to 7 by the addition of acid or alkali as required, and the volume of each made up to 2 ml. with Earle's saline. Each of these 10 aliquots and another aliquot of the original seed virus were titrated in the same batch of human amnion cultures. The results are given in Table 37. Frater virus was stable from pH 4 to pH 8, and showed greater inactivation in highly alkaline solutions than in highly acid solutions.

## TABLE 37

### Residual virus activity after exposure to various

### levels of pH

| Original | Heat control | pH | | | | | | | | |
|----------|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 4.5 | 4.5 | 2.5 | 3.8 | 4.2 | 4.5 | 4.2 | 2.5 | 4.2 | 2.2 | 0.8 |

### Stability in diluents other than maintenance medium

Glycerol. An experiment was carried out to find if storage of

Frater virus in 50% glycerol would increase its stability at room

temperature. It was of practical value to obtain this information

in order to find the best means of transmitting the virus by post to

other laboratories. Virus seed in maintenance medium was mixed with

an equal volume of glycerine and left at 20-25°C for 3 months. The

original virus titre was $10^{-5.5}$, after 6 weeks the titre was $10^{-2.5}$,

and after 3 months no live virus could be detected. This may be com-

pared with the results for virus stored at room temperature without

added glycerine. All virus activity in this case had been lost in 7

weeks. Although the times of sampling were not exactly the same,

glycerol does appear to increase the stability of Frater virus on

storage.

Distilled water. In the preparation of Frater virus for electron

microscopy, which is discussed later, the virus had to be suspended

in distilled water. As a preliminary, therefore, its stability in

distilled water was determined. 0.2 ml. of seed virus was added to

19.8 ml. of distilled water and as a control similar volumes of virus
and PBS were mixed. Both were kept for 21 hours at 18°C (room tem-
perature), when sodium chloride was added to the distilled water
sample to restore its isotonicity. Both samples were titrated in
amnion cultures. The titre of the virus suspended in distilled water
was $10^{-3.2}$ and that of the control sample $10^{-3.5}$. The virus is thus
as stable for short periods in distilled water as in buffered saline
solution.

### Ether resistance

A preliminary test of the ether resistance of the virus was done
by mixing 0.5 ml. of virus suspension with 0.5 ml. of ethyl ether.
The mixture was well shaken and left on the bench for 2 hours, excess
either was removed, and the suspension stored at 4°C overnight. A
control suspension of virus without ether was treated in the same way.
Both samples were titrated for virus activity and both had exactly
the same titre of $10^{-3.5}$.

The test was repeated by a somewhat different method. A mix-
ture of 1 ml. virus suspension and 4 ml. ethyl ether was shaken in
a bijou bottle and kept at 4°C for 21 hours. The mixture was reshaken
and the ether allowed to evaporate by leaving the mixture in an open
Petri dish on the bench for 30 minutes. A control suspension without
ether was held at the same temperatures for the same periods. Both
ether-treated virus and the control virus were titrated in amnion cul-
tures. The $TCD_{50}$ of treated virus was $10^{-4.2}$ and that of the control
specimen was $10^{-4.5}$, for practical purposes the same. Frater virus
is therefore an ether-stable virus.

## Ultracentrifugation

In the investigation of methods of concentrating Frater virus seed for electron microscopy tissue culture fluid was ultracentrifuged. Two tubes each containing 12 ml. of fluid were centrifuged for 2 hours at 40,000 r.p.m. (144,000 g.) in the type 40 rotor of a model L Spinco ultracentrifuge. After centrifugation the supernatant was carefully removed in separate 2 ml. batches from the tops of the tubes downwards. The first sample from each tube was pooled, the second from each was pooled and so on. The seventh sample consisted of the pooled reconstituted pellets of sediment from the two tubes. The original titre of the uncentrifuged virus suspension was $10^{-5.5}$. The titre of each of the first 4 samples was $10^{-2.5}$, that of the fifth was $10^{-3.5}$, and that of the sixth was $10^{-4.5}$. The titre of the reconstituted pellet was $10^{-6.5}$. Most of the virus had been deposited in the pellet by this cycle of centrifugation.

## Ultrafiltration

Gradocol membranes. Membranes of sizes suitable for the measurement of viruses by ultrafiltration were not commercially available when this work was done. I was able to obtain single gradocol membranes of average pore diameter 100 mμ, 50 mμ, and 10mμ from Dr. P. Wildy. The origin of these membranes, which had been stored in his laboratory for some time, was uncertain and it seemed unwise, therefore, to assume that the average pore diameters stated, were necessarily exact. I devised an experiment which would give a cross-check on the pore sizes of the membranes by filtering 3 other viruses of known size through each membrane in addition to Frater virus. As

only one membrane of each size was available, it was essential to choose viruses which could be titrated in host systems insensitive to the others because each virus filtrate was liable to be contaminated with the virus suspensions filtered previously. The viruses chosen were vaccinia virus which was titrated in hens' eggs, phage K (Hotchin, 1954) titrated in its propagating strain of staphylococcus, and poliovirus type 1 titrated in HeLa cell cultures. The diameters of these are: vaccinia virus 250 m$\mu$, phage K 80 m$\mu$, and poliovirus 30 m$\mu$. With each membrane the virus suspensions were passed through in the order Frater virus, poliovirus, phage K, and vaccinia virus. Frater virus was titrated in human amnion cultures. The host system of any one of the other 3 viruses was insusceptible to any of the viruses passed through the membrane before that particular virus. Before the virus suspensions were filtered, 4 to 5 ml. of PBS containing penicillin and streptomycin were passed through the membrane. Each virus suspension was 2 ml. in volume and each was collected after filtration in a separate tube.

After filtration each virus filtrate was titrated for infectivity in the appropriate host system. The results are shown in Table 38. The results show that the pore diameters of the membranes were within the range stated. Frater virus is clearly smaller than phage K and must thus be less than 80 m$\mu$ in diameter.

## TABLE 38

### Filtration of Frater and other viruses through

### gradocol membranes (Titres as $\log_{10}$ TCD$_{50}$)

| Virus | Titre of original pool | Titre after filtration through membrane | | |
|---|---|---|---|---|
| | | 100m$\mu$ | 50m$\mu$ | 10m$\mu$ |
| Vaccinia | 4.5 | nil | nil | nil |
| Phage K | 5.5 | 5.5 | 2.0 | nil |
| Polio | 3.5 | 3.5 | 3.5 | nil |
| Frater | 4.0 | 3.0 | 3.5 | nil |

Gesellschaft membranes.  The experiments with gradocol membranes
did not allow the size of Frater virus to be estimated within narrow
enough limits.  With the assistance of Dr. P. Wildy I was able to
obtain another type of fine-pore membrane manufactured by Membran
Filter Gesellschaft, Gottingen.  The pore sizes of these membranes
were stated to be 10-20 m$\mu$, 20-35 m$\mu$, 35-100 m$\mu$ for the 3 sizes
covering the required range.  These membranes could not be obtained
until the time at which I left the laboratory.  My colleague, Dr.
M. Timbury, actually carried out the filtration experiments with
them.  As I planned and prepared the experiments before my depar-
ture and advised Dr. Timbury in correspondence on details of pro-
cedure, it seemed permissable to include them in this thesis al-
though circumstances had prevented me from performing them personally.

The results from gradocol membrane filtration suggested that

Frater virus might be of the same general range of size as the
enteroviruses. In the experiments with Gesellschaft membranes the
Brunenders strain of type 1 poliovirus was filtered through mem-
branes of the same sizes as were used for filtering Frater virus in
order to provide a standard of comparison with a virus whose size
has been accurately determined. The Frater virus filtrates were
titrated for infectivity in amnion cultures and the poliovirus fil-
trates in HeLa cell cultures. The results are shown in Table 39.

TABLE 39

Filtration through Gesellschaft membrane filters

(titres as $\log_{10} TCD_{50}$)

| Virus | Titre of original pool | Titre after filtration through membrane | | |
|---|---|---|---|---|
| | | 35-100m$\mu$ | 20-35m$\mu$ | 10-20m$\mu$ |
| Frater | 4.5 | 4.1 | 1.1 | nil |
| Polio. | 4.0 | 3.5 | 0.6 | nil |

The smallest-pore membrane retained both viruses completely,
and the 20-35 m$\mu$ membrane held back all but a small proportion of
infective particles of each virus. The fall in titre after passage
through this membrane was almost identical with both viruses, which
strongly suggests that Frater virus is the same size as poliovirus.

Electron Microscopy

The information on size and shape which may be obtained by
electron microscopy can be of great assistance in characterising an

unusual virus. As I had no experience of this type of work I could not do it myself and I was most fortunate in obtaining the help of colleagues who carried out this examination for me. These colleagues have given me permission to quote their findings and although it might be more logical to present them in the discussion in Chapter XXVIII it is more convenient to do so in the present chapter in which all the other physical properties of the virus are described.

High-titred seed of Frater virus was obtained by passing the virus through human amnion cultures, finally growing it in human thyroid cultures, and concentrating this seed by ultracentrifugation. The subsequent preparation of the virus by the negative staining method of Brenner and Horne (1959) was done by Dr. W.C. Russell, Department of Virology, Glasgow University. The virus was mixed with an approximately equal volume of 1% ammonium acetate and centrifuged at 35,000 r.p.m. for 1 hour. The pellets were suspended in a small volume of 1% ammonium acetate and the suspension was clarified by spinning at 4,000 r.p.m. for 10 minutes. The supernatant was mixed with various amounts of phosphotungstic acid at pH 5 and pH 7 and sprayed on carbon-coated grids. These were later examined by Dr. D.H. Watson and Dr. R.W. Horne at the Cavendish Laboratory, Cambridge, in a Siemans Elmiskop microscope at an instrumental magnification of 40,000.

The virus particles were of uniform size and presented well-marked hexagonal profiles. This is seen in Figures 18 and 19. Measurements of 13 virus particles gave an average face-to-face diameter of 27 m$\mu$ and a corner-to-corner diameter of 29.5 m$\mu$.
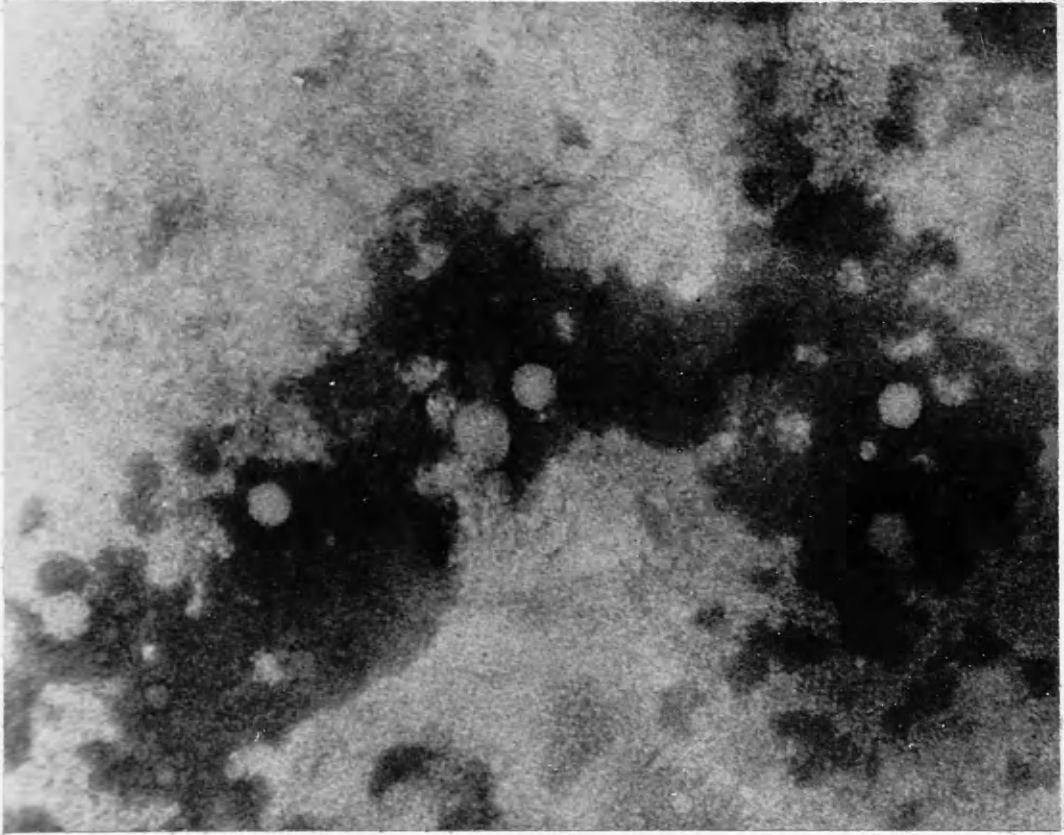
Figure 18   Electron micrograph showing several Frater virus
particles.  Phosphotungstic acid negative staining
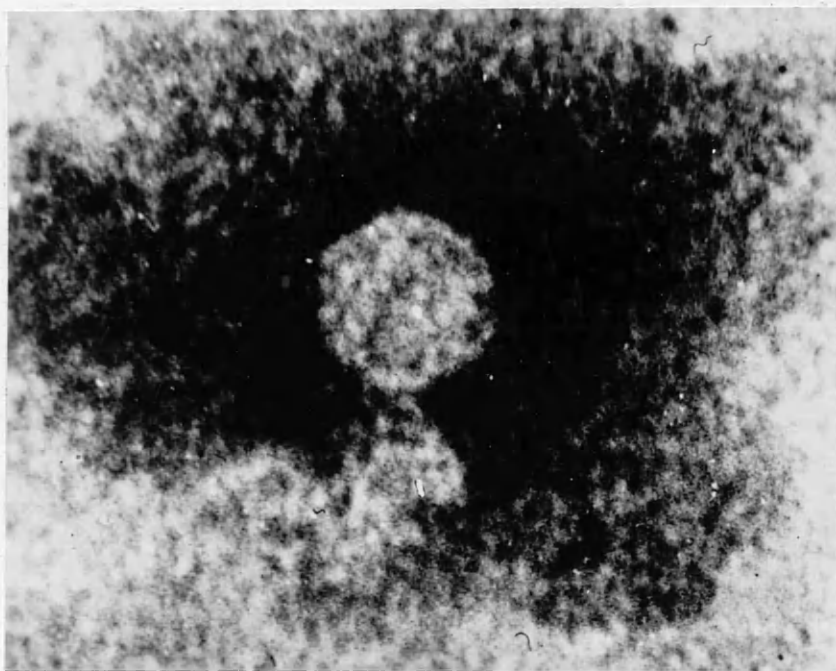method.  x175,000.

<u>Figure 19</u>   Enlargement from same photograph as Figure 1. showing
marked hexagonal profile of Frater virus.   x700,000.

No evidence was obtained of facets or capsomeres.

## CHAPTER XVIII

## DISCUSSION

The earlier work on Frater virus reported in Parts 1 and 2 of the thesis suggested that it was most probably an enterovirus. It was shown to occur in the human bowel and to be capable of causing aseptic meningitis. Its seasonal incidence was in the summer and early autumn. It was cytopathogenic for human and monkey cells in culture, and the cytopathic effects produced in the cultures were the same as those of known enteroviruses. The investigations described in Part 3 confirmed that Frater virus was indeed an enterovirus.

The detailed nuclear and cytoplasmic changes produced by Frater virus in monkey kidney cells were exactly like those which have been described with polioviruses and most types of ECHO viruses (Beale et al., 1956; Shaver, Barron, and Karzon, 1958). Frater virus, like the enteroviruses, was resistant to treatment with ethyl ether. The stability of Frater virus at the middle range of pH and the extent of its drop in infectivity at very acid and very alkaline levels, are similar to the findings of Philipson (1958) with the U strain of ECHO type 11 when tested under similar conditions. The stability of type 2 poliovirus at various pH levels has been investigated by Bachrach and Schwerdt (1952) who found that the stability was greatest over the same range as was found with Frater virus. As their experiments were carried out not at 37°C but at 4°C the actual details are not comparable.

The periods of survival of Frater virus at different temperatures

are within the ranges to be expected with enteroviruses. Its stability during repeated cycles of freezing and thawing resembles that of ECHO type 9 virus (Tyrrell et al., 1958). At room temperature and at 56$^O$C its survival was similar to that of Philipson's U virus. The experiment on the inactivation of Frater virus at 37$^O$C was done under the same conditions as the experiments carried out by Lehmann-Grube and Syverton (1959) with prototype strains of ECHO viruses. Over a period of 10 days the fall in infectivity of Frater virus was very similar to the results obtained by these workers with the prototype strain of ECHO type 4 virus.

Filtration through gradocol membranes only allowed the size of Frater virus to be estimated as less than that of phage K, the largest diameter of which has been shown by Hotchin (1954) to be 82 m$\mu$. The filtration of Frater virus and poliovirus through the series of Gesellschaft membranes gave a much closer estimate of the size of Frater virus. Filtration of Frater virus and poliovirus through the 20-35 m$\mu$ pore membrane produced a similar reduction in titre of both viruses, showing that they must be of similar size. The size of poliovirus has been thoroughly investigated. By electron microscopy, Sabin, Hennessen and Warren (1954) and Horne and Nagington (1959) found the diameter of poliovirus to be about 30 m$\mu$. Melnick et al., (1951) by measuring its rate of sedimentation in the ultracentrifuge, calculated the size as 28 or 30 m$\mu$, and Benyesh et al., (1958) using the inactivation of poliovirus by ionizing radiations as the method of measurement found its size to be 27 to 30 m$\mu$. Various groups of workers, basing their estimates on the results of

filtration through gradocol membranes, have placed poliovirus in a rather smaller range of size from 13 to 24 m$\mu$, (Benyesh et al., 1958; Melnick et al., 1951; Sabin, Hennessen, and Warren, 1954). However, considerable doubt has been cast by later workers (Markham, Smith, and Lea, 1942; Black, 1958) on the validity of the formulae proposed by Elford (1933) for calculating the size of a virus from the pore diameter of the filter membrane. The size of poliovirus is now therefore accepted as about 30 m$\mu$, and Frater virus can be assumed from my filtration results to be closely similar in size.

This estimate of the size of Frater virus agrees extremely well with the measurements of 27 m$\mu$ from face to face and 29.5 m$\mu$ from corner to corner which were obtained by electron microscopy. These measurements may be assumed to be particularly accurate because the negative staining method of Brenner and Horne (1959) was employed and this method does not distort the virus morphology. The hexagonal outline observed, resembles that described by Horne and Nagington (1959) in the poliovirus, and probably implies an icosahedral structure with 5:3:2: cubic symmetry in Frater virus similar to that of poliovirus.

All these properties of Frater virus were those to be expected in an enterovirus. Serologically it was not one of the poliovirus types, and unlike polioviruses it was not cytopathogenic for HeLa cell cultures. Unlike the Coxsackie viruses, Frater virus was not pathogenic for suckling mice, and also it was not neutralised by antisera to the B strains and type A9 Coxsackie viruses, which are types commonly cytopathogenic for human and monkey tissue cultures.

By exclusion, therefore, it appeared to be an ECHO virus and in fact it satisfied all the criteria of the Committee on the ECHO Viruses (1955) for inclusion in this group. Frater virus was cytopathogenic for human and monkey cells in culture, it was not a poliovirus or a Coxsackie virus, and it did not appear to be related to herpes virus, adenovirus, or influenza viruses, which may sometimes be isolated in tissue cultures from the human alimentary tract. Frater virus was also shown to be neutralised by many individual human sera and by several samples of human gamma-globulin. A further feature of resemblance between Frater virus and ECHO viruses is the ability of Frater virus to produce plaques under an agar overlay. Timbury (1962) found that Frater virus produced in human amnion monolayers round, well defined plaques with irregular borders which in 3 days were approximately 2 mm. in diameter. The plaques observed with Frater virus were more like those of other ECHO viruses in shape than like Coxsackie B virus plaques. All Timbury's work was performed with fresh human amnion monolayers. Monkey kidney cells were not used.

My own serological investigations failed to show any relationship between Frater virus and ECHO types 1 to 27. Moreover, its cytopathic effects in tissue cultures did not at all suggest that it was type 28 ECHO virus (Pelon, 1961). Dr. A.D. Macrae, Virus Reference Laboratory, Colindale, examined seed of the prototype strain of Frater virus which I sent to him, with his antisera to ECHO virus types 1 to 27. These sera were generally of higher titre than those which I had used. He failed to obtain evidence of neutralisation with his antisera (Macrae, 1960).

My investigations showed that Frater virus had all the characteristics of an ECHO virus but differed antigenically from all the accepted prototype strains. This did not prove that it was an entirely new type of ECHO virus because some variant strains within existing types show marked antigenic differences from their prototypes. Philipson's U virus strain of ECHO type 11 and strains of ECHO type 4 were examples of this. Whether Frater virus was a new type or was a variant of an accepted type, further serological investigations were required and these could only be done by the members of the Committee on the Enteroviruses. In September 1960 I therefore sent seed of the Frater prototype strain to Dr. J.L. Melnick, the Chairman of the Committee. I have very recently been informed (Wenner, 1962) that the Committee have accepted Frater virus and have assigned a number to it. As the Committee are proposing a new scheme of nomenclature for all the enteroviruses, it is likely that Frater virus may not be named as a type of ECHO virus but as a type of enterovirus. The acceptance of Frater virus is of some local interest because it makes Frater virus the first prototype in the ECHO group to be originally isolated in a European laboratory.

The work reported in this thesis extended well beyond the investigation which was my original objective, the comparison of human amnion and thyroid with monkey kidney cultures. I was able to make a number of fairly definite conclusions; on the place of amnion and thyroid cultures in a diagnostic laboratory, on the aetiological connection of Frater virus and aseptic meningitis, and on the nature of Frater virus itself. The work on other aspects of the virus,

such as the antigenic variation between strains, the nature of its differing infectivity for different types of cells, and its epidemiology in other areas and at other times than Scotland in 1959, has done no more than point out to me directions in which further work should be done. Most important, the way in which my investigations developed has clearly illustrated the common experience that even the apparently most routine investigation may unexpectedly lead on to enquiries very different from those which were the starting point.

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