UNIVERSITY OF EDINBURGH

"The Application of Tissue Culture Methods to the Study of the Vesicular Diseases of Animals"

Thesis presented for the Degree of Doctor of Philosophy

by

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INTRODUCTION

and

REVIEW OF LITERATURE

Much research in all scientific disciplines is directed to finding a working model in which the various factors can be defined and the findings related to the actual problem. Virus research is no exception, and with human and animal viruses much time has been spent in finding susceptible small laboratory animals, tissue culture methods and *in vitro* tests, which can be used easily in the laboratory. With the vesicular diseases of domestic animals, foot-and-mouth disease, vesicular stomatitis and vesicular exanthema, the finding of a suitable system to be used in the laboratory is even more important, since these diseases affect horses, cattle, sheep and pigs, expensive animals requiring expensive accommodation. And so in these diseases tissue culture has taken a place among the methods that have been investigated.

Maitland and Maitland (1931) and Hecke (1930,1931) were the first to use tissue cultures for the cultivation of the virus of foot-and-mouth disease. Maitland and Maitland were unable to grow the virus in chick embryo cultures, but with the pads, lips, tongue and hairy skin of embryo guinea pig tissue in guinea pig serum cultivation of the virus was successful through seventeen passages. On one occasion they were able to grow the virus in embryo kidney and also found that minced adult kidney tissue in

Tyrodes solution and guinea pig serum allowed survival of the virus. The virus did not change in any way on cultivation. Hecke also found chick embryo tissue cultures unsuitable for virus growth but was able to carry the virus through seventeen passages in tissue cultures of embryo guinea pig skin. Later Hecke (1932a & b) studied various guinea pig tissues for their ability to support growth of the virus of foot-and-mouth disease. Embryonic skin, oesophagus, lung and adult testicle were suitable; occasional growth was found in adult mammary gland and kidney. With other organs cultivation was unsuccessful. He believed that the tissue should be of epithelial character.

The work of Maitland and Maitland and of Hecke does not appear to have been followed up immediately, probably because they used guinea pig strains in guinea pig tissues, and difficulty may be experienced in adapting cattle and pig strains to the guinea pig. Antibiotics had not been introduced and about that time many workers turned to using chick embryo techniques.

In 1947, after the advent of antibiotics, Frenkel reported the growth of the virus of foot-and-mouth disease in tissue cultures of the surviving epithelium of cattle tongues. By a suitable technique he was able to remove the lower layers of the cattle tongue epithelium, wash them free of blood and suspend them in a nutrient medium. Virus was then added, and a high titre was obtained after 18 - 24 hours. The method was essentially the same as that of the Maitlands. It was developed on a large scale for the cultivation of the virus of foot-and-mouth disease (Frenkel,

1953, 1954, Frenkel and Ribelin, 1956), and after inactivation with formalin the culture virus was made into a vaccine which has been used with success. Henderson and Galloway (1953) showed that a vaccine prepared from culture virus was as effective as a vaccine prepared from cattle virus. Studies of the growth of the virus in the suspended cattle tongue epithelium cultures have been carried out by a number of workers including Henderson (1953 a & b), Brooksby and Wardle (1954), Mackowiak, Girard, Camand and Hirtz (1955) and Ubertini, Nardelli, Barei and Santero (1956), all of whose results gave a similar picture of virus growth. Cartwright, Pay and Henderson (1957) studied the infectivity curve of the virus in single cells obtained by trypsinisation of cattle tongue epithelium and were able to follow the pattern of multiplication and yield of virus very closely. The cattle tongue epithelial cultures have been used for virus titration. Brooksby and Wardle (1954) incubated pieces of epithelium with dilutions of the virus in cups on perspex plates and detected the presence of virus by means of a complement fixation test after incubation for a suitable period at 37°C. They also used this method for serum neutralisation tests and this was confirmed by Gillespie and Frenkel (1955) and by van Bekkum (1957).

The work of Enders, Robbins and Weller (1949 a & b, 1950) led to a renewed interest in tissue culture by workers on human viruses. They discovered that poliomyelitis viruses multiplied in non-neural tissues with cytopathogenic effect. The viruses were grown on tissue explants in plasma in roller tubes, but with the findings that suitably cleaned glass surfaces could be used for the growth of cells

and that trypsin could be used for obtaining cell suspensions the use of tissue culture widened considerably. Using these methods Dulbecco (1952), Dulbecco and Vogt (1954) introduced the plaque technique for virus assay. In this technique the cytopathogenic effect was localised by addition of an agar overlay and thus plaques of necrotic débris were formed. By use of cultivation on glass and the trypsin technique, Sellers (1955) and Bachrach, Hess and Callis (1955) were able to grow monolayers of pig, ox embryo and calf kidneys and show that the virus of foot-and-mouth disease grew in them with cytopathogenic effect. This finding was confirmed by Wesslen and Dinter (1956), Petermann, Lang and Mackowiak (1956) and Guillot (1957). The kidney tissue cultures have been used for virus titration using the presence of cytopathogenic effect to indicate growth of the virus (Bachrach, Hess and Callis, 1955, Wesslen and Dinter, 1956) and by this means investigation has been made into the growth of the virus and the action of heat, formalin and other physical and chemical agents on it (Bachrach, Breese, Callis, Hess and Patty, 1957, Wesslen and Dinter, 1957). Suggestions have been made that the virus grown in kidney cultures should be used as a vaccine and the antibody levels in cattle inoculated with such a vaccine have been followed by Dinter and Wesslen (1958).

Sellers (1955) showed that plaques could be produced by the virus of foot-and-mouth disease on pig kidney monolayers and that this provided an accurate method of titration. Bachrach, Callis, Hess and Patty (1957) demonstrated that it was possible to obtain

plaques on calf kidney monolayers and they described some of the conditions necessary for their formation. Dinter and Sibalin (1958) also described plaque formation by a number of strains from cattle and cattle tongue epithelium cultures on calf kidney monolayers. Using cultivation on glass and the trypsin technique Pay has grown tissue cultures from bovine amnion (Pay, 1956) and cattle tongue epithelium (Pay, 1957), and shown that the virus of foot-and-mouth disease multiplied in them but with cytopathogenic effect only in the case of certain cells in the tongue epithelium monolayer.

The history of the use of tissue cultures in the study of the virus of vesicular stomatitis resembles that of the virus of foot-and-mouth disease. Cox, Syverton and Olitsky (1933) described a method for cultivation of the virus of vesicular stomatitis in suspensions of minced chick embryos in Tyrodes solution. However, Burnet and Galloway (1934) showed that the virus could be cultivated in the chick embryo and use was made of this method for virus growth and titration. With the development of cultivation on glass and the trypsin technique, McClain and Hackett (1955) and Sellers (1955) described the growth and cytopathogenicity of the virus of vesicular stomatitis for chick embryo, ox kidney, calf kidney and pig kidney monolayers. Sellers was also able to obtain plaques in these systems. Cooper (1955) introduced a method of obtaining plaques in cell suspensions of chick embryo cells. Growth and cytopathogenicity of the virus for guinea pig kidney, cattle tongue epithelium and other tissue cultures were reported by Bachrach, Callis and Hess (1955, 1956). Work of a more fundamental

nature has since been done by Cooper (1957 a,b & c) and by Franklin (1958) on the growth of the virus in chick embryo cells.

No small animal host has been found to be susceptible to the virus of vesicular exanthema and tissue culture methods have been used. Brooksby (1954) reported attempts to grow the virus in surviving epithelium cultures of pig tongue. McClain, Madin and Andriese (1954) were able to grow the virus in embryonic pig skin fragments in embryo clots and in other organs as well as in pig kidney and pig testis monolayers with cytopathogenic effect (Madin and Traum, 1955). Bankowski and Pfeiffer (1955) also cultivated the virus in pig embryo tissues, and Stuart and Bankowski (1957) reported the growth of the virus in plaques of pig embryo skin placed on the chorioallantoic membrane of chick embryos. Sellers (1955) was able to confirm that the virus of vesicular exanthema grew on pig kidney monolayers with cytopathogenic effect and was able to produce plaques. McClain, Hackett and Madin (1958) have subsequently described the different types of plaque formed.

From the above review of the literature it is evident that tissue culture does provide a suitable laboratory technique for the study of the vesicular viruses. Of the cultures tested the most suitable have come from the tongue and the kidneys.

INTRODUCTION TO WORK TO BE REPORTED IN THE THESIS

Cells from chick embryos have long been known to grow well in tissue cultures and since the virus of vesicular stomatitis caused death of the chick embryo it was decided to see if the virus would grow on chick embryo monolayers with cytopathogenic effect. This was successfully achieved. The virus of vesicular stomatitis grew with cytopathogenic effect not only on chick embryo monolayers but also on ox embryo, calf, ox, pig and monkey kidney monolayers. In addition, it was possible to produce plaques on all these monolayers. This led to attempts to grow the virus of foot-and-mouth disease on such monolayers. No growth was found on chick embryo monolayers; in mouse embryo cultures doubtful results were obtained. In ox embryo, calf and ox kidney monolayers growth of the virus was demonstrated together with cytopathogenic effect but many cells in the monolayer were unaffected. In the pig kidney monolayers the virus multiplied and at the same time destroyed the majority of cells; hence by use of the agar overlay method plaques were obtained. The virus of vesicular exanthema was also successfully propagated in the pig kidney monolayers with cytopathogenic effect and plaques were obtained. Much of this work has already been reported (Sellers, 1955).

Since the main work of the Research Institute, (Animal Virus Diseases), Pirbright is research on foot-and-mouth disease, it was decided to concentrate on work with that virus. Pig kidney was chosen as the tissue culture system, since pig kidneys were readily

available and they supported the growth of the virus with good cytopathogenic effect; however, calf, ox and lamb kidneys were also used on occasions.

In the thesis, therefore, I shall be illustrating by special reference to kidney tissue cultures and the virus of foot-and-mouth disease the several ways by which tissue culture methods can be applied to the study of the vesicular diseases of animals.

The findings will be given under six sections as follows:-

I. The production of pig kidney monolayer tissue cultures for use in studies on the virus of foot-and-mouth disease.

II. Factors affecting the titration of the virus of foot-and-mouth disease on pig kidney monolayers by the plaque technique and comparison with other methods of titration.

III. The behaviour of strains of the virus of foot-and-mouth disease in kidney monolayer tissue cultures.

IV. The growth and multiplication of the virus of foot-and-mouth disease in pig kidney tissue cultures.

V. Studies on the vaccination of guinea pigs with an inactivated vaccine prepared from virus grown in pig kidney monolayer tissue cultures.

VI. Brief reference will also be made to the use of pig kidney monolayer tissue cultures for diagnosis of vesicular diseases and for assay of neutralising antibody.

MATERIALS AND METHODS

VIRUSES

The following strains of the virus of foot-and-mouth disease were used:-

Vallée O type

Strain M 11. This strain was recovered from an outbreak in cattle in Mexico. It was subsequently passaged in cattle for 12 passages (M11 cattle), in pigs (4 passages) and in pig kidney tissue cultures, 100 and more passages, (M11 pig kidney). The cattle virus has also been passaged in unweaned mice (M11 young mouse), in 90 day old mice (M11 old mouse) and in chicks and chick embryos (M11 egg or M11 chick embryo).

Strain Ven 1. This strain was recovered from an outbreak of the disease in Venezuela. It was passaged in cattle for 15 passages (Ven 1 cattle), in tissue cultures of surviving cattle tongue epithelium (Ven 1 cattle tongue epithelial culture) and chick embryos. (Ven 1 egg or Ven 1 chick embryo).

Strain Dutch O This strain was received from Holland. It had been passaged in cattle (Dutch O cattle) and had had over 200 passages in tissue cultures of cattle tongue epithelium for vaccine production. (Dutch O cattle tongue epithelial culture).

Strains 01112, 01114, 643. These strains were recovered from outbreaks of the disease in Great Britain occurring in pigs. They were passaged in pig kidney monolayer tissue cultures for up to 10 passages.

Strain V1. This strain was passaged solely in the guinea pig and was used for the production of Type O hyperimmune guinea pig serum.

Vallée A type.

Strain 119. This strain was recovered from an outbreak of the disease in cattle (119 cattle) in Great Britain. It has been passaged in cattle for 82 passages, in 7 day old mice (119 young mouse), in 90 day old mice (119 old mouse) and in chick embryos (119 chick embryo or egg).

Waldmann C type.

Strain GC. This strain was passaged solely in the guinea pig and was used for the production of Type C hyperimmune serum.

Strain C 997. This strain was recovered from an outbreak of the disease in Great Britain occurring in cattle and pigs. It was passaged in tissue cultures of cattle tongue epithelium for 38 passages and subsequently received over 80 passages in pig kidney monolayer tissue cultures (C 997 pig kidney).

SAT 2 type.

Strain Rho 1. This strain was recovered from outbreaks of the disease in Rhodesia. It was passaged in cattle (Rho 1 cattle) and in chick embryos (Rho 1 chick embryo or egg) and also received alternate passage in chick embryos and mice (Rho 1 egg/mouse).

Strain K/3/57. This strain was recovered from an outbreak in Zebu cattle in Kenya. It was passaged in cattle (K3 cattle), in pig kidney monolayer tissue cultures (K3 pig kidney) and in tissue cultures of cattle tongue epithelium (K3 cattle tongue

epithelial culture).

Strain K/121/56. This strain was recovered from an outbreak of the disease in Zebu cattle in Kenya. It was passaged in pig kidney monolayer tissue cultures.

Asia type.

Strain Ind 2. This strain had been passaged in laboratory guinea pigs in India. It was subsequently passaged in guinea pigs and in pig kidney monolayer tissue cultures.

STORAGE AND PREPARATION OF VIRUS

Epithelium from cattle, pigs or guinea pigs was stored at 4°C in 50% glycerol phosphate. Infective mice and chick embryos were kept whole at -20°C. For use 1/10 to 1/25 suspensions of epithelium, mouse carcase or chick embryo carcase or heart in 50% Hartley's broth, 50% M/25 phosphate buffer (pH 7.6) were prepared and sterilised by filtration through a Seitz EK filter.

Surviving cattle tongue epithelial cultures grown by the method of Brooksby and Wardle (1954) or Cartwright, Pay and Henderson (1957) were stored at 4°C or -20°C. The supernatant fluid or a suspension of ground up tissue in M/25 phosphate was used.

Virus from kidney tissue cultures infected with virus in the presence of Earle's, 0.5% lactalbumen hydrolysate, 0.01% yeast extract with or without 5% ox serum was collected as supernatant fluid 7 hr., 18 hr., 24 hr. or later after infection. The virus was stored at 4°C. if to be used shortly; otherwise it was stored in the presence of 10% ox serum at -20°C., whereby the virus titre was maintained for at least 4 months.

ANTISERA

Antisera used for the identification of the strains of the virus of foot-and-mouth disease were prepared routinely at the Institute by hyperimmunisation of guinea pigs (Brooksby, 1952).

Other antisera were prepared from blood obtained from cattle, pigs and guinea pigs infected with virus or immunised with attenuated or inactivated vaccines. They were kept at 4°C. or -10°C. until used.

GLASSWARE

Good quality glassware (Pyrex or glassware made by the United Glass Bottle Manufacturers Limited) was used for the tissue culture work. It was carefully washed by the following method. Infected glassware was autoclaved and then brushed in running hot tap water. It was then brushed in hot water containing 0.5% Calphos (Joseph Crosfield and Sons, Ltd., Warrington.) and Calgon, rinsed ten times in hot tap water and three times in softened water. After soaking overnight in deionised water it was rinsed in hot double deionised water, dried and sterilised. Where it was difficult to remove cell debris, the glassware remained overnight in 2% cold Solgon (Joseph Crosfield and Sons Limited, Warrington) in tap water, and then subjected to the procedure described.

SOLUTIONS

Analar grade chemicals were used wherever possible, and the water was prepared by double deionisation.

The following solutions were used:-

Phosphate buffered saline (PBS - Dulbecco and Vogt, 1954)

Phosphate buffered saline, from which the calcium and magnesium chlorides had been omitted. (Ca and Mg free PBS).

0.25% Trypsin (Difco) in PBS or in Ca and Mg free PBS.

0.02% Disodium versenate (BDH) in Ca and Mg free PBS (versene).

Earle's saline.

Double strength Earle's saline (2 x Earle's).

Hank's saline.

Earle's saline containing 0.5% lactalbumen hydrolysate (Nutritional Biochemicals Company, Inc.) and 0.01% Yeast Extract (Nutritional Biochemicals Company, Inc. or Difco), (EYL).

Hank 's saline containing 0.5% lactalbumen hydrolysate and 0.01% Yeast Extract. (LYH).

Synthetic medium (C. Waymouth, 1955).

M/25 Phosphate buffer (pH 7.6)

Hartley's Broth.

Neutral Red, 1:20,000 in Earle's saline.

Antibiotics were added to the solutions as follows:-

Penicillin	100 units/ml.
Streptomycin	100 units/ml.
Polymyxin	50 units/ml.
Mycostatin	40 units/ml.

The Ca and Mg free PBS and the versene were sterilised by 15 minutes autoclaving at 15 lb pressure.

The other solutions were sterilised by being forced under

5 lb positive pressure through a Seitz EK filter. All solutions were kept at 4°C. until use.

Normal ox, calf and pig serum was separated from blood obtained from the abattoir. Horse serum came from a gelding kept at the Institute. The serum was sterilised by filtration through a Seitz EK filter and then kept at -10°C. until used.

The agar overlay used in plaque titration contained one part washed Difco agar (2.5% in double deionised water), one part double strength Earle's saline and one part 10% ox serum in EYL. The agar was melted and then mixed with the other components and finally held at 43°C. in a water bath until used for overlaying. 3.6 ml. was the usual volume poured on a 6 cm. Petri dish.

TISSUE CULTURES

9 - 10 day old chick embryos from eggs bought from a dealer were used for preparation of chick embryo cultures. Mouse embryos were obtained from pregnant mice bred in the colony maintained at the Institute and were about 11 - 14 days old. Calf, ox, lamb and pig kidneys came from the abattoir.

ANIMALS

The unweaned mice (5 - 7 days old) were bred at the Institute. They were a 'P' strain of Swiss mouse obtained originally from the Medical Research Council Laboratories, Hampstead.

The virus was inoculated by the method described by Skinner (1951) using serial dilutions in PBS or M/25 phosphate buffer.

White guinea pigs used for titration and vaccination came from

the Institute colony and were 300 - 400 g. weight. Titration of the virus was carried out by intradermal inoculation of the right hind pad with serial dilutions of virus.

The cattle were Devon steers 1½ - 2 years old and were known to have 'clean' history with regard to foot-and-mouth disease. The virus was titrated by intradermal inoculation of the tongue using the method of Henderson (1949).

The pigs were of mixed breed about 4 months old and were infected by intradermal inoculation of the tongue and snout.

For production of chick embryo virus 11 - 14 day old fertile eggs (White or Black Leghorn) were inoculated intravenously, and the embryo harvested at death (Skinner, 1954).

CALCULATION OF VIRUS TITRE

Virus titres were calculated by the method of Reed and Muench (1938) and expressed as ID_{50}/ml. In some instances ID_{50}/ml. were converted into infectious units per ml. (IU/ml) by assuming that according to the Poisson distribution 1 ID_{50} = 0.7 IU.

Plaque titres were calculated according to the following formula:-

Titre as plaque forming units per ml. (pfu/ml.)

$$= \frac{\text{average number of plaques per plate}}{\text{volume of inoculum x dilution of virus}}$$

STATISTICAL METHODS

The methods given by Mainland (1952) and by Snedecor (1956) were followed for calculation of variance and standard deviation and for

the analysis of variance on plaque counts. In the analysis of variance a square root transformation was applied to the plaque counts (see Mainland, p.290).

RESULTS

I. THE PRODUCTION OF PIG KIDNEY MONOLAYER TISSUE CULTURES FOR USE IN STUDIES ON THE VIRUS OF FOOT-AND-MOUTH DISEASE.

The importance for virus growth of the state and nature of the cells in a monolayer cannot be underestimated. Hence it was necessary to examine every stage in the technique of monolayer production in order that the monolayers might be of suitable standard for titration of virus and antiserum by the plaque method and for provision of virus stocks for vaccine and for research purposes. The criteria used for assessing the suitability of monolayers were the state of the cells and extent of the monolayer as judged by microscopic examination, the pH of the medium and the number of cells obtained from the monolayers at subculture. The techniques used were based on those elaborated by Dulbecco and Vogt (1954) and by Youngner (1954) for the preparation of monkey kidney cell suspensions for growth of monolayers on glass.

Method of preparation

The kidneys came from pigs (60 - 120 lb weight) killed for pork and were received 1 - 2 hours after death. Usually they were used for tissue culture immediately, but it was found that they would give satisfactory cultures if stored untouched in PBS overnight at 4°C. Kidneys from bacon pigs (160 - 180 lb weight) were found to be unsatisfactory either because of the damage in processing or because the pigs were older.

The perirenal fat and kidney capsule were dissected away, and the cortex was separated from the medulla and minced into pieces

3 - 4mm square. The mince was washed free of blood and debris with warm PBS, placed in several 250 ml. conical flasks and warm (37°C) trypsin added. After 15 minutes at 37°C, the supernatant fluid was discarded, fresh trypsin was added, and the flasks were placed on magnetic stirrers, which caused the kidney fragments to rotate. After 10 - 15 minutes stirring, the supernatant was discarded and fresh warm trypsin added and stirring continued. The supernatants after the fourth stirring were collected since they contained mostly cell clusters and cells instead of blood cells and debris as found in the previous supernatants. LYH + 5% ox serum was added in equal volume to the supernatant fluid, which was then centrifuged for 4 minutes at 900 r.p.m. in an M.S.E. Minor Centrifuge. The supernatant fluids of the centrifuge pots were discarded and the pellets, resuspended in LYH + 5% ox serum, were centrifuged for 4 minutes at 600 r.p.m. in an M.S.E. Minor Centrifuge. The packed cell volume was estimated and the cells resuspended in 150 times their volume of growth medium. The ox serum added to the LYH served the double purpose of helping to inactivate the trypsin and of protecting the cells on centrifugation. The collection of supernatants from the 250 ml. flasks, the centrifugation and washing were carried out until a sufficient volume of packed cells had been obtained.

0.25% trypsin in PBS was usually used for the production of cell suspensions; the concentration of trypsin could be reduced to 0.15% with no delay in the rate of production. Trypsin in Ca and Mg free PBS gave no advantage, although the absence of calcium and magnesium salts in trypsin solutions has been shown to assist the

action of trypsin (Rinaldini, 1954). Trypsin in Hank's saline tended to lead to the formation of glutinous clusters of cells and cell debris which were difficult to centrifuge. Other methods of trypsinisation such as overnight trypsinisation at 4°C (Bodian, 1956) did not give such good results as the method described. Often overdigestion by the trypsin occurred and glutinous masses were formed. The cells derived from them grew and formed a monolayer but much debris was seen on the glass.

The 1:150 dilution of the packed cell volume in growth medium led to the formation of monolayers in 4 - 6 days, and this was found to be the optimum dilution; with a packed cell volume diluted 1:100 or less the medium was soon exhausted, much debris was seen and growth was poor; with a packed cell volume diluted greater than 1:200 the monolayers took longer to form and contained many fibroblasts.

The diluted cell suspension was dispensed into Roux bottles (100 ml.), 20 oz (60 ml.), 12oz (30 ml.), 8 oz (15 ml.) and 4 oz (10 ml.) medicinal flat bottles, and into 10cm diameter Petri dishes (12 ml.) and 6cm diameter Petri dishes (5 ml.). The bottles were sealed with caps or rubber bungs and placed at 37°C in an incubator. On occasions they were closed with sterile cotton wool plugs and placed together with the Petri dishes at 37°C. in well humidified incubators, which received a constant flow of 4% CO_2 in air.

Usually some cells and cell clusters settled out on the glass within 24 hours and started to grow; by 48 - 72 hours patches of epithelial type cells could be seen and a monolayer was formed in

4 - 6 days. Not all the cells settled out on the glass; indeed it was possible to remove the supernatant fluid on the second and third day, place the fluid in another bottle, and a monolayer was formed in that bottle in 4 - 5 days. At first a medium change in the cultures was carried out on the second, third or fourth day, but it was found possible to omit the change and the cultures were left unchanged for six days without apparent effect on the cells.

The nature of the growth medium

In the first experiments 7.5% ox serum in a synthetic medium devised by Dr. C. Waymouth (1955) was used. The synthetic medium consisted of a balanced salt solution containing 16 amino acids, glutamine, hypoxanthine and some B-group vitamins. The cells grew and formed monolayers but the medium was expensive and difficult to prepare. It was compared with Earle's + 0.5% lactalbumen hydrolysate + 7.5% ox serum and Hank's + 0.5% lactalbumen hydrolysate + 7.5% ox serum and these two media were found to give superior growth. The effect of the addition of yeast extract to the Earle's + 0.5% lactalbumen hydrolysate + 7.5% ox serum and to the Hank's + 0.5% lactalbumen hydrolysate + 7.5% ox serum was then investigated. Concentrations of yeast extract of 0.03%, 0.1% and above were toxic to the cells; at a concentration of 0.01% the cells grew better than when the yeast extract was omitted.

The type and concentration of serum was varied. Ox and calf serum were equally suitable for the cells. Growth of the cells in 2%, 5%, 7.5%, 10%, 15%, 20% and 30% ox serum in EYL or LYH was carried out and 7.5 - 10% was found to be the optimum concentration.

With insufficient serum the cells often failed to form a monolayer; with too much serum (20% and above) granularity in the cells was observed. Pig serum (5 - 10%) caused the death of many cells in the cell suspensions and on the few occasions when monolayers did form, the cells were fragile. The batch of horse serum used (5 - 10%) proved toxic to the cells.

Experiments were carried out to compare the balanced salt solutions and to compare sealed tissue cultures with tissue cultures grown in a constant flow of 4% CO_2 in air. Cultures were grown in:-

(i) LYH + 7.5% ox serum in sealed bottles:

(ii) LYH + 7.5% ox serum; the sodium bicarbonate was replaced by 0.01 M tris-hydroxyaminomethylmethane buffer, and the cultures were grown in Petri dishes or open bottles in an ordinary incubator:

(iii) EYL + 7.5% ox serum in bottles which were gassed with 4% CO_2 in air and sealed:

(iv) EYL + 7.5% ox serum in bottles and Petri dishes exposed to a constant flow of 4% CO_2 in air.

The results showed that cultures from bottles or Petri dishes grown in EYL + 7.5% ox serum in a constant flow of 4% CO_2 in air gave the best growth and yielded on an average the greatest number of cells when the cells were removed from the glass by versene treatment. The cells were also the most susceptible to the strains of virus used. From the cultures grown in EYL + 7.5% ox serum in gassed and sealed bottles and from cultures grown in LYH + 7.5% ox serum in sealed containers the yield of cells was about the same or less than from the cultures grown in the constant flow: the cells were not so sensitive to virus action. When Tris buffer was

substituted for the bicarbonate the cultures grew as well as the others for three days, then became acid, ceased to grow and degenerated. Unfortunately, owing to lack of space cultures could not always be grown in EYL + 7.5% ox serum in the constant flow of 4% CO_2 in air, and so the cells were grown in a medium consisting of 2 parts LYH, 1 part EYL and 7.5% ox serum. They gave a slightly better yield than cultures grown in LYH + 7.5% ox serum and did not go so acid.

In some experiments, the bicarbonate content of the medium was varied and the cultures made acid (pH 6.8) or alkaline (pH7.8) by variation in the amount of carbon dioxide given. It was found that during the first 48 hours acid conditions in the medium favoured the attachment to the glass and the initial growth of cells, whereas alkaline conditions tended to destroy the cells. After three days acid conditions especially when combined with low concentrations of bicarbonate caused the patches of cells to retract, come off the glass and degenerate. However, with a bicarbonate concentration of 0.22% and at a pH of 7.2 - 7.4, the cells attached and grew well.

From these results it was concluded that the superiority of the cultures grown in the constant flow of 4% CO_2 in air was due to the constancy of the pH of the medium. The pH was sufficiently acid at the beginning for the cells to attach to the glassware and grow and there was sufficient bicarbonate to act as a buffer and take part in the metabolic processes once the culture was established. Where LYH containing Tris buffer was used the medium became too acid and there was insufficient bicarbonate. The success of Tris buffer initially can probably be ascribed to bicarbonate present in the

serum and in the cells.

The yield of cells from monolayer tissue cultures.

Despite the adherence to the same techniques from week to week, variation in growth of the monolayers and in yield of cells from monolayers at subculture was found. Part of this variation might be ascribed to differences in the batch of serum or of trypsin used, but undoubtedly part could be ascribed to variation in the kidneys. Table 1 shows the yield of cells from monolayers grown from different kidneys; the monolayers had been grown in EYL + 7.5% ox serum in a gassed and sealed system (Roux bottles) and in a constant flow of 4% CO_2 in air (Petri dishes 10cm.). It can be seen that the monolayers from one kidney yielded fewer cells at subculture than the other despite the similar conditions under which they were grown.

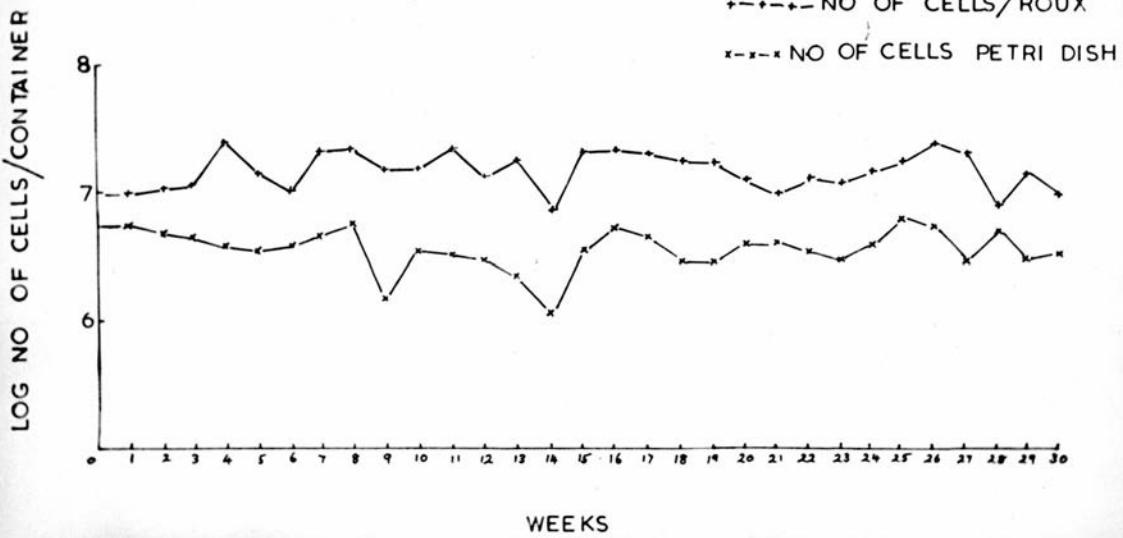
TABLE 1 VARIATION IN THE CELL YIELD OBTAINED AT SUBCULTURE FROM SIX-DAY OLD MONOLAYERS GROWN FROM DIFFERENT PIG KIDNEYS

	Number of Cells	
Source of monolayer	Kidney 1	Kidney 2
Roux bottles	1.37×10^7/Roux	8.4×10^6/Roux
10 cm. Petri dishes (PD)	2.8×10^6/PD	1.8×10^6/PD

Records were kept of the cell yield from Roux bottles and from Petri dishes at the time of subculture each week and these are illustrated graphically in Figure 1. It is apparent that the yield varied from week to week but the variation in yield was not always in the same direction for both Roux bottles and Petri dishes. This

FIGURE 1

Yield of pig kidney cells per Roux bottle and per 10cm. Petri dish obtained at the time of subculture each week.


+–+–+– NO OF CELLS/ROUX
x–x–x NO OF CELLS PETRI DISH
LOG NO OF CELLS/CONTAINER
8
7
6
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
WEEKS

indicates that factors other than the kidneys were affecting the cell yield. The mean number of cells obtained each week from the different cultures was calculated over a period of ten months and was found to be 1.37×10^7 cells from Roux bottles (standard deviation 3×10^6 cells) and 3.72×10^6 cells from 10 cm. Petri dishes (standard deviation 1.9×10^6 cells).

Subculture of monolayer tissue cultures

One of the objects of growing monolayers was to provide suitable cultures for virus titration by the plaque technique. However, the time taken for the formation of a complete monolayer varied from kidney to kidney and from week to week, and it was not always possible to ensure that plates for titration were ready on a given day. Therefore, the plates were made by subculture of the cells in the Roux bottles and the 10 cm. Petri dishes.

The culture supernatant fluid was removed and the cultures washed with Ca and Mg free PBS; 0.25% trypsin in Ca and Mg free PBS was added and the cultures incubated at 37°C for 15 - 30 minutes. The cells that came off the glass as a result of the trypsin action were collected, washed free of trypsin by centrifugation in LYH + 5% ox serum, counted and then resuspended in EYL + 7.5 - 10% ox serum and placed in 6 cm. Petri dishes in the 37°C incubator, where they were exposed to a constant flow of 4% CO_2 in air. The trypsin method of cell removal proved unsuitable, since, on many occasions, the cells remained attached to the glass. The reason for this was never discovered. When 0.02% disodium versenate in Ca and Mg free PBS (versene) was substituted for the trypsin, the cells came off the glass quickly, the majority of the cell suspension consisted of single cells and the yields were higher than after trypsin treatment.

It was convenient to have the monolayers for use from two days after subculture onwards. To achieve this experiments were carried out on the optimum concentration of cells to be seeded on 6 cm. Petri dishes. 5×10^5 cells/ml. was found to be the concentration which ensured a monolayer in 48 hours under most conditions. However, concentrations as low as 2×10^5 cells/ml. led to the formation of a monolayer in 48 hours, if the cells were in good condition.

Summary and conclusions.

The technique that made best use of the available facilities and provided a consistent supply of monolayer tissue cultures for virus growth and assay was as follows. Cell suspensions obtained from pig kidneys by a process of repeated trypsinisation were diluted 1:150 in growth medium. The growth medium for cultures in sealed bottles consisted of two parts LYH, 1 part EYL + 7.5 - 10% ox serum; for cultures in the air - 4% CO_2 incubator the medium consisted of 7.5 - 10% ox serum in EYL. Cultures were incubated at 37°C. for 4 - 6 days, when they were used for virus growth or were subcultured by the versene method for the provision of plates for titration. The optimum concentration of cells at subculture was 5×10^5 cells/ml., and these cultures were grown in 7.5 - 10% ox serum in EYL in the air - 4% CO_2 incubator. Monolayers were thus ready for use at 48 hours after subculture and up to five days later.

II. FACTORS AFFECTING THE TITRATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE ON PIG KIDNEY MONOLAYERS BY THE PLAQUE TECHNIQUE AND COMPARISON WITH OTHER METHODS OF TITRATION.

The demonstration that the virus of foot-and-mouth disease multiplied with cytopathogenic effect on pig kidney monolayers led to the use of the monolayers for production of plaques by the Dulbecco (1952) method (Sellers, 1955). In this method the monolayer is infected with a suitable dilution of virus and, after an adsorption period, covered with an agar overlay. The cultures are then incubated for a given period, during which time the cell initially infected is destroyed and releases virus which infects and destroys the surrounding cells thus leading to the formation of a plaque. The plaques are made visible by staining with an intravital dye, usually neutral red. The method is accurate and sensitive but is subject to variation and in this section the results of investigations into the factors that affect plaque formation by the virus of foot-and-mouth disease on pig kidney monolayers will be given.

Four strains of the virus of foot-and-mouth disease were used:-

Strain M11 pig kidney: This strain was purified by plaque isolation at the nineteenth passage and was maintained by rapid passage in pig kidney monolayer tissue cultures. The virus had a titre of 5×10^6 - 3×10^7 pfu/ml. when collected 7 hours after infection and at least 10^6 pfu/ml. when collected 18 hours after infection. It gave rise to regular shaped plaques having a diameter of 5 - 8 mm., although there were occasional smaller plaques. Virus from the 60th passage onward was used.

Strain M11 old mouse. This strain was passaged for 190 passages in 90 day old mice; it was then grown for two passages in pig kidney monolayer tissue cultures, where it had titres of 10^5 - 10^6 pfu/ml. when collected at 24 hours after infection. It gave rise to irregular shaped plaques 1 - 4mm. in diameter.

Strain Ven 1 cattle tongue epithelial culture. Virus from the 100th surviving cattle tongue epithelium culture was grown for two passages in pig kidney monolayers, where it had titres of 10^5 - 10^6 pfu/ml., when collected 24 hours after infection. It gave rise to irregular shaped plaques 1 - 2mm. in diameter with an occasional larger plaque 3 - 5mm. in diameter.

Strain C997 pig kidney. This strain was grown in pig kidney monolayer tissue cultures for over 50 passages. It had a titre of 5×10^6 - 3×10^7 pfu/ml., when collected at 7 hours and over 10^6 pfu/ml., when collected at 18 hours. It gave rise to regular shaped plaques 5 - 8mm in diameter, although there were occasional smaller plaques.

In general the monolayer cultures were prepared by the method described in Section I, but variations were made in media and in cultural conditions, in the method of infection and the period of adsorption. Changes were also made in the composition of the overlay, and occasionally neutral red was included in the overlay at a concentration of 1:37,000. Usually it was poured on the plates 48 - 96 hours after infection as a 1:20,000 solution in Earle's saline. Note was made of the number, size and shape of the plaques as well as the appearance of the cells in the culture.

Optimum number of cells in the secondary monolayer.

It was found (Section I) that the optimum concentration of cells to be seeded on 60mm. Petri dishes at subculture was 5×10^5 cells/ml. If the cell concentration was below this it was often insufficient to give rise to a complete monolayer by 48 hours and hence the plaques formed by the virus were irregular in shape, varied in size and were fewer in number compared with complete monolayers.

Variation between kidneys and cultural conditions during growth of the primary monolayer.

It was shown in Section I that variation in the kidneys and in the cultural conditions had an effect on the nature of the monolayer and on the yield of cells obtained at subculture. These effects persisted during the growth and infection of the secondary monolayers even though from the time of subculture the cultures were treated alike in every respect. The differences in kidneys and cultural conditions were reflected in the number of plaques obtained after infecting the secondary monolayers with the same virus dilution at the same time. The following experiments illustrate this.

In Table 2A, it can be seen that there was a significant difference in the number of plaques on plates derived from different kidneys and on plates derived from Roux bottles and from Petri dishes (10 cm.), ($P<.01>.001$). The difference between the number of plaques on plates derived from different kidneys is even more apparent in Table 4. In Table 2B, plates derived from different kidneys showed no difference in the number of plaques but there was a significant difference ($P<.001$) between the number of plaques obtained on plates derived from Petri dishes and the

number of plaques obtained on plates derived from Roux bottles. It can be seen that in the experiments described the number of plaques was greater on the plates derived from Petri dishes (grown in a constant flow of 4% CO_2 in air) than on plates derived from Roux bottles (sealed), and this was found on most occasions. It was not found possible to predict which kidney would give the larger number of plaques.

Some primary cultures were grown at a low pH (6.6 - 6.8) in the presence of 10% CO_2 in air, and Table 3 shows that the number and size of the plaques on plates derived from the acid cultures were greatly reduced compared with those grown under normal pH, even though the secondary monolayers were kept under identical conditions. A comparison was also made between plates derived from cultures grown in a synthetic medium (Waymouth, 1955) containing 7.5% ox serum and plates from cultures grown in EYL + 7.5% ox serum. The EYL + 7.5% ox serum was superior to the synthetic medium (Table 3).

TABLE 2 EFFECT OF VARIATION OF KIDNEY AND CULTURAL CONDITIONS DURING THE GROWTH OF PRIMARY MONOLAYERS ON THE NUMBER OF PLAQUES FORMED ON SECONDARY MONOLAYERS.

Virus - M11 Pig kidney

A) Cells grown in Pig kidney

	1	2	3
Roux	26,27,35 (29.3)	12,15,18 (15)	20,33,35(29.3)
Petri dish	33,39,40 (37.3)	19,20,37 (23.3)	40,40,43(41)

Kidneys $P < .01 > .001$

Roux-Petri dish $P < .01 > .001$

B) Cells grown in Pig kidney

	1	2
Roux	25,32,38 (31.7)	30,31,31 (30.7)
Petri dish	36,40,42 (39.3)	44,44,48 (45.3)

Kidneys $P > .05$

Roux-Petri dish $P < .001$

The figure in the bracket is the mean plaque count.

TABLE 3 EFFECT OF ACID pH AND SYNTHETIC MEDIUM DURING GROWTH OF PRIMARY MONOLAYERS ON NUMBER AND SIZE OF PLAQUES FORMED ON SECONDARY MONOLAYERS Virus - M11 pig kidney

Relative virus concentration	Normal pH		Acid pH(6.6 - 6.8)	
	Number	Size (diam)	Number	Size(diam)
1/1	Confluent		8	1 - 3mm.
1/5	33	4 - 8mm.	0	
	Synthetic Medium (Waymouth,1955)		EYL + OS	
1/1	48		uncountable	
1/10	6		31	

The figure given for the plaque count is the mean of three plates. The size of the plaque represents the diameter at 48 hours.

It can thus be seen that some cultural conditions and some media may lead to the production of more plaques than others. This might be brought about either by encouragement of the growth of more epithelial cells or by affecting the metabolism of the cells so that they may allow infection and multiplication by the virus to proceed to plaque production.

Washing of plates before addition of virus

When the plates were washed once with PBS, PBS + 1% ox serum or Hank's saline before addition of virus a higher number of plaques was obtained than if the plates were left unwashed ($P < .001$).
It can also be seen in the table that there is a difference in the number of plaques obtained on one kidney as compared with the other

(P <.01 > .001). (Table 4). Bachrach, Callis, Hess and Patty(1957), however, found that with the calf kidney monolayers washing made no difference to the number of plaques formed.

TABLE 4 EFFECT ON THE PLAQUE COUNT OF WASHING PLATES ONCE BEFORE ADDITION OF THE VIRUS

Virus - C997 Pig kidney

Plates	Kidney 1	Kidney 2
Washed	19,21,23,26,27,32 (24.7)	26,29,30,31,34,35 (30.8)
Unwashed	10,11,15,15,17,22 (15)	13,15,23,25,26,28 (21.7)

Washed - unwashed plates P <.001

Kidneys P <.01 > .001

The figure in the bracket is the mean plaque count. The plates were washed with PBS.

Variation in diluting fluid and volume of virus added to plate

Varying the diluting fluid from PBS with or without ox serum and EYL or LYH with or without ox serum had no consistent effect on the number of plaques formed (Table 5).

TABLE 5 EFFECT ON PLAQUE COUNT OF VARIATION IN DILUTING FLUID

Virus - M11 pig kidney

	PBS	PBS+OS	EYL	EYL+OS	LYH	LYH+OS
Experiment 1	8,8	11,13	15,22	12,15	11,14	6,11
Experiment 2	24,27	15,17	19,21	23,29	16,20	26,29

OS = ox serum

When the volume of virus fluid put on the plate for adsorption was varied from 0.1 ml. to 1.0 ml., no difference was seen in the number of plaques formed (Table 6). Bachrach, Callis, Hess and Patty

(1957), however, found that smaller volumes led to the formation of a greater number of plaques on calf kidney monolayers.

TABLE 6 EFFECT ON NUMBER OF PLAQUES OF VARIATION IN VOLUME OF VIRUS FLUID USED TO INFECT PLATES

Virus - M11 Pig kidney

Volume of virus fluid	Number of plaques	Mean
0.1 ml.	11,12,14,25	15.5
0.4 ml.	10,15,15,16	14
1.0 ml.	12,15,17,25	17,25

(0.1 ml. of virus dilution in PBS added to 0.3 ml. and 0.9 ml. PBS to make volumes of 0.4 ml. and 1.0 ml.)

Period of virus adsorption

The results obtained when the plates were washed after varying periods of virus adsorption immediately before addition of the overlay compared with plates left unwashed can be seen in Table 7. On the unwashed plates the number of plaques was the same from immediately after addition of the virus to 60 minutes later. On the washed plates 27% of the virus found on the unwashed plates was adsorbed immediately, and this represented half the amount of virus that remained adsorbed on the plates after washing from 15 minutes onwards.

TABLE 7

NUMBER OF PLAQUES OBTAINED BY WASHING AND OVERLAYING AT INTERVALS AFTER THE ADDITION OF VIRUS

Virus - M11 Pig kidney

Time (mins.) after addition of virus	Number of plaques on Unwashed plates (a)	Washed plates (b)	Percentage b/a
0	26	7	27%
15	33	17	51.5%
30	37	18	48.7%
45	30	19	63.5%
60	37	17	46%

The results are a mean of three experiments.

Composition of the overlay

When Neutral Red was included in the overlay at a concentration of 1/37,000 the number of plaques was reduced. (Table 8). The effect of the inclusion of neutral red was more striking when a strain of virus which formed small irregular plaques was used, and it can also be seen that the reduction of plaques was greater with plates from kidneys which, with the normal overlay, gave less plaques than with plates from kidneys that gave a larger number of plaques.

TABLE 8 EFFECT ON THE NUMBER OF PLAQUES FORMED OF THE PRESENCE OF NEUTRAL RED (1:37,000) IN THE OVERLAY

VIRUS	OVERLAY	
	+ Neutral Red	- Neutral Red
M11 pig kidney	10,11,13,14,14,15,17 (13.4)	19,23,24,26,26,27,28,29 (25.5)

	Kidneys 1 and 2		Kidneys 3 and 4	
	Overlay			
	+ Neutral Red	- Neutral Red	+ Neutral Red	- Neutral Red
M11 old mouse	0,0,0,0, (0)	16,20,30,33 (25)	24,25,44,49 (35.8)	60,63,70,75(67)

The figure in the bracket is the mean.

From Table 9 it can be seen that addition of neutral red to the culture 30 minutes before infection with virus reduced the plaque count to the same extent as inclusion in the overlay. Where neutral red was added to the cell layer beforehand as well as to the overlay, the reduction in plaque number was even greater.

TABLE 9 EFFECT OF NEUTRAL RED ADDED BEFORE OR AFTER INFECTION OF MONOLAYER BY VIRUS

Virus - M11 Pig kidney

Monolayer pre-treated with		Neutral Red in overlay	No Neutral Red in overlay.
Neutral Red (1:37,000	Kidney 1	20, 40 (30)	46, 57 (51.5)
	2	5, 8 (6.5)	20, 24 (22)
Nil	Kidney 1	42, 55 (48.5)	68, 71 (69.5)
	2	14, 30 (21)	41, 42 (41.5)

The figure in the bracket is the mean.

The effect of neutral red is presumably brought about by a toxic effect on the cells, as has been found by Waterson (1958) for fowl plague virus and chick embryo cells. The toxic effect appears to be greater on the less sensitive monolayers and with a strain of virus that produces smaller plaques. The number of plaques formed appears to be the same whether neutral red is added before or after the virus, and presumably this means its effect is intracellular and that it reduces the ability of certain cells to allow multiplication of virus to proceed.

Composition of gaseous phase

Change in the relative concentration of the gases in the flushing mixture had an effect on plaque size. Two sets of plates gave similar plaque counts, but the set which had been incubated in 1.5% CO_2 in air had a plaque size of 3 - 5mm. in diameter whereas the other incubated in 4% CO_2 in air had a plaque size of 4 - 9mm. diameter. The cultures with the lower concentration of CO_2 were more alkaline.

Time of staining for plaques

With strains of the virus of foot-and-mouth disease that formed large plaques there was an increase in size and number of plaques from the first to the second day after overlay but thereafter in size but not in number. With the strains that formed small plaques increase in size and number was noted up to the fourth day after infection. Hence with the strains forming large plaques staining of the overlays was carried out on the second day whereas with the small plaque strains staining of the overlay was not carried out until the fourth day after infection.

Comparison of the number of plaques obtained on monolayers with the number of plaques obtained by the cell suspension method

Cooper (1955) introduced a modification of the plaque technique by the use of cell suspensions instead of monolayers. He found that the cell suspension method was more sensitive than the monolayer method for the virus of vesicular stomatitis on chick embryo cells.

In carrying out the comparison the cells for the monolayers and for the cell suspension came from the same source and had had the same treatment. 0.4 ml. of virus suspension was added to 1 ml. of cell suspension containing 10^7 cells and then mixed with 4 ml. warm agar overlay and poured on a pre-set agar plate. 0.4 ml. of the same virus suspension was added to monolayers and the agar overlay added in the usual way. The cultures were stained with neutral red at 48 hours.

In Table 10 can be seen the results of two experiments. They showed that in detection of virus the cell suspension method was more sensitive than the monolayer method by a factor of 1.6 to 2.8. This may have been due to the greater surface area of cell exposed and due to the fact that in the cell suspension method the virus had a greater chance of encountering a cell and was not subject to disintegration as much as virus taken up into the monolayer overlay. The cell suspension method, however, required more cells than the monolayer method.

TABLE 10 COMPARISON BETWEEN PLAQUE COUNTS OBTAINED BY THE CELL SUSPENSION METHOD WITH THOSE BY THE MONOLAYER METHOD

Virus - C997 Pig kidney

	Cell suspension	Monolayer
Experiment 1	80,83,86,92 (85.5)	26,29,30,31,34,35 (30.8)
Experiment 2		
Relative virus concentration		
$^1/_1$	24,27,34,37 (30.5)	8,17,21,24 (17.5)
$^1/_2$	10,12,15,29 (14.25)	3, 7, 7,18 (8.75)

The figure in the bracket is the mean plaque count.

Error to be expected in the plaque technique

When all the factors affecting the plaque count have been accounted for, there still remains an error due to innate variation. The distribution of plaques is generally agreed to follow a Poisson distribution as has been shown for bacteriophage (Luria, 1953), for poliomyelitis viruses (Dulbecco and Vogt, 1954) and for the virus of foot-and-mouth disease on calf kidney monolayers (Bachrach, Callis, Hess and Patty, 1957). In the Poisson distribution the variance is about the same value as the mean. In order to see if this was so with the virus of foot-and-mouth disease on pig kidney monolayers titrations were carried out on 40 plates on three different days and the means and variances calculated. (Table 11).

TABLE 11 THE NUMBER OF PLAQUES, MEAN AND VARIANCE IN TITRATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE ON PIG KIDNEY MONOLAYERS

Virus - M11 pig kidney

Dilution of Virus	Number of plaques
1. 1:2 x 10^4	9,10,12,12,12,15,15,16,16,17,17,18,18,18, 18,18,18,19,19,19,20,20,20,20,21,21,21, 21,21,22,22,22,23,23,23,24,25,25,26,31.
Mean 19.175	Variance 19.994
2. 1:2 x 10^4	7,18,19,20,21,21,21,22,22,22,23,24,24, 24,24,24,24,25,25,25,25,25,26,26,26,26, 27,28,28,28,28,29,29,29,29,29,30,31,31,32.
Mean 25.25	Variance 14
3. 1:2 x 10^4	9,11,13,14,15,15,15,15,15,16,16,16,16,16,17, 17,18,18,18,18,18,19,19,19,19,19,19,19,19, 20,22,22,23,23,24,24,25,27,29,33.
Mean 18.75	Variance 22

The three groups of 40 plates for each titration were grown under identical conditions, but the cells on the groups of plates were derived from different kidneys. It can be seen that the variance is above the mean in two titrations but below it in the other titration. Thus it appears suitable to assume a Poisson distribution in the calculation of the error.

The conventional standard of error is usually 5 per cent. This is equivalent to $\pm$ 2 x standard deviation, which is equal to $\pm$ 2 x $\sqrt{\text{variance}}$, i.e. in a Poisson distribution $\pm$ 2 x $\sqrt{\text{mean}}$.

If there are 36 plaques on a plate the error would be ± 12. If more than one plate were used the error would be $\frac{\pm 2 \times \sqrt{\text{mean}}}{\sqrt{\text{number of plates}}}$; thus if there were a mean of 36 plaques on 4 plates, the error would be $\frac{\pm 2 \times \sqrt{36}}{\sqrt{4}}$ which is equal to ± 6.

Relationship between concentration of virus and number of plaques

A linear relationship is usually found between the concentration of virus and the number of plaques indicating that one virus particle or an indivisible group of virus particles is responsible for initiating a plaque. The linear relationship can be seen in Table 12 and in Table 10.

TABLE 12 RELATIONSHIP BETWEEN CONCENTRATION OF VIRUS AND NUMBER OF PLAQUES. Virus - M11 Pig kidney

Relative concentration of virus	Number of Plaques	Expected mean	Observed mean
1/1	39,42,50	38	44
1/2	12,18,18,19	19	17
1/4	9,10,10	9.5	9.7

This relationship does not always hold. For example, in the titration of Ven I cattle tongue epithelial culture strain the increase in number of plaques was threefold compared with a tenfold increase in virus concentration. (Table 13).

TABLE 13 RELATIONSHIP BETWEEN CONCENTRATION OF VIRUS AND NUMBER OF PLAQUES Virus - Ven I cattle tongue epithelial culture.

Relative concentration of virus	Number of plaques	Expected mean	Observed mean
1/1	20,30	55	25
1/10	7,10	5.5	8.5

Where there is overcrowding of plaques on a plate, difficulty in counting is experienced and deviation from proportionality can be expected. However, that does not appear to be so with strain Ven I virus, and a tentative explanation might be that with strain Ven I a virus not adapted to the cells was used, and, consequently, there might have been a mixed population. There is the possibility of aggregation of virus particles, However, if more than one virus particle were required to form a plaque, a tenfold reduction in virus concentration would result in a much greater than tenfold reduction in the number of plaques.

Comparison of the plaque method with other methods of titration

Comparison was made between titres obtained by the plaque method and titres by other methods of titration. Table 14 shows two comparisons; the relationship between plaque count and mouse titre for a number of strains can be seen in Section III, Table 17.

TABLE 14 COMPARISON OF TITRES OBTAINED BY DIFFERENT METHODS OF TITRATION

Source of Virus		PFU/ml.	Mouse IU/ml.	Cattle IU/ml.
M11	Cattle	6.1	6.0	6.4
	Pig kidney	6.3	7.4	6.2
	Old Mouse	4.3	6.7	0
VenI	Cattle	6.6	7.7	6.3
	Chick embryo	5.8	7.0	0

The numbers represent the $\log_{10}$ titre in infectious units (IU) per ml. (1 ID_{50} = 0.7 IU).

In some instances the plaque count gave the highest titre, at other times mouse inoculation, and at other times the cattle tongue inoculation; it would appear that the result depended on the degree of adaptation to the host of the strain used. However, there might also be other factors involved due to the plaque technique.

Titrations of virus suspensions were carried out by the plaque count method, by intraperitoneal inoculation of unweaned mice and by growth in rocked cell suspensions derived by versene treatment from pig kidney monolayers. The cell suspensions were rocked for 48 hours and then tested for the presence or absence of virus by mouse or pig kidney tissue culture inoculation. The results are given in Table 15. It can be seen that good agreement was obtained by all methods in the case of the large plaque strain M11 pig kidney, but that in the small plaque strains M11 old mouse and Ven I cattle tongue epithelial culture the plaque counts were lower than the titres obtained by the other methods. This suggests that with the small plaque strains the pig kidney cells are as susceptible as the

unweaned mouse, but that the addition of an overlay affects the formation of a plaque from the initial infection possibly by a damaging effect of the overlay on the cells.

TABLE 15 COMPARISON OF TITRES BY PLAQUE COUNT, BY MOUSE INOCULATION AND BY INOCULATION OF PIG KIDNEY CELL SUSPENSIONS.

Virus	Mouse ID_{50}/ml.	Cell suspension ID_{50}/ml.	PFU/ml.
Ml1 Pig kidney	5.7	6.2	6.1
Ml1 old mouse	6.8	6.0	4.9
Ven I cattle tongue epithelial culture	6.2	5.8	3.2

The numbers represent the $\log_{10}$ titre per ml.

Summary and Conclusions

The results indicate that differences in number and size of plaques can occur as a result of variation in techniques used during growth of the cells and assay of the virus. The differences in plaque number and size are more apparent where the virus titrated has not been adapted to the pig kidney tissue cultures and any untoward effect is likely to have a greater influence where the time taken to form a plaque is longer.

The following technique was adopted as the standard technique in carrying out titrations by the plaque method. Monolayers were prepared by the method described at the end of Section I. They were washed once with PBS, and then 0.4 ml. of the virus dilution in PBS was added and the monolayer incubated for 30 minutes at 37°C. At the end of the adsorption period overlay of the composition described in Materials and Methods was added and the cultures incubated at 37°C. in a constant flow of 4% CO_2 in air. At 48 hours

in the case of strains forming large plaques or at 96 hours in the case of strains forming small plaques the cultures were removed from the incubator and 4 ml. of a 1:20,000 solution of neutral red in Earle's saline added. After 2 hours further incubation the plaques were counted and measured and the titre calculated. If isolation of plaques was required, instead of the neutral red in Earle's saline, a second overlay containing neutral red (1:37,000) was added and the plaque was subcultured by taking up a cylinder of agar over the plaque, adding it to a pig kidney monolayer and harvesting the culture after 18 - 42 hours.

To allow for variation in the plaque count as a result of using different kidneys the cells obtained at subculture were mixed but at the same time the plates were kept in groups in order to avoid loss through contamination or other causes. The experiments were designed in such a way as to do the same assays on the same group of plates, and variation between groups of plates was determined by assay of a stock virus kept at -20°C.

III. THE BEHAVIOUR OF STRAINS OF THE VIRUS OF FOOT-AND-MOUTH DISEASE IN KIDNEY MONOLAYER TISSUE CULTURES.

The virus of foot-and-mouth disease is well known for its variability. In addition to immunological differences there are naturally occurring strains which are more virulent for cattle than for pigs and vice versa (Brooksby, 1950), and passage of the virus in laboratory hosts may lead to modification of the original virus. In the course of work described in Section II it was noticed that there were differences in behaviour in pig kidney monolayers between the strains forming large plaques and the strains forming small plaques. It was, therefore, decided to investigate the behaviour of strains from different sources not only in pig kidney monolayers but also in monolayers derived from calf, ox and lamb kidneys. The interest was in the degree of multiplication, the degree of cytopathogenic effect and the type of plaque formed. As passage of the virus in the mouse and chick embryo diminished the infectivity of virus for cattle without loss of antigenicity (Skinner - personal communication) attention was paid to the mouse and chick embryo passaged strains in order to correlate their behaviour in tissue culture with their behaviour in cattle and pigs.

The growth and cytopathogenicity of cattle and kidney strains of the virus on kidney monolayer cultures.

The optimum conditions for growth of the pig kidney monolayers and for infection of them by pig kidney strains of the virus have been described in Section II.

In the case of the monolayers derived from 7 day old calf

kidneys it had been found that although strain M11 and strain C997 multiplied in the monolayer with cytopathogenic effect, there were cells which were unaffected by the virus and which continued to metabolise as shown by the acid change of pH in the medium (Sellers, 1955). It was later found that in cultures 6 - 8 days old cytopathogenic effect was less and the number of fibroblastic cells greater than in cultures 4 - 5 days old; the virus titre reached ($>10^6$ pfu/ml.) was the same whatever the age of the culture. The unaffected cells were tested for the presence of virus by removal from the glass and titration on pig kidney monolayers; no virus was found. It was also found impossible to reinfect the unaffected cells by addition of fresh virus of the same strain. It was concluded that these cells had not been infected by the virus or had been infected but had been unable to release virus. By changing the medium used for initiating the cultures from EYL + 10% ox serum to EYL + 5% ox serum and by infection of the cultures in the presence of EYL or of EYL + 5% pig serum (i.e. without ox serum) it was found possible to grow a monolayer which on infection with a cattle or a kidney strain of virus would show maximum cytopathogenic effect.

Since the ox serum contained no neutralising antibodies against the virus, it was concluded that the presence of excess ox serum in the medium before and after infection led to the production of an insusceptible cell population, which, unaffected by the virus, continued to multiply and form a monolayer Wesslen and Dinter(1956) found a similar effect due to the presence of bovine amniotic fluid in the medium. As a result of these findings, ox serum was omitted during the period of infection when ox kidney and lamb kidney monolayers were used.

The growth and cytopathogenicity of strains of the virus on kidney monolayer cultures.

The growth and cytopathogenicity of strains of the virus of foot-and-mouth disease from different sources were compared on pig, calf, ox and lamb kidney monolayers. The cultures, which had been grown in 4 oz medicinal flat bottles, were washed with PBS and then virus and fresh medium (EYL) added. The cultures were observed daily for cytopathogenic effect and samples of supernatant fluid were collected and titrated by inoculation of unweaned mice. The strains used came from suspensions of cattle tongue epithelium, from pig kidney culture supernatants and from suspensions of virus after passage in mice and after passage in chick embryos. The strains passaged in mice and chick embryos were also tested for non-infectivity on the tongues of susceptible cattle.

In Table 16 are given the results of the growth and cyto-pathogenicity of strain 119 in kidney monolayers as well as the infectivity for cattle tongue. It can be seen that virus from the 82nd cattle passage multiplied to the same degree in pig, calf, ox and lamb kidney cultures and also produced a good cytopathogenic effect. After 238 passages in the unweaned mouse the multiplication and cytopathogenic effect were similar to that of the cattle strains in the pig kidney monolayers but the cytopathogenic effect was less in the calf, ox and lamb kidney monolayers. Virus of the 238th passage in 90 day old mice multiplied well in the lamb kidney cultures but poorly in the pig, the calf and the ox kidney cultures. The cyto-pathogenic effect was less in the ox kidney cultures. Virus of the 70th egg passage multiplied to the same extent as the virus of the

238th passage in 90 day old mice in pig and calf kidney monolayers, but there was poor multiplication and cytopathogenic effect in the lamb kidney monolayers and no multiplication in the ox kidney monolayers.

On the basis of the growth in ox kidney monolayers the virus from the 238th passage in unweaned mice was less virulent than the cattle virus, and the results in cattle show that of the 8 cattle infected with the 238th unweaned mouse passage two showed no lesions four had lesions on the tongue and two had lesions on the tongue and feet. All 8 would have been expected to show lesions on the tongue and feet if cattle virus had been given. From the results in pig and calf, and especially ox kidney, monolayers the viruses from the 238th 90 day old mouse passage and from the 70th egg passage were less virulent than the 238th young mouse passage and the cattle virus, and this was also apparent from results in cattle. None out of 8 cattle given the 238th 90 day old mouse virus and only one out of 8 given the 70th egg passage virus showed lesions on the tongue. From the ox kidney and sheep kidney monolayer results it would have been expected that the 70th egg passage virus would be less infective than the 238th 90 day old mouse passage. However, it might be said that the one animal that did show lesions was particularly susceptible. It can thus be seen that there is some correlation between the growth and cytopathogenicity in kidney cultures especially ox and calf and the degree of non-infectivity based on the development of tongue lesions in cattle.

TABLE 16 MULTIPLICATION AND CYTOPATHOGENICITY OF VARIOUS STRAINS OF THE VIRUS OF FOOT-AND-MOUTH DISEASE, TYPE A 119, IN PIG, CALF, OX AND LAMB KIDNEY MONOLAYER CULTURES

Strain & Origin	Hours after Infection	Pig		Calf		Ox		Lamb		Lesions in Cattle	
		Titre	CPE	Titre	CPE	Titre	CPE	Titre	CPE	Tongue	Feet
Cattle 82nd	24	6.0	+++	5.8	+++	5.0	++	5.8	+++	8/8	8/8
passage	48	3.3	+++	3.3	+++	3.5	++	4.8	+++		
10^6											
7 day old mice	24	7.5	+++	6.0	++	4.5	+	6.0	++	6/8	2/8
238th passage	48	4.5	+++	4.8	++	3.5	++	4.0	++		
$10^{6.5}$	72	-		3.8	++	3.5	++	2.3	++		
90 day old mice	24	3.3	+++	3.0	++	2.2	(+)	5.5	++	0/8	0/8
238th passage	48	4.0	+++	4.0	++	2.2	+	3.5	++		
$10^{5.8}$	72	-		1.5	++	1.5	+	1.5	++		
70th egg	24	4.5	+++	3.8	+	<1.5	-	3.5	+	1/8	1/8
passage	48	4.8	+++	4.5	++	<1.5	-	<1.5	+		
$10^{6.1}$	72	-		2.2	++	<1.5	-	<1.5	+		

The titres are given as the $\log_{10}$ mouse ID50/ml. The titre of the inoculum in the cultures and to the cattle is given in Column 1. In the 'lesions in cattle' columns the denominator represents the number of cattle inoculated, and the numerator the number showing lesions. CPE = cytopathogenic effect.

Tests for growth and cytopathogenicity were carried out on pig kidney, cattle and mouse, and egg adapted strains of strain M11. Pig, calf and lamb monolayers were equally susceptible to the pig kidney, cattle and unweaned mouse strains, but in the ox kidney monolayers growth and cytopathogenicity of the unweaned mouse virus was reduced. Multiplication of virus adapted to 90 day old mouse and to chick embryos was similar in the pig kidney and lamb kidney cultures, but growth and cytopathogenic effect was much reduced in the calf and ox kidney cultures. It was thus possible to rank the strains for infectivity for calf and ox kidney cultures in the following order - cattle and pig kidney, young mouse, old mouse and egg. In infectivity tests on cattle tongues a similar ranking was found for the strains.

Similar results were also obtained with strains of Rho I when tested in kidney monolayers. The calf and ox kidney cultures were good indicators of the degree of non-infectivity of the strains for the cattle tongue.

Only two experiments using egg and mouse adapted strains have been carried out in pigs. With M11 old mouse strain the virus infected two pigs out of two given undiluted virus and one out of two given virus diluted 10^{-1}. A Rho I egg strain, which multiplied and was cytopathogenic in pig kidney tissue cultures, infected pigs irregularly. No tests in sheep have been carried out.

Conclusions

All the strains tested grew to a greater or lesser degree in pig kidney cultures causing a cytopathogenic effect whether they were derived from cattle, mice, eggs or tissue cultures. The same

was found with lamb kidney cultures. In the calf, and, particularly, the ox kidney cultures strains found to be attenuated for cattle by passage through mice and eggs multiplied to a less extent and did not produce so much cytopathogenic effect. Pay (1957 and personal communication) has found that some strains attenuated for cattle multiplied with reduced or no cytopathogenic effect on monolayer cultures of cattle tongue epithelium while others failed to multiply. This indicates that cattle tongue tissue cultures, ox and calf kidney cultures, may form a useful means of assessing the probable degree of non-infectivity for cattle of attenuated strains. The final tests for non-infectivity would, however, have to be carried out in cattle, sheep and pigs.

Type of plaques formed on pig kidney monolayer tissue cultures by various strains of the virus of foot-and-mouth disease.

When strains of the virus of foot-and-mouth disease from various sources were titrated by the standard plaque technique on pig kidney monolayers (see Summary and Conclusions to Section II), differences in size and shape could be seen in the plaques. The plaques might be large, medium or small with a regular or irregular shaped edge. In Table 17 is given a list of strains titrated by the plaque method. The table also includes the percentage of plaques falling within certain sizes, the shape of the plaques and an indication of the efficiency of plaque titration compared with mouse titration.

TABLE 17 TYPE OF PLAQUES FORMED ON PIG KIDNEY MONOLAYER TISSUE CULTURES

VIRUS Type strain and Source	Percentage of plaques found in following sizes Large >5mm. diam.	medium 3-5mm. diam.	small 1-2mm. diam.	Shape	Ratio plaque titre / mouse titre
O M11 cattle	4	60	36	Irregular	1
M11 pig kidney	80	19	1	Regular	0.1 - 2
M11 old mouse	-	10	90	Irregular	0.005 - 0.01
O Ven I cattle	2	44	54	Irregular	1
Ven I cattle epithelial culture	-	12	88	Irregular	0.001
Ven I egg	-	80	20	Irregular	1
O Dutch cattle	84	16	-	Regular	0.1 - 1
Dutch cattle tongue epithelial culture	84	16	-	Regular	0.1 - 1
O 1112 pig	80	20	-	Regular	2 - 10
O 1114 pig	80	20	-	Regular	-
O 643 pig	84	16	-	Regular	1
O V1 guinea pig	76	24	-	Regular	-
C GC guinea pig	-	36	64	Irregular	0.1
C 997 pig kidney	80	16	4	Regular	1
SAT 2 Rho I cattle	-	84	16	Irregular	0.3 - 1
Rho I egg/mouse	-	7	93	Irregular	0.02
RhoI egg	-	40	60	Irregular	0.1

Table 17 - continued

SAT 2	K/3/57 cattle	80	16	4	Regular	1 - 3
	K/3/57 pig kidney	80	16	4	Regular	1 - 3
	K/3/57 cattle tongue epithelial culture	3	20	77	Large regular Small irregular	0.1
SAT 2	K/121/56 pig kidney	-	32	68	Irregular	10 - 50
ASIA 1						
	Ind. 2 12th guinea pig passage	68	20	12	Regular	-
	Ind. 2 15th guinea pig passage	-	-	100	Regular	-

Of the strains shown as producing large plaques, strains 643, O 1112 and O 1114 were recovered from field outbreaks occurring in pigs and produced large plaques immediately on titration in pig kidney monolayers. Strain K/3/57 caused a severe disease in pigs as well as in cattle; strain C997 was recovered from an outbreak in which pigs and cattle were infected at the same time. With strain M11 the cattle virus gave rise to medium irregular plaques with an occasional larger plaque; after 4 passages in pigs and passage in pig kidney monolayers it gave rise to large regular plaques with occasional medium and small regular plaques. After 58 passages in pig kidney tissue cultures the pig kidney strain was compared with the cattle strain for virulence and invasiveness in cattle and pigs. The ability to initiate infection was the same with both strains; on cattle tongues the titres were $10^{6.6}$ and $10^{6.4}$ ID_{50}/ml and on pig tongues the titres were $10^{3.5}$ and $10^{4.3}$ ID_{50}/ml for the cattle and pig kidney strains respectively. However, after initial infection the course of the disease changed. With the cattle strain in cattle there were severe lesions on the lips and and four feet whereas with the pig kidney strain in two cattle only on four feet out of eight was a vesicle found. With the cattle strain in pigs, small lesions appeared on the feet, but with the pig kidney strain lesions were seen on the feet, the hocks and the teats. From these results it might be tentatively said that strains producing large plaques will cause severe disease in pigs once they are infected and that strains recovered from pigs will give large plaques. Of the other strains that form large plaques, two, strain V1 and strain Ind 2 came from guinea pigs and one, strain Dutch 0, from cattle

and from surviving cattle tongue epithelium tissue cultures.

It is interesting to note that the strains passaged in mice and eggs and shown to have reduced infectivity for cattle were among those that produced medium or small irregular plaques and in many cases had a lower titre by the plaque method than by mouse inoculation. This may indicate that the strains have a reduced infectivity for pigs. As mentioned before a Rho I attenuated strain infected pigs irregularly and in the case of the M11 old mouse strain the disease in three pigs after infection was mild with six feet out of a possible twelve showing small vesicles.

Passage of the virus through various hosts and tissue cultures modified the type of plaques formed. It has already been mentioned how M11 cattle strain, which produced medium irregular plaques, gave rise to M11 old mouse strain producing small plaques and the M11 pig kidney strain producing large plaques. With Ven 1 the cattle strain produced mainly medium and small plaques with an occasional larger one, and the titre was the same by the plaque method as by mouse inoculation. After 100 passages in surviving cattle tongue epithelium cultures it was difficult to obtain plaques. On a plate there might be 1 to 3 large plaques and many very small indefinite plaques. The plaque count titre was lower than the mouse titre. Ven I egg passaged virus, however, gave rise to medium and small plaques and titres by plaque count and by mouse inoculation were the same.

The cattle strain of K/3/57 was found to give large plaques and continued to do this on passage in pig kidney tissue cultures. It was, however, found difficult to adapt it to large scale

surviving cattle tongue epithelial cultures; but after a passage in the mouse and further cultivation in the tongue cultures, it grew well. By titration of the cattle tongue epithelial fluid from such cultures it could be seen that there was a mixed population consisting mainly of small plaques visible 3 to 4 days after infection but with an occasional large plaque.

Titrations of several strains of Rho I virus were compared on pig kidney monolayers. The cattle strain gave rise to plaques 3 - 5mm. in diameter with an occasional small plaque; on passage through eggs the virus gave rise to plaques 2 - 4.5mm. in diameter with an occasional smaller plaque; after alternate passage through eggs and mice the virus produced plaques 1mm. in diameter with an occasional one 2 - 3mm. in diameter.

Strain Ind 2 after 12 passages in guinea pigs gave rise on pig kidney monolayers to a virus population producing mainly large plaques with an occasional smaller plaque. Subculture of the large plaque led to a pure clone producing only large plaques. At the 15th passage in guinea pigs the plaque population was mainly small plaques and on subculture of a small plaque a clone was obtained which gave rise to small plaques only.

Conclusions

The strains of the virus of foot-and-mouth disease have been shown to give rise to different types of plaques. It appears that large plaque strains tended to produce a more severe disease in pigs, but sufficient attenuation for cattle of a strain passaged in pig kidney cultures did not occur at the passage level tested. Mouse and egg passaged strains produced small plaques and were found to

have reduced infectivity for pigs. The plaque population of a strain was found to vary after passage through different hosts. These findings may be of use in the selection of a suitable large plaque strain for making an inactivated vaccine, and in testing the homogeneity of an attenuated strain as regards its plaque forming particles.

PLATES

PLATES ILLUSTRATING THE TYPES OF PLAQUE FORMED BY DIFFERENT STRAINS OF THE VIRUS OF FOOT-AND-MOUTH DISEASE ON PIG KIDNEY MONOLAYER TISSUE CULTURES.

The plaque assays were carried out on 6cm. Petri dishes. At 48 hours or later the plates were stained with a solution of 1/20,000 Neutral Red in Earle's saline. After 2 hours incubation at 37°C., the supernatant fluid and overlay were carefully removed and the cells fixed in 10% formol saline. 24 hours later they were washed with tap water and stained with polychrome methylene blue for 1 - 2 minutes. The dye was removed, the plates allowed to dry and photographed.

Magnification - actual size.

PLATE 1

Large regular plaques formed by strain M11 pig kidney.

PLATE 2

Large and medium regular plaques formed by strain C997 pig kidney.

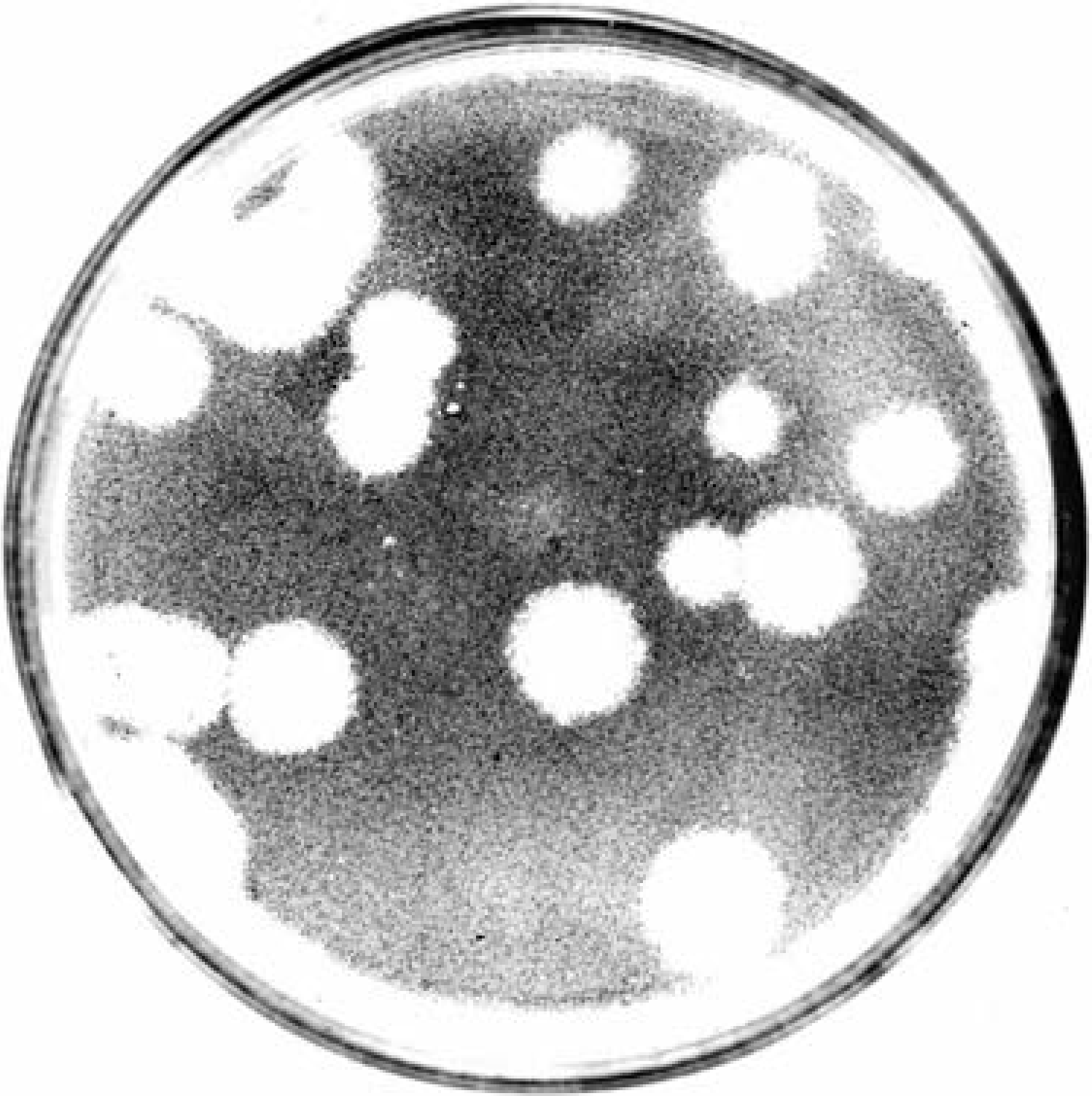


PLATE 3

Medium and small irregular plaques formed by strain K/121/56 pig kidney.

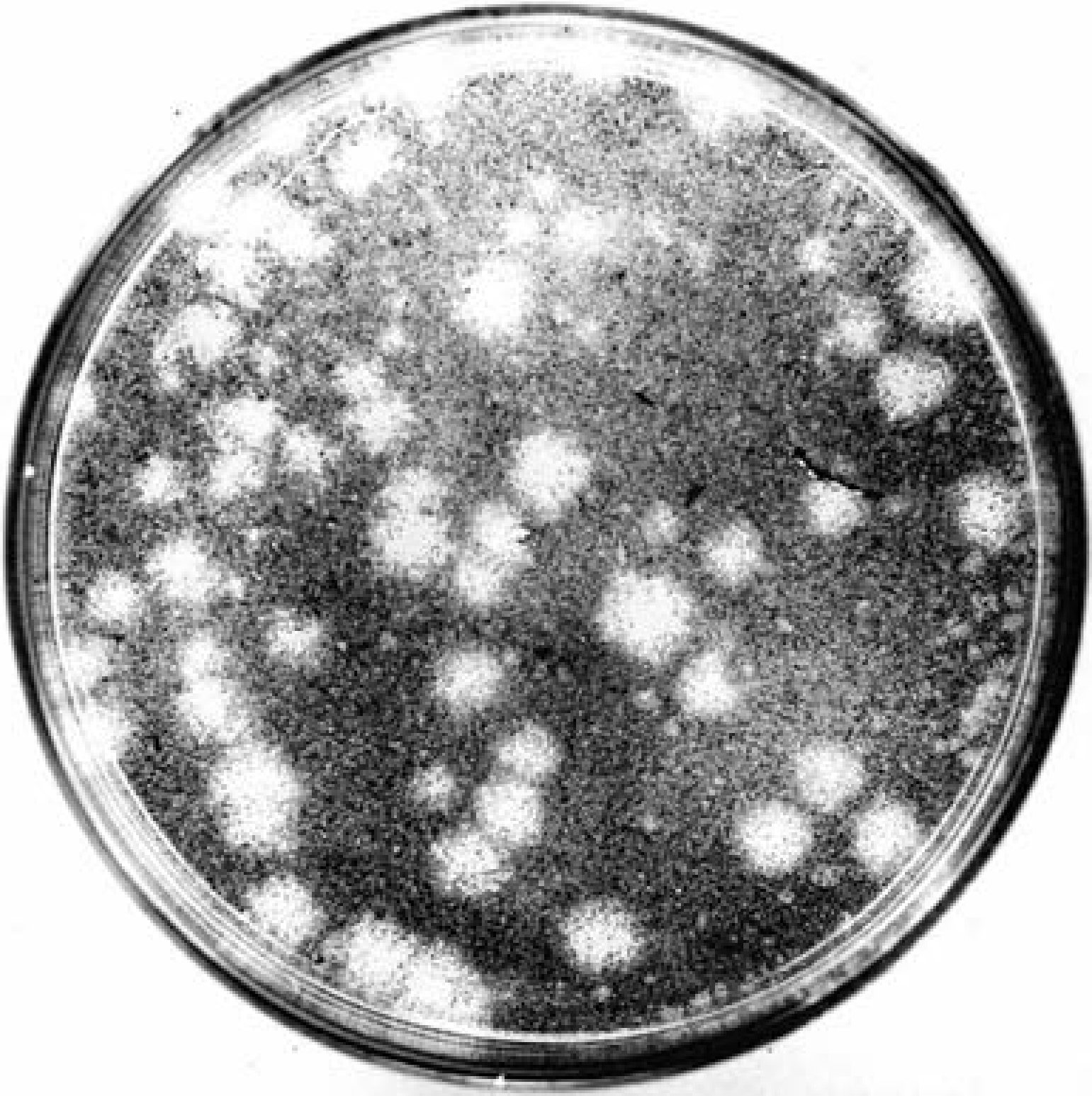


PLATE 4

Medium and small irregular plaques formed by strain Ven I chick embryo.

PLATE 5

Medium and small irregular plaques formed by strain Rho I egg.

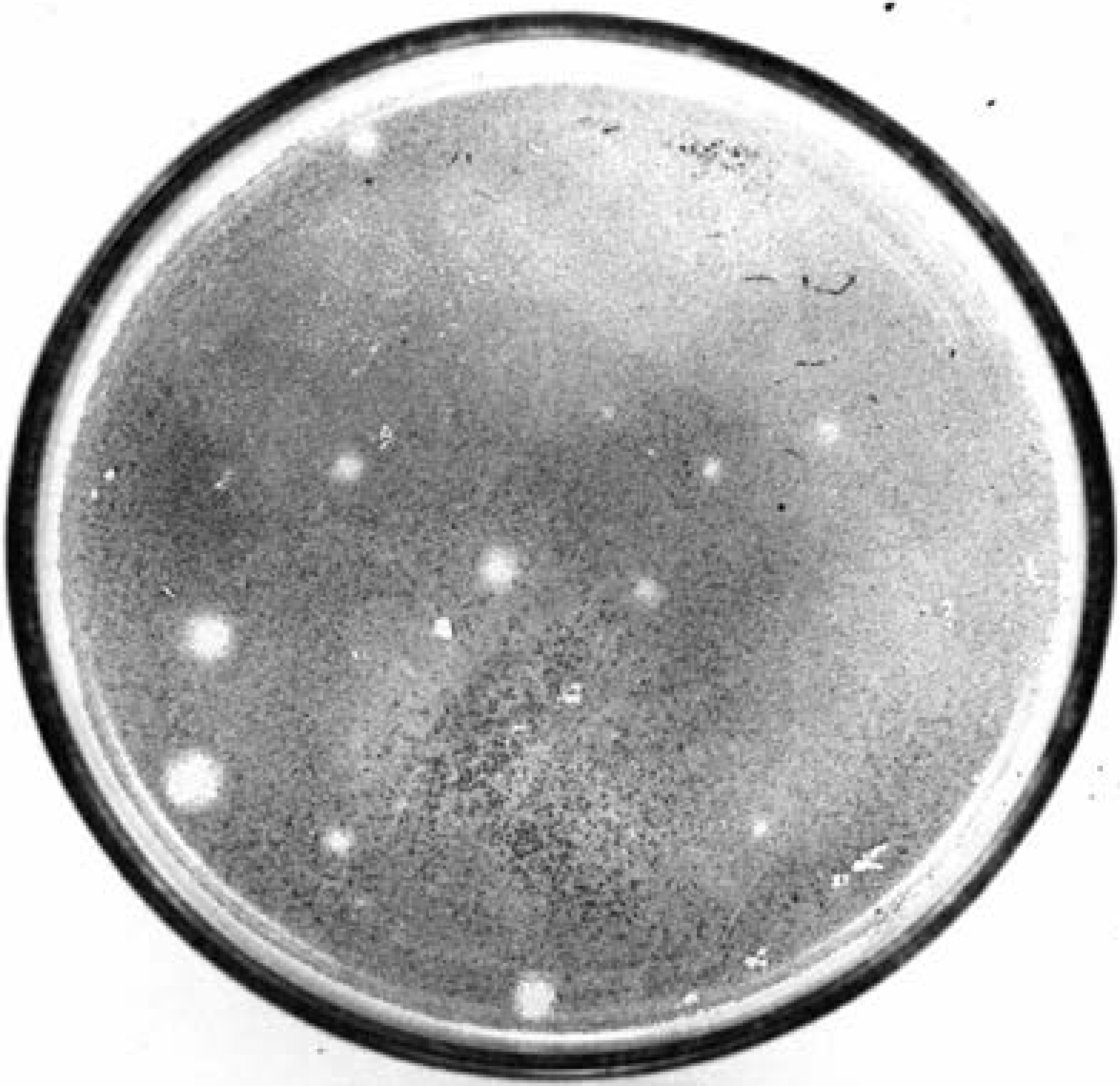
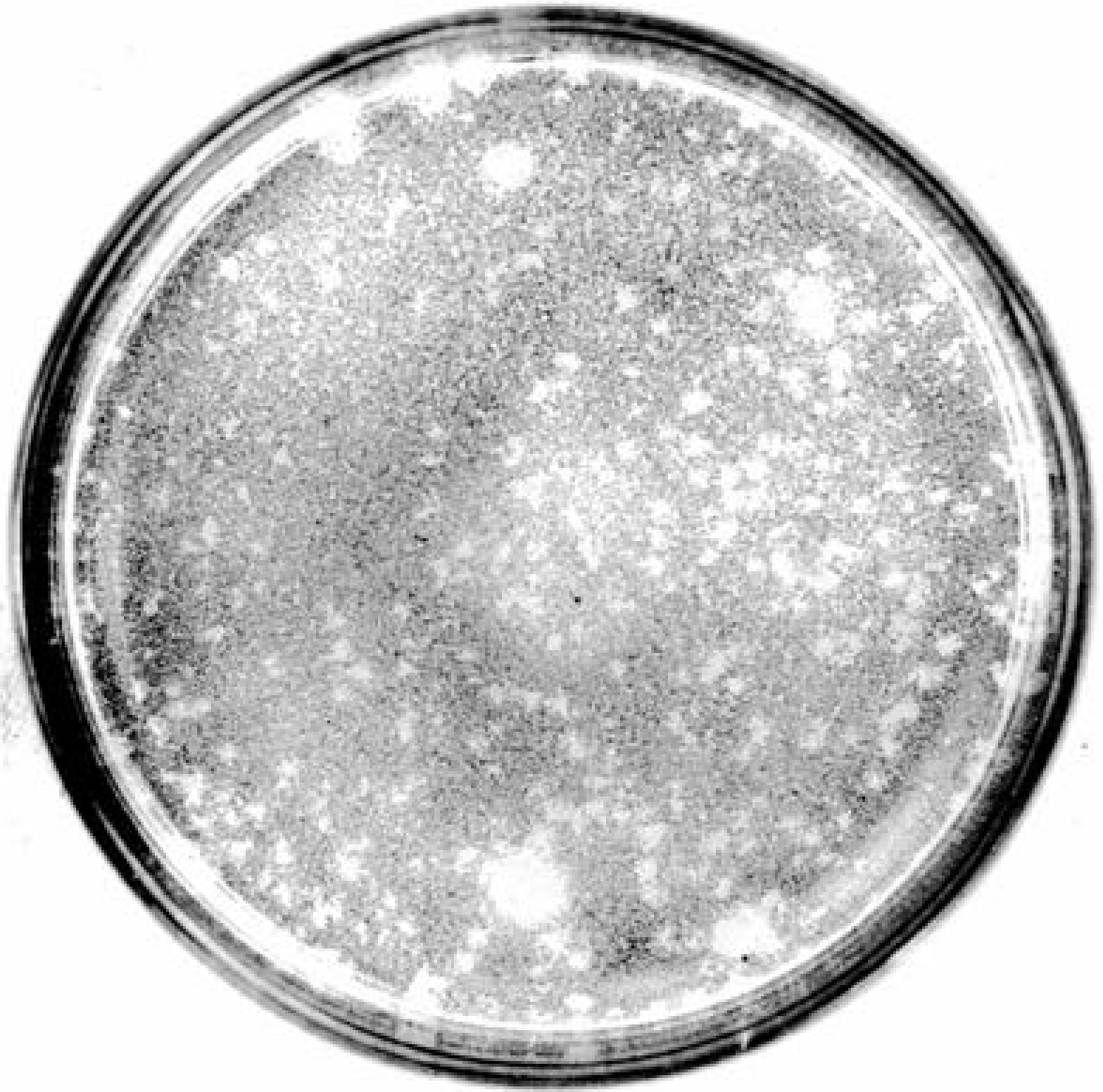


PLATE 6

Small irregular plaques with occasional medium to large regular plaques formed by strain K/3/57 cattle tongue epithelial culture.



IV. THE GROWTH AND MULTIPLICATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE IN PIG KIDNEY TISSUE CULTURES.

In the experiments described in the paper written in 1955, (Sellers, 1955) and in Sections II and III of this thesis, the virus was harvested at 7 hours, 16 hours, 18 hours, 24 hours or later after infection of the pig kidney tissue cultures. It was, however, necessary for production of high titre stocks for research and for vaccines to find out more about the adsorption of the virus on pig kidney cells, the latent period and the rate of virus growth, the titres reached at the different times and the amount of virus produced per cell. In this section the results of investigations into the multiplication of the virus of foot-and-mouth disease in pig kidney tissue cultures are recorded.

For the work four strains of the virus of foot-and-mouth disease were used, namely strain M11 60th - 120th pig kidney passage, strain 0997 50th - 80th pig kidney passage, strain K/3/57 7th - 14th pig kidney passage and strain K/121/56 6th - 12th pig kidney passage. The experiments were carried out on primary or secondary pig kidney monolayers or on cell suspensions derived from primary or secondary pig kidney monolayers. The cultures were grown in 8 oz. medicinal flat bottles. The virus and infected cells were titrated by the plaque technique (Section II).

Adsorption of the virus of foot-and-mouth disease on cell suspensions.

Stidies of the adsorption of the virus of foot-and-mouth disease were carried out on cell suspensions and on monolayers; in most of the experiments, however, cell suspensions were used since the

number of cells could be determined more accurately than in monolayers.

Cell suspensions were prepared by the removal of cells from primary or secondary monolayers with 0.25% trypsin in Ca and Mg free PBS or with versene, as described for the subculture of cells in Section I. After washing free of trypsin or versene the cells were resuspended in Earle's saline containing lactalbumen hydrolysate and yeast extract (EYL) and counted in a haemocytometer. 0.5 ml of virus of known titre was added to 2 ml of cell suspension having a concentration of $2 - 5 \times 10^6$ cells/ml. After incubation at 37°C for 30 minutes the cells were washed three times with fresh medium, counted again and diluted for assay of the number of infective cells on pig kidney monolayers.

In Table 18 can be seen the results of several experiments. At a ratio of one virus particle added to 30 or more cells there was apparent complete recovery of virus in infected cells. With ratios of 1 virus particle added to 4 cells up to 35 particles to one cell, it was not possible to infect every cell that could be infected, if a Poisson distribution of virus particles is assumed; at the highest input of virus to cells, namely, 17 - 35 particles per cell the proportion of cells infected was 37%. It must be remembered that the haemocytometer count included all intact cells, and it was possible that some of the cells were insusceptible, for example, fibroblasts, or were unable to allow virus multiplication to proceed after adsorption due to damage in preparation or to metabolic insufficiency. It was observed that in monolayers infected with sufficient virus nearly all the cells showed cytopathogenic action

so that it could be concluded that most cells were susceptible provided the conditions were right. Another possibility is that some cells required infection by more than one virus particle. The titrations of the infected cells were carried out by plating the cells onto monolayers; some of the cells might have been taken up in the overlay and thus been unable to release virus; by using cell suspension methods of titration of virus it was possible to increase the plaque count by a factor of 1.6 to 2.8. If it is assumed that the same increase holds for titration of infected cells by the cell suspension method, then the percentage of infected cells would be 61 - 100% at a ratio of 35 particles per cell and 26 - 45% for a ratio of 1 virus particle per cell. In a comparable study using trypsinised cattle tongue epithelium cells infected with strain Ven I, Cartwright, Pay and Henderson (1957) found that only 1% of their cells became infected when 10 ID_{50} were available per cell. The tongue epithelium cells, however, were a mixed cell population of varying degrees of viability.

Some experiments were carried out on the adsorption of virus on pig kidney monolayers. The monolayers were washed free of old medium 0.5 - 1 ml. of virus of know titre was added and adsorption carried out for 30 minutes at 37°C. The unadsorbed virus was then removed, the monolayer washed three times with PBS, and the cells taken off the glass by trypsin or versene treatment. The cells were then counted and diluted for assay of the number of infective cells on pig kidney monolayers. It was found that when one virus particle was added per 22 cells, 5 - 6% of the cells in the monolayer became infective. This indicates apparent complete recovery of virus.

TABLE 18 PERCENTAGE OF INFECTED CELLS IN PIG KIDNEY SUSPENSIONS AFTER 30 MINUTES ADSORPTION AT 37°C. WITH VARYING AMOUNTS OF VIRUS

Virus - M11 Pig kidney

Ratio of virus added per cell	Percentage observed infected cells	Percentage expected infected cells +
35: 1	37%	
17: 1		
8: 1	25%	
4: 1	19%	
2: 1	15%	
1: 1	16%	63%
1: 2	11%	39%
1: 4	8%	12%
1: 48	2%	2%
1: 1900	0.07%	0.05%
1: 7000	0.008%	0.014%
1: 70,000	0.002%	0.0014%

+ Percentage non-infected cells = 100 e^{-m}, where m = virus/cell ratio.

Growth curves of the virus of foot-and-mouth disease in cell suspensions

The object of studying the growth of virus in cell suspensions was to determine the time of virus release, the length of the latent period, the slope of the growth curve, the titres reached and the amount of virus produced per cell.

Cell suspensions were prepared by the removal of cells from primary or secondary monolayers with trypsin or with versene (see Section I). After washing they were resuspended in EYL at a concentration of $2 \times 10^6 - 5 \times 10^6$ cells per ml. in a volume of 2 - 3ml. 0.5 ml. of virus of varying titre was added and adsorption carried out for 30 minutes at 37°C. The cell suspension was then diluted with fresh EYL and centrifuged three times with resuspension in EYL to remove unadsorbed virus. Finally, the cells were resuspended in 10 - 50 ml. of EYL and kept at 37°C. Samples were taken at intervals for plaque assay of whole culture and of supernatant fluid after centrifugation. The cells were also counted in a haemocytometer and usually had a final concentration of $10^5 - 4 \times 10^5$ per ml.

In Fig. 2 the curve marked (a) shows a typical growth curve obtained when the ratio of virus input to cells was one to one. After the end of the adsorption period of 30 minutes the titre of the whole culture remained constant for 2 hours. Between 2 and 3 hours there was a rise which was maintained up to the fourth hour. If not all the cells were initially infected there was a further increase, and a high titre, $10^6 - 10^7$ pfu/ml., was reached at 7 hours. The latent period i.e. the time from the beginning of the adsorption

FIGURE 2

Growth curves of the virus of foot-and-mouth disease, strain M11, in pig kidney cell suspensions:-

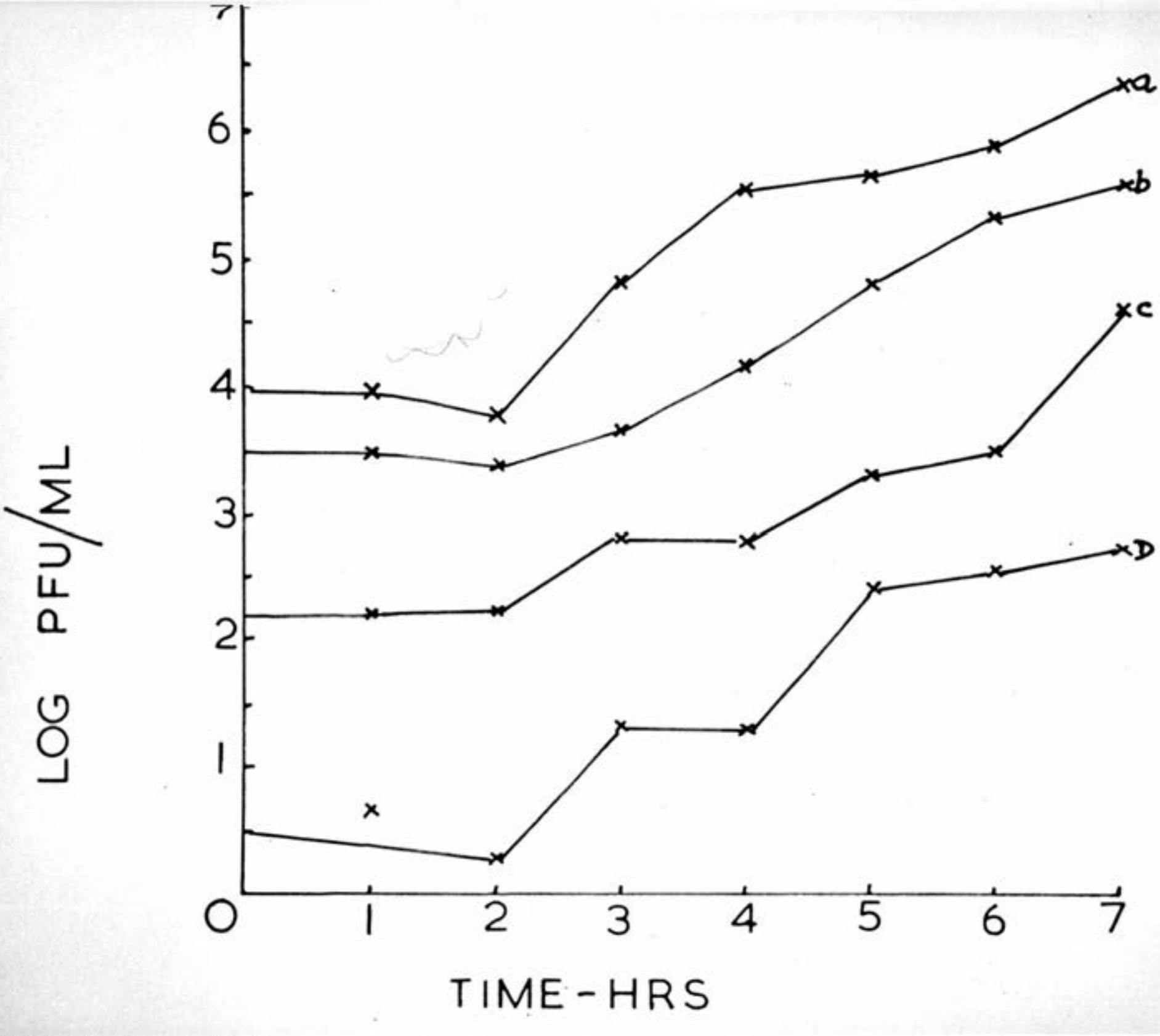
a) infected with virus/cell ratio 1:1

b) " " " " " 1:40

c) " " " " " 1:1,600

d) " " " " " 1:64,000

(The titres are those of the whole culture)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TIME-HRS
0
1
2
3
4
5
6
7
a
b
c
D

period until the time when the infected cell has released on the average one virus particle, was about 2½ - 3 hours. A rise in virus titre of the supernatant fluid took place between the first and second hour after the adsorption period indicating that the time of release of the first virus particles was about 2 hours from the beginning of the adsorption period. Over a series of experiments the mean yield per infected cell was about 188 plaque forming virus particles.

In Fig. 2, curves (b), (c) and (d) represent curves obtained when the cells were infected with different amounts of virus, the virus-cell ratio being considerably less than unity. The length of the latent period appeared to be the same in every case, namely, 2½ - 3 hours. The growth of the virus proceeded in some cases in steps, this being more evident in the cell suspension receiving the least amount of virus.

In Fig. 3, a comparison was made of the growth curves obtained with different media but with the same virus inoculum. It can be seen that although the same number of cells were infected initially the latent period was prolonged and the initial rise was later in the cell culture kept in a medium deficient in amino-acids and growth factors (medium, 50% M/25 phosphate buffer, 50% glucosol solution - Brooksby and Wardle, 1954); again a second virus rise was absent in the deficient culture.

Growth curves of the virus of foot-and-mouth disease in monolayers

The aim of studying the growth of the virus in monolayers was to find out about the shape of the curve, the titres reached at the various times and the amount of virus produced per cell.

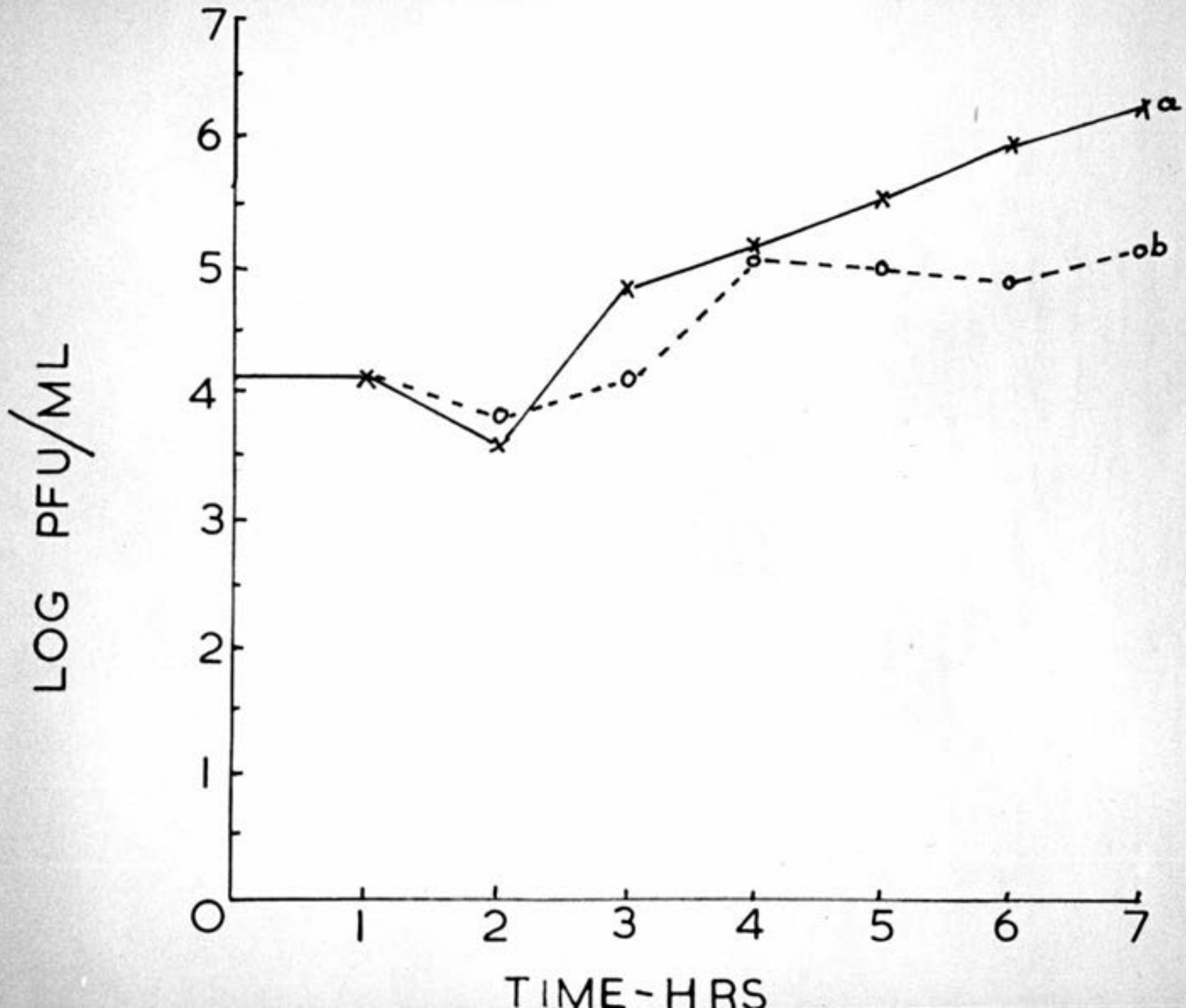
FIGURE 3

Growth curves of the virus of foot-and-mouth disease, strain M11, in pig kidney cell suspensions:-

a) cells after infection kept in Earle's saline containing 0.5% lactalbumen hydrolysate and 0.01% yeast extract.

b) cells after infection kept in 50% glucosol solution and 5% M/25 phosphate buffer (pH 7.6).

(The titres are those of the whole culture)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TIME-HRS
0
1
2
3
4
5
6
7
a
b

Primary or secondary monolayers in 8 oz. medicinal flat bottles were washed free of ald medium with two washes of PBS. 0.5 - 1 ml. of the virus undiluted or diluted in PBS was added and adsorption carried out for 30 minutes at 37°C. Unadsorbed virus was then removed, the monolayer washed three times with PBS and 10 - 15 ml. of prewarmed EYL added, the culture being replaced at 37°C. Samples of supernatant fluid were taken at intervals for plaque assay and the cultures were examined under the microscope at that time. For routine passage of virus or for growth curves carried on over several days a dilution of virus was added to cultures, which had already been washed and given fresh medium.

The shape of the growth curve of the virus on monolayers was similar to that in cell suspensions (Fig.4). Counts of cells removed from monolayers by versene treatment indicated that there were about 3×10^6 cells on a monolayer; consequently, where all the cells were initially infected the latent period was about $2\frac{1}{2}$ - 3 hours from the beginning of the adsorption period and the time of release $1\frac{1}{2}$ - 2 hours. This was similar to the finding with cell suspensions. After the cells had started to release virus there was a rise and the titre reached a maximum of 5×10^6 - 3×10^7 pfu/ml. between 7 and 8 hours. Destructive changes in the cells were seen from $2\frac{1}{2}$ hours after infection onwards, a time coinciding with the end of the latent period, and by 7 hours most cells were rounded up and were coming off the glass. Where only a proportion of cells were infected initially the time of release was later and the titre rose by a series of steps, which merged together to reach a maximum

FIGURE 4

Growth curves of the virus of foot-and-mouth disease, strain M11, in pig kidney monolayer tissue cultures:-

a) infected with a virus/cell ratio of 1.5: 1

b) " " " " " " 1.5:1,000

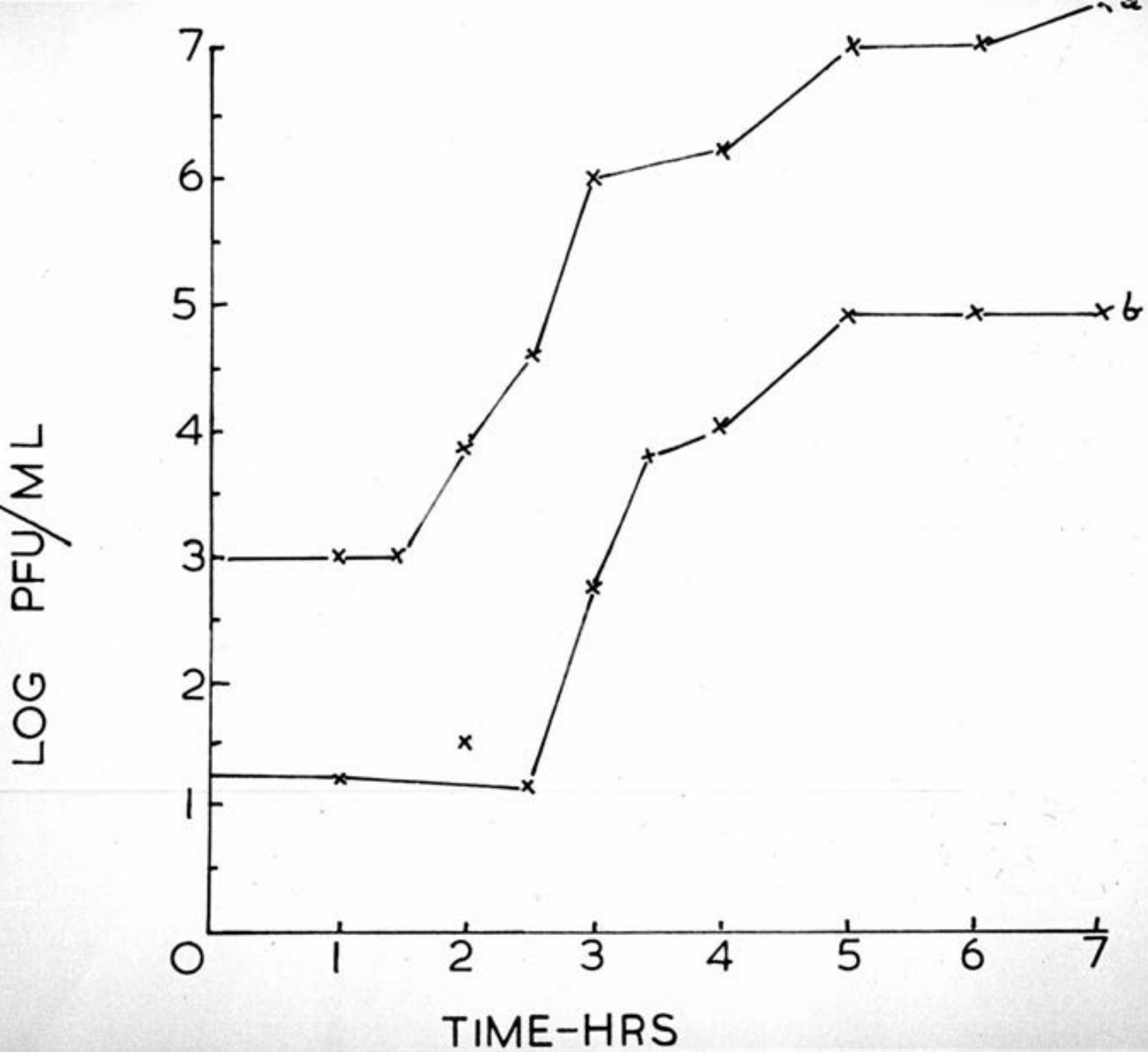
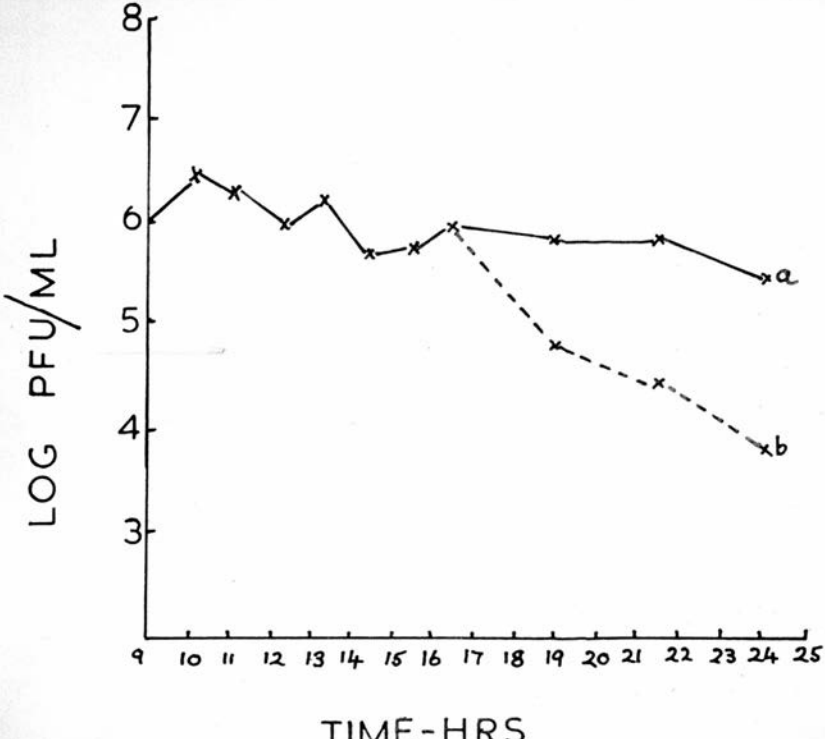

LOG PFU/ML
TIME-HRS
0
1
2
3
4
5
6
7
b

FIGURE 5

a) Growth curve of the virus of foot-and-mouth disease, strain M11, in pig kidney monolayer tissue cultures from the 9th to the 24th hour after infection.

b) Represents the curve obtained when cells and cell debris were removed.


8
7
6
5
4
3
LOG PFU/ML
a
b
9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
TIME-HRS

at about 9 - 12 hours (Fig. 4, curve (b)). In a series of experiments the mean yield of infective virus per cell was 200 plaque forming particles.

The titre levelled out with a gradual decrease from the 9th to 24th hour after infection (Fig. 5). If, however, at 16 hours the culture was centrifuged free of cells and debris and the supernatant fluid incubated at 37°C, the fall in virus titre was greater than in the control cultures indicating either that there was still release of virus from the cells or that the cell protein in the medium was protecting the virus. The fall in titre was continued until 90 hours (Fig. 6, Section V.), and even after 11 days at 37°C. infective virus was recovered from the culture.

Summary and conclusions

In the adsorption experiments 37% of the cells were infected after 30 minutes with a virus input of 17 - 35 pfu/cell; with a virus input of 1 pfu/cell 16% of the cells became infective. With virus/cell ratios lower than 1 to 30 full recovery of inoculated virus was obtained. It is thus apparent that it is difficult to ensure that all cells become infected and this may be a disadvantage in studies of the intracellular growth of virus and in genetic studies. However, better techniques of cell handling may improve the cell infection rate.

The length of latent period and the shape of the curve were similar in monolayers and in cell suspensions. The final titres reached at 7 hours were different being higher in the monolayers. The titre in the monolayers ($10^{6.7}$ - $10^{7.5}$ pfu/ml) was lower than that obtained in trypsinised cattle tongue cells at 6 hours.

$10^{7.5}$ - $10^{8.0}$ mouse ID_{50}/ml (Cartwright, Pay and Henderson, 1957) and than that obtained in calf kidney cells at 12 hours, $10^{8.6}$ pfu/ml (Bachrach, Callis Hess and Patty, 1957). These differences may be related to the type and number of cells and to the volume of supernatant fluid as well as to the strain of virus used.

The yield of infective virus particles per cell was about 200 plaque forming units for monolayers and for cell suspensions. However, the latent period and the amount of virus produced but not the number of cells initially infected may be affected by the composition of the medium. Deficiencies in amino-acids and growth factors led to a lengthening of the latent period and a reduction in titre at 7 hours.

For production of high titre stocks the following procedure was, therefore, adopted. Monolayers grown as described in Section I were used either as primary or secondary cultures. The cells were washed to remove old medium; undiluted virus 0.5 - 1.0 ml. was added and adsorption carried out at 37°C. for 30 minutes. The monolayer was then washed free of unadsorbed virus and fresh medium (EYL) added. The cultures were incubated at 37°C. and collected 7 - 8 hours later, when the cytopathogenic effect could be seen in most cells.

Often however, the virus was passaged by addition of a suitable dilution of virus to monolayers already given fresh medium, and in these instances the virus was collected at 16 hours, 18 hours, 24 hours or later depending on the inoculum given. The method of infection of monolayers and the time of harvest of virus for inactivated vaccine production will be dealt with in the next section.

V. STUDIES ON THE VACCINATION OF GUINEA PIGS WITH AN INACTIVATED VACCINE PREPARED FROM VIRUS GROWN IN PIG KIDNEY MONOLAYER TISSUE CULTURES

One of the main objects in the control of foot-and-mouth disease is the production of an effective vaccine whether living or inactivated; for this a source of virus is required. It was, therefore, decided to see if virus grown in pig kidney tissue cultures was suitable. It has been shown in Section III that strain M11 virus was not sufficiently attenuated for cattle and pigs by passage in pig kidney cultures, altheugh it may be possible to produce living vaccine by growth of virus attenuated by mouse and chick embryo passage in pig kidney monolayers. In this section production of an inactivated vaccine from virus grown in pig kidney monolayers is considered. The object was to see if the virus after inactivation could stimulate the production of antibodies and enable the vaccinated animals to resist challenge with the homologous virus.

For the work four strains of the virus of foot-and-mouth disease were used. They were strain M11 82 - 120th passage in pig kidney monolayers, strain C997 63 - 75th passage in pig kidney monolayers, strain K/3/57 7th - 11th passage in pig kidney monolayers and strain K/121/56 7th - 11th passage in pig kidney monolayers. Growth of the virus was carried out on pig kidney monolayer tissue cultures and the medium consisted of EYL (85%), $^{M}/_{25}$ phosphate buffer (pH 7.6) (10%) and ox serum (5%). Tests of the vaccine were carried out in guinea pigs.

Infection of guinea pigs with virus grown in pig kidney monolayers

In the first experiment virus grown in pig kidney monolayers was tested for its capacity to infect guinea pigs. K/121/56 and K/3/57 strains caused lesions at the site of inoc ulation on the hind pad, but only occasional generalisation to other sites was observed. After inoculation of M11 and C997 strains lesions were observed at the site of inoculation on the right hind pad, on the other pads and sometimes on the tongue. A comparative titration of strain C997 in unweaned mice, on pig kidney plates and in guinea pigs was carried out and the results are shown in Table 19. It can be seen that based on the results of primary lesions alone the virus titre in guinea pigs was of the same order as that obtained by mouse inoculation or on plates; the virus titre based on the number of guinea pigs showing generalisation was slightly lower. It must be emphasised that these results were obtained on guinea pigs of 300 - 400g weight. Older guinea pigs or guinea pigs kept on wire mesh were not suitable as the pads tended to be hard and resisted infection, titres a hundred-fold lower being obtained. M11 strain gave similar results when inoculated into guinea pigs. It was, therefore, decided to use strains M11 and C997 grown in pig kidney tissue cultures for preparation of vaccine and for the challenge of vaccinated guinea pigs.

TABLE 19 RESPONSE IN GUINEA PIGS TO INOCULATION OF VARYING DILUTIONS OF PIG KIDNEY GROWN VIRUS - strain C997

Virus dilution	Local lesions	Generalised lesions
10^{-6}	0/8	0/8
$10^{-5.3}$	4/8	2/8
$10^{-4.6}$	8/8	7/8
$10^{-3.9}$	8/8	7/8
$10^{-2.6}$	8/8	8/8
$10^{-1.3}$	8/8	8/8

Titre based on local lesions in guinea pigs -$10^{6.3}$ guinea pig ID_{50}/ml.

Titre based on generalised lesions in guinea pigs -$10^{5.9}$ guinea pig ID_{50}/ml

Titre based on plaque counts in pig kidney monolayers - $10^{6.2}$ pfu/ml

Titre based on inoculation of unweaned mice -10^{6} mouse ID_{50}/ml.

0.1 ml. given to guinea pigs intradermally on right hind pad.

Numerator represents number of guinea pigs showing lesions

Denominator represents number of guinea pigs inoculated.

Inactivation of the virus of foot-and-mouth disease by formalin

The inactivation of the virus of foot-and-mouth disease was carried out by formalin treatment of culture virus adsorbed on aluminium hydroxide gel. The method was based on that described by Galloway and Henderson (1953).

Supernatant fluids of infected cultures were harvested and pooled. The fluid was centrifuged to remove cell debris and the supernatant collected. Sometimes the supernatant was filtered through a Seitz EK pad, which had been pre-washed with 10% ox serum in phosphate buffered saline. To two parts of supernatant fluid was added three parts of aluminium hydroxide gel and enough 40% formalin to give a final concentration of 1:2,000. Incubation was then carried out at 26°C. at pH 7.2 - 7.4. The preparation was tested for non-infectivity in mice and guinea pigs, since undiluted aluminium hydroxide interfered with the cytopathogenic effect of the virus on tissue cultures. In Table 20 the titres obtained during inactivation of the virus are recorded. It can be seen that at 48 hours no virus was detected in ten mice inoculated with 0.03 ml. undiluted vaccine by the intraperitoneal route. It was decided to terminate the incubation at 26°C after 4 days, at which time the vaccine was tested for non-infectivity by intraperitoneal inoculation of mice and intradermal inoculation of guinea pig pads.

(It is interesting to note that when the titres given in Table 20 are plotted as log titre against log time a straight line is obtained. The straight line cuts the 0 titre level at 40 hours. By termination of the incubation at 96 hours it is apparent that no virus could be detected in about 1 ml. of undiluted vaccine, if the

straight line is prolonged below the 0 line to -1.5 log titre.)

TABLE 20 INACTIVATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE BY FORMALIN AFTER ADSORPTION ON ALUMINIUM HYDROXIDE

Virus - M11

Time after addition of formalin (Hours)	Titre (log mouse ID_{50}/0.03 ml.)
0	5.5
5	3.0
24	0.8
48	0^+
72	0^+
96	0^+
120	0^+

$^+0$/10 mice dead when given undiluted vaccine.

The vaccination of guinea pigs with inactivated vaccine prepared from virus grown in pig kidney monolayers

After the vaccine had been prepared from culture grown virus and had been shown to be non-infective, it was inoculated as a dose of 1 ml. subcutaneously into the thigh of guinea pigs, which were then examined daily. After 2 - 3 weeks blood samples for neutralisation tests were taken and the guinea pigs challenged by the inoculation of about 0.1 ml of pig kidney culture virus intradermally on the right hind pad. The guinea pigs were examined daily for the next 8 days and the lesions at the site of inoculation and on the other pads and on the tongue noted. Neutralisation tests were carried out by the inoculation of mice with preincubated mixtures

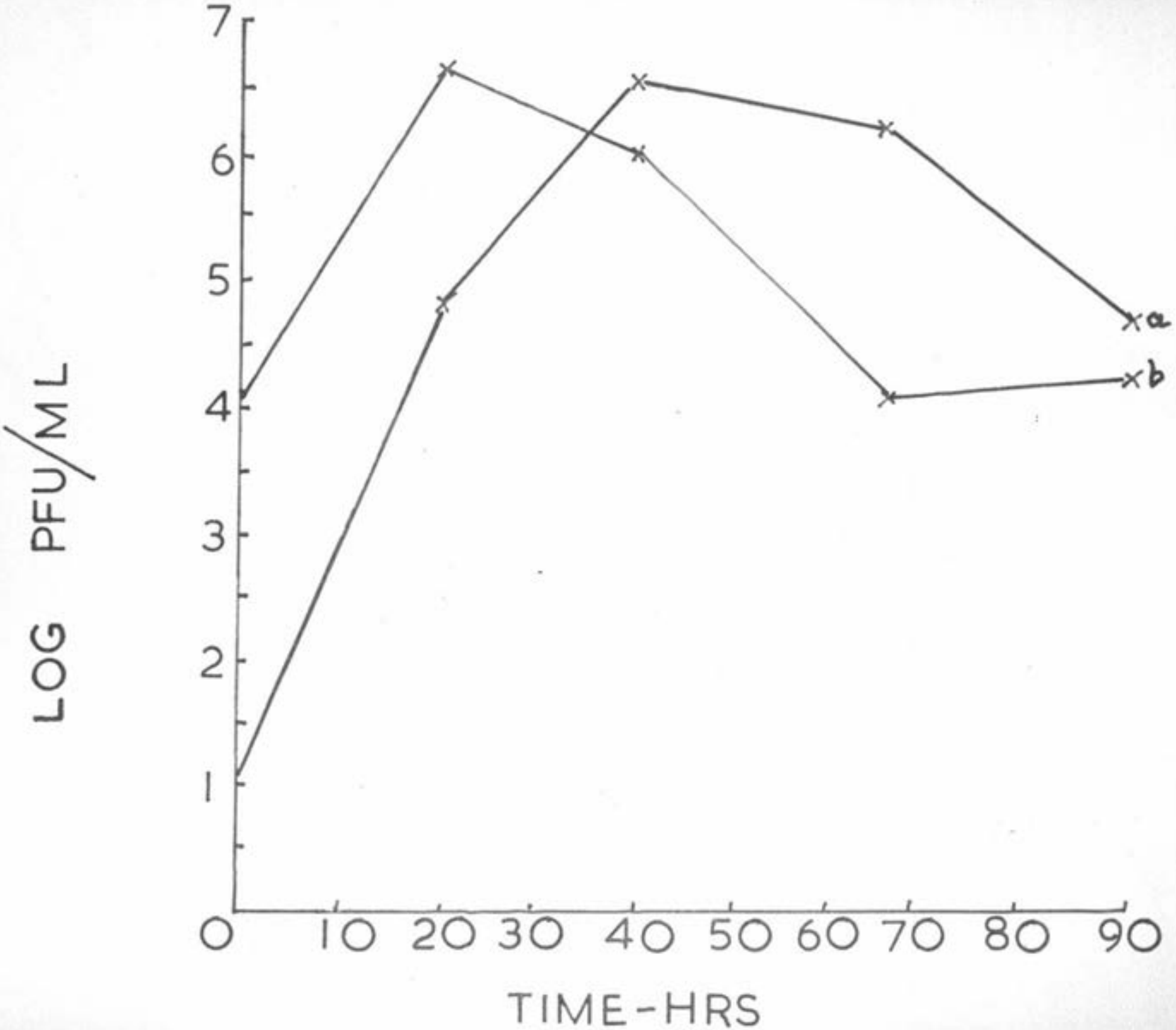
of 1/10 serum and tenfold dilutions of virus.

Preliminary experiments showed that vaccines made from culture virus harvested 24 - 48 hours after inoculation stimulated the production of antibodies and protected the guinea pigs when challenged with the homologous pig kidney grown virus. Some of the vaccinated guinea pigs showed lesions at the site of challenge, others did not. It was, therefore, decided to investigate the optimum time for harvest of culture virus and the best methods of challenge and to devise a test of potency for the vaccine.

Culture virus was harvested 18,42,66 and 90 hours after addition of seed virus. Vaccines were prepared and given to guinea pigs, which were subsequently challenged 3 weeks later with the culture virus. The results are given in Table 21. Although the difference was not significant vaccines made from virus harvested from 42 hours onwards appeared to give better protection than vaccine of the 18 hour harvest, although the virus titre at 18 hours was the same as that at 42 hours and greater than those at 66 and 90 hours. This experiment was repeated using virus harvested from two sets of cultures which had been seeded with two levels of virus differing a thousandfold in titre. Vaccines were made from the virus collected at 18, 42, 66 and 90 hours, and the growth curves showing the titres can be seen in Fig. 6. Vaccines prepared from 18 and 42 hour harvests of cultures given the larger inoculum gave better protection than vaccines prepared from 66 and 90 hour harvests; and vaccines prepared from 42 and 66 hour harvests of cultures given the smaller inoculum gave better protection than vaccines from the 18 and 90 hour harvests. It was observed that in

FIGURE 6

Growth curves of the virus of foot-and-mouth disease, strain C997, in pig kidney monolayer tissue cultures. (b) cultures infected with a thousandfold less virus than (a) cultures.


LOG PFU/ML
7
6
5
4
3
2
1
0
10
20
30
40
50
60
70
80
90
TIME-HRS
a
b

cultures given the low inoculum the cytopathogenic effect was not complete at 18 hours and thus all the cells had not released virus. The maximum cytopathogenic effect was observed from 18 to 42 hours depending on the size of the inoculum; hence in the remainder of the experiments an inoculum of 10^4 pfu was added per culture and the cultures were harvested at 24 hours when the cytopathogenic effect was complete. The poor response to challenge of guinea pigs vaccinated with the 90 hour cultures could probably be attributed to denaturation of the antigen; the ox serum (5%) was therefore included in the medium in order to minimise denaturation.

TABLE 21 RESPONSE IN GUINEA PIGS TO VACCINES PREPARED FROM VIRUS COLLECTED AT DIFFERENT TIMES AFTER INOCULATION OF CULTURES

Virus - M11

Time of collection Hours	Virus Titre (log pfu/ml.)	Response in guinea pigs to challenge with culture virus (10^4 guinea pig ID_{50})	
		Local lesions	Generalised lesions
18	6.9	7/7	3/7
42	6.5	5/6	0/6
66	3.9	8/8	1/8
90	2.6	7/7	1/7

In columns 3 and 4, the numerator represents the number of animals showing lesions, and the denominator the number of animals vaccinated.

Investigations were made into the effect on vaccinated guinea pigs of challenge with varying doses of pig kidney culture virus. Vaccinated guinea pigs were challenged with two levels of pig kidney virus, which in controls had a titre of $10^{2.3}$ and $10^{4.3}$ ID_{50} based

on the development of primary lesions and $10^{1.5}$ and $10^{3.5}$ ID_{50} based on the development of generalised lesions. It can be seen that the vaccine prevented development of primary lesions in $^{7}/_{10}$ guinea pigs challenged with the lower level of virus and that generalisation did not occur at either challenge level (Table 22). At these challenge levels and with undiluted vaccine it was not possible to assess the potency of a vaccine.

TABLE 22 RESPONSE IN GUINEA PIGS VACCINATED WITH PIG KIDNEY VACCINE TO CHALLENGE WITH DIFFERENT LEVELS OF CULTURE VIRUS

Virus - C997

Challenge Dose			
$10^{2.3}$ ID_{50}	$10^{1.5}$ ID_{50}	$10^{4.3}$ ID_{50}	$10^{3.5}$ ID_{50}
Local lesions	generalised lesions	Local lesions	generalised lesions
$^{3}/_{10}$	$^{0}/_{10}$	$^{10}/_{10}$	$^{0}/_{10}$

Numerator represents number of guinea pigs showing lesions

Denominator represents number of guinea pigs inoculated.

Accordingly culture virus was prepared and made into vaccines using undiluted harvest virus and harvest virus diluted $^{1}/_{3}$, $^{1}/_{9}$ and $^{1}/_{27}$. Three weeks later the guinea pigs were challenged with pig kidney culture virus at levels $10^{1.4}$, $10^{2.7}$, 10^{4} and $10^{5.3}$ ID_{50} as determined by local and generalised lesions in control guinea pigs. The results can be seen in Table 23. As far as development of local lesions is concerned, it can be seen that all vaccines were able to protect against challenge with 1.4 guinea pig ID_{50}, but it was not possible to differentiate between the four levels of vaccine on the basis of primary lesions. As far as the occurrence

of generalisation it can be seen that in the 1/27 and 1/9 vaccines there was a difference between the response in guinea pigs challenged with $10^{1.4}$ and $10^{2.7}$ ID_{50} and the guinea pigs challenged with 10^4 and $10^{5.3}$ ID_{50}. In guinea pigs given undiluted and 1/3 vaccines there was no difference in the response. In response to challenge of 10^4 and $10^{5.3}$ ID_{50}, there was a difference in the development of generalised lesions between the guinea pigs given 1/9 and 1/27 vaccine and guinea pigs given 1/3 and undiluted vaccine. Thus it might be possible to assess the potency of a vaccine on the basis of protection of guinea pigs against generalisation when given 10^4 to $10^{5.3}$ guinea pig ID_{50}. The results are summarised in Table 24. From this table the dilution of vaccine that will protect 50% of the guinea pigs from generalisation when challenged with 10^4 ID_{50} or more can be calculated by the method of Reed and Muench (1938) as 1/11.

TABLE 23 RESPONSE IN GUINEA PIGS GIVEN VACCINES PREPARED FROM DIFFERENT DILUTIONS OF CULTURE VIRUS TO CHALLENGE WITH DIFFERENT LEVELS OF CULTURE VIRUS

Virus - C997

Dilutions of virus forming vaccine	Challenge dose guinea pig log ID_{50}	Response in guinea pigs local lesions	Response in guinea pigs generalised lesions
$^1/_{27}$	1.4	$^0/_4$	$^0/_4$
	2.7	$^1/_4$	$^0/_4$
	4	$^4/_4$	$^3/_4$
	5.3	$^4/_4$	$^2/_4$
$^1/_9$	1.4	$^0/_4$	$^0/_4$
	2.7	$^4/_4$	$^0/_4$
	4	$^4/_4$	$^2/_4$
	5.3	$^4/_4$	$^2/_4$
$^1/_3$	1.4	$^0/_4$	$^0/_4$
	2.7	$^4/_4$	$^0/_4$
	4	$^4/_4$	$^0/_4$
	5.3	$^4/_4$	$^0/_4$
Undiluted	1.4	$^0/_4$	$^0/_4$
	2.7	$^2/_4$	$^0/_4$
	4	$^4/_4$	$^1/_4$
	5.3	$^4/_4$	$^0/_4$

Numerator represents number of guinea pigs showing lesions.

Denominator represents number of guinea pigs inoculated.

TABLE 24 RESPONSE IN GUINEA PIGS GIVEN VACCINES MADE FROM DIFFERENT DILUTIONS OF CULTURE VIRUS TO CHALLENGE WITH 10^4 and $10^{5.3}$ ID_{50} (Derived from Table 23)

Vaccine dilution	Response of guinea pigs to challenge dose of 10^4 and $10^{5.3}$ ID_{50} Generalised lesions	
	Protected	Unprotected
1/27	3	5
1/9	4	4
1/3	8	0
Undiluted	7	1

Hence by the method of Reed and Muench (1938) the dilution giving 50% protection is 1/11.

Another experiment was carried out on similar lines in which guinea pigs were given vaccines prepared from undiluted, 1/4 and 1/16 culture virus and challenged with 10^4 and 10^5 guinea pig ID_{50} (Table 25). From these results the dilution of the vaccine that will protect 50% of the guinea pigs from challenge with 10^4 and 10^5 guinea pig ID_{50} can be calculated as 1/8. Neutralisation tests were carried out on serum obtained from vaccinated guinea pigs before challenge, and the log neutralisation index was 1.6 with the 1/16 vaccine, 2.5 with the 1/4 vaccine and 2.7 with the undiluted vaccine. This indicates that a difference in antibody response occurred between vaccines made from undiluted and 1/4 culture virus and the vaccine made from 1/16 culture culture virus as well as a difference in response to challenge. On the basis of these results a tentative scheme for potency may be put forward; namely, the potency of a

given vaccine is that dilution of the vaccine which will protect 50% of the guinea pigs from generalisation when challenged with virus of titre 10^4 or higher.

TABLE 25 RESPONSE IN GUINEA PIGS GIVEN VACCINE MADE FROM DIFFERENT DILUTIONS OF CULTURE VIRUS TO CHALLENGE WITH 10^4 and $10^5 ID_{50}$

Virus - C997

Vaccine dilution	Response of guinea pigs to challenge dose of 10^4 and $10^5 ID_{50}$ Generalised lesions	
	Protected	Unprotected
1/16	5	15
1/4	17	3
Undiluted	17	3

Hence by the method of Reed and Muench (1938) the dilution giving 50% protection is 1/8.

Summary and conclusions

Virus grown in pig kidney monolayer tissue cultures was inactivated by adsorption on aluminium hydroxide and treatment with 0.05% formalin for 4 days at 26°C. The vaccine thus produced stimulated the production of antibodies and protected guinea pigs from generalisation when challenged with homologous culture virus. Investigations were made into the optimum time of culture harvest, and it was concluded that it was best to collect the culture virus when cell destruction was at a maximum and before the antigen was denatured. The time varied between 18 and 42 hours depending on the

amount of virus added to the culture. It appeared that for the strains used a challenge dose of at least 10^4 guinea pig ID_{50} should be given, since below that amount the response at the site of inoculation and in the development of generalised lesions in guinea pigs vaccinated with one vaccine level may vary with the size of the challenge dose. With a challenge dose of $10^4 ID_{50}$ and above variation in response in guinea pigs given the same vaccine did not appear to occur, but there was variation in the degree of generalisation in guinea pigs given different dilutions of vaccine. Hence it is tentatively suggested that the potency of a vaccine be defined as that dilution of vaccine which will protect 50% of the guinea pigs from generalisation when challenged with 10^4 guinea pig ID_{50}. This appeared a suitable definition for the two strains of virus used, but it may have to be modified for other strains.

The experiments have thus shown that pig kidney monolayers can be used as a source of virus for inactivated vaccines and that althpugh the ultimate test of effectiveness of a foot-and-mouth disease vaccine for use in the field must be carried out in cattle, sheep and pigs, it is possible to use guinea pigs for exploratory and comparative studies on vaccine preparations.

VI. THE USE OF PIG KIDNEY MONOLAYER TISSUE CULTURES FOR THE DIAGNOSIS OF VESICULAR DISEASES AND FOR THE ASSAY OF NEUTRALISING ANTIBODY.

The cytopathogenic effect of the virus of foot-and-mouth disease on pig kidney monolayers has been shown to be prevented by the presence of specific immune serum (Sellers, 1955). The same finding is true in the pig kidney monolayers for the viruses of vesicular stomatitis and vesicular exanthema. It is thus apparent that pig kidney monolayers provide a method of differentiating between the three diseases. The unknown virus sample is grown in pig kidney monolayers in the presence of normal serum and of antisera prepared against the virus of foot-and-mouth disease, the virus of vesicular stomatitis and the virus of vesicular exanthema. In the tissue culture showing no cytopathogenic effect the antiserum has neutralised the virus sample, and thus the virus is identified. In a similar manner the pig kidney monolayers can be used for the typing of strains of virus and identification of serum from animals involved in outbreaks of vesicular disease.

A quantitative neutralisation test has also been developed. The presence of virus in previously incubated virus-antiserum mixtures is detected by the cytopathogenic effect on pig kidney monolayers or by the plaque assay method. The results of such investigations using the plaque assay method will be published elsewhere (Capstick, Sellers and Stewart, to be published). The methods should prove useful in measuring antibody levels in convalescent or vaccinated animals and for classification of virus strains within the virus types.

DISCUSSION

The results obtained with kidney tissue cultures and the virus of foot-and-mouth disease have shown that it has been possible by kidney tissue culture methods to provide a regular supply of suitable monolayer cultures for titration of virus and neutralising antibody and for the study of the behaviour of the virus strains in tissue culture with especial reference to their growth, cytopathogenicity and plaque forming ability. In addition it has been possible in tissue cultures to investigate the adsorption and multiplication of the virus on pig kidney cell suspensions and monolayers, to obtain virus stocks suitable for production of an inactivated vaccine which has protected guinea pigs, and to identify types and strains of the virus by serum neutralisation tests. These results will be discussed in relation to the problems of control of foot-and-mouth disease in the field and to fundamental studies of the virus, both of which are interdependent.

In the field the main problems appear to be recognition of the disease, identification of the type and strain involved and control of the disease by suitable means such as slaughter or vaccination. A knowledge of the incidence and geographical distribution of the disease is helpful in planning and control. Initial diagnosis must obviously be based on clinical signs, but it is difficult in pigs to differentiate between the three vesicular diseases, foot-and-mouth, vesicular stomatitis and vesicular exanthema. By neutralisation tests using pig kidney cultures it is possible to identify the virus responsible for the disease since all three viruses cause cytopathogenic effects in pig kidney cells. For rapid identification of the virus of foot-and-mouth disease it is better to rely on

complement fixation tests and if they fail, inoculation of susceptible cattle (Brooksby, 1958). Brooksby and Rogers (1957) have described their methods for identification of virus types and have shown the usefulness of mouse inoculation and inoculation of surviving cattle tongue epithelial cultures for recovery of virus present in insufficient amount in the original sample. The mouse carcase suspension or tissue culture supernatant can subsequently be typed by a further complement fixation test. Pig kidney cultures may also provide another means of virus recovery, which can subsequently be typed by complement fixation or neutralisation tests in monolayers. This would be especially suitable where the outbreak occurred in pigs, since from the results given in Section III, virus obtained from pig epithelium grew well in pig kidney monolayers. For preliminary work on identification of new types the cultures may be a useful addition to complement fixation tests, but the final proof of the new type would have to be made by cross immunity tests in cattle and guinea pigs. However, neutralisation tests in pig kidney cultures as well as complement fixation should provide methods of classification of the different variants that exist among the seven types of the virus, and thus enable a rational choice to be made of strains to be included in a vaccine. Capstick, Sellers and Stewart (to be published) have suggested that variants may be classified either by measuring the rate of inactivation of different virus strains by antiserum (K values) as in bacteriophage work (Adams, 1950) and with poliomyelitis viruses (McBride, 1959) or in cross-neutralisation tests by measurement of virus survival after neutralisation has been completed.

For preparation of a vaccine against foot-and-mouth disease a source of virus is required. The virus may be harvested from the animal after infection or prepared from tissue cultures, and may be inactivated by chemical or physical means or used as a living attenuated vaccine. A successful inactivated vaccine prepared from culture virus has been used extensively in Holland and elsewhere Frenkel 1947, 1953) and this vaccine is made from virus grown in tissue cultures of surviving cattle tongue epithelium. Experiments on inactivated vaccine prepared from virus grown in calf kidney cultures have been reported by Dinter and Wesslen (1958). Another suitable source of culture virus for vaccine production appears to be pig kidney tissue cultures.

Production of an inactivated vaccine in tissue culture involves growth of the cells, growth of the virus in the cells, titration of the virus harvest, inactivation of the virus and tests of non-infectivity and of potency on the completed product. In Section I, methods of production of pig kidney monolayers were described and in Sections IV and V, it was shown that the virus of foot-and-mouth disease multiplies in the cultures and this virus of good titre and antigenicity may be obtained. In Section II the method of titration of the virus by the plaque technique was given. For non-infectivity tests pig kidney monolayers proved unsuitable, since aluminium hydroxide was toxic for the cells. It may be possible to add aluminium hydroxide after formalin inactivation of the virus is completed, and thus the non-infectivity tests could be performed in tissue cultures. It would still be advisable to test the final product for non-infectivity in susceptible large animals. It was

shown in Section V that a vaccine prepared from virus grown in pig kidney monolayers stimulated production of antibodies in guinea pigs and protected the guinea pigs when challenged with homologous virus. Whether such vaccine would be suitable for cattle, sheep and pigs must await further trials. A test of potency using a challenge level of virus and dilutions of vaccine was devised in guinea pigs, but it must also be pointed out that antibody levels obtained by neutralisation tests in pig kidney cultures on the sera of vaccinated animals can be used as a measure of the potency of a vaccine. Compared with vaccines prepared from virus grown in cultures of surviving cattle tongue epithelium, pig kidney grown virus vaccines have advantages and disadvantages. Cattle tongue epithelium cultures are prepared easily, and the period of culture and growth of virus are over by 24 hours, whereas it may be seven days before the growth of pig kidney cultures and harvest of the virus is completed. However, the non-viral protein of the pig kidney virus is less than that of the cattle tongue virus and it may be possible to produce a greater bulk of vaccine from kidney cultures for a given amount of tissue. The cytopathogenic effect of the virus on pig kidney cells can be followed microscopically and the virus can be harvested when the cytopathogenic effect is complete with the knowledge that there is much antigen in the culture, whereas on tongue tissue cultures the action of the virus on the cells cannot be followed easily and reliance must be placed on titration of the culture virus. The relative antigenicity of the two types of vaccine has still to be compared.

Cattle virus given 58 passages in pig kidney monolayer tissue

cultures did not prove suitable as an attenuated vaccine although a slight reduction of virulence for cattle occurred. It is possible that further passage might increase attenuation but there is the danger that virulence for pigs might increase. In Section III, all strains attenuated by passage in mice and chick embryos were shown to grow in pig kidney monolayers and it may be possible to use pig kidney monolayers as a source of mouse and chick embryo passaged virus for production of attenuated vaccine. It has been demonstrated in Section III that there appears to be a correlation between the growth and cytopathogenicity of the mouse and chick embryo passaged strains in ox and calf kidney cultures and the degree of attenuation of the strains for cattle, and this may be of help in assessing the probable degree of non-infectivity for cattle of attenuated strains.

Knowledge of virus titre is important in fundamental research on the virus as well as in vaccine production and in Section II the plaque assay method using pig kidney monolayers was considered in detail. A technique was evolved that has been shown to give consistent and reliable results. It is apparent that variation in the kidneys and cultural conditions has an effect on the number and type of plaques formed. This effect may not be so apparent using comparable numbers of observations in end point methods of titration and hence by the plaque method accurate measurement of virus titre is possible and small differences between titres can be shown to be significant. In the case of some strains of the virus of foot-and-mouth disease titres by the plaque technique have not been so high as by other methods of titration. As seen in Section II this may have been due to the presence of the agar overlay, since with

several strains rocked pig kidney cell suspensions were as sensitive as mouse inoculation.

An advantage of the plaque technique is that on each plate in the titration information is obtained on the size and shape of the plaque formed as well as on the number of plaques. It has been possible to classify the types of plaques formed and this has been applied to determining the plaque characteristics of strains and the degree of homogeneity of a virus population as regards its plaque forming particles. It has been found that virus attenuated for cattle by mouse and chick embryo passage had a low titre by the plaque technique and usually produced small plaques; use may be made of this finding in assessing the degree of attenuation and in the detection of contamination of the attenuated virus by virus forming large plaques which might be virulent for cattle. In selection of a strain for use in inactivated vaccine production it may be advisable to select a large plaque strain, since strains producing large plaques appear to produce severe disease in pigs and may prove more antigenic than strains producing small plaques. If the strain selected grows well in calf and ox kidney monolayers and produces a good cytopathogenic effect, then it is likely it would be a suitable choice for use as an inactivated vaccine in cattle and pigs. In a specific problem, where choice of a strain can be made for convenience, it would be an advantage to use a large plaque strain, since plating efficiency would be high, extent of variation due to cultural conditions would be less than with a small plaque strain and titrations could be completed in two days. In Sections IV and V, the usefulness of the choice of the large plaque

strains M11 and C997 was demonstrated in multiplication and vaccination studies.

It is possible to recover virus from a plaque and by three successive isolations to obtain relatively pure virus clones. This has obvious application in the selection and purification of strains of the virus of foot and mouth disease for genetic studies and for study of immunological relationships between the strains. It also ensures a homogeneous virus population for studies on growth and behaviour of the virus.

Analysis of virus growth in tissue culture systems has been shown with many viruses to give useful information on cell adsorption and penetration of the virus, on the intracellular formation of infective nucleic acid and virus and on the release of the virus from the cell. Preliminary work on the adsorption and multiplication of the virus of foot-and-mouth disease in cell suspensions and in monolayers has been given in Section IV, and it has been shown that with high input of virus not all the cells became infected and able to release virus after an adsorption period of 30 minutes; the latent period, the rate of virus multiplication and the yield of virus per infected cell appeared to be the same in cell suspensions and monolayers. It is of interest to note the rapidity of virus growth and the early appearance of cytopathogenic effect. Virus of titre $10^{6.7}$ - $10^{7.5}$ pfu/ml was obtained in monolayers 6 - 8 hours after infection. This work should form a useful basis for more detailed studies of virus-cell relationships especially the intracellular growth of the virus. Perhaps for this work it would be an advantage to use cell lines established by the method of Westwood, Macpherson and Titmuss (1957) and grown as

clones by the method of Puck, Marcus and Cieciura (1956).

Virus of good titre harvested from pig kidney monolayers should prove useful in several ways in addition to a source of virus for vaccines. As well as being used for infection of cell suspensions and monolayers for analysis of virus-cell relationships and further production of virus stocks it could be used as a source of virus for neutralisation tests and as starting material for the study of the effects of chemical and physical agents on the virus; it could be used for analysis of the virus by ultracentrifugation and electron microscope studies. As the virus can be grown in a chemically defined medium of salts, amino-acids and growth factors, a comparatively pure product can be obtained, which can be concentrated and analysed chemically as well as being used for recovery of infective ribonucleic acid (Brown, Sellers and Stewart, 1958).

Tissue cultures are, however, at a disadvantage compared with animal inoculation in cases where microbial contamination occurs and in cases where chemical or physical agents injurious to the cells are used. Antibiotics can control contamination to a large extent and chemical agents can be removed by dialysis or neutralisation, but often in these cases the recovery and titration of virus may have to be carried out by inoculation of susceptible animals.

It may thus be seen that tissue culture methods as illustrated by pig kidney monolayers and the virus of foot-and-mouth disease have wide application in the study of the vesicular diseases of animals. It should be emphasised that tissue culture is a laboratory technique and should be used in conjunction with other

techniques such as complement fixation, gel diffusion and animal inoculation for studies on the virus and control and prevention of disease. Regarded in this light it provides an interesting and useful working model, in which the various factors can be defined and the findings related to the problem encountered.

SUMMARY

The application of tissue cultures in the study of vesicular diseases of animals has been illustrated by reference to pig kidney tissue cultures and the virus of foot-and-mouth disease.

Investigation was carried out on the most suitable method of producing monolayer tissue cultures from pig kidneys by the trypsinisation technique and it was found possible to produce monolayers in suitable quality and quantity. The monolayers were used for titration of the virus of foot-and-mouth disease and study was made of the factors affecting the formation of plaques on the monolayers. It was found that the number, size and shape of the plaques formed were affected by the state of the cells, the nature of the overlay, the conditions of culture incubation and the strain of virus used. By adherence to a standard technique and by comparison of titres obtained from assay of a standard virus on different sets of cultures it was possible to obtain accurate and useful results.

Strains of the virus of foot-and-mouth disease, which had been passaged through mice and chick embryos did not multiply or cause cytopathogenic effect in calf and ox kidney monolayers to the same extent as strains passaged in cattle or in kidney cultures and it was possible to correlate their behaviour with non-infectivity tests in cattle. All strains of the virus tested grew in pig kidney monolayers and caused cytopathogenic effect but gave rise to plaques of varying size and shape. The plaques arising from the different strains were compared and it was found that the relative number of the different plaque sizes varied with the origin of the virus and

that the plaque population changed on passage in different cultures or animals.

The adsorption and multiplication of the virus was followed in pig kidney cell suspensions and monolayers. It was found difficult to infect all the cells after 30 minutes adsorption with a high input of virus. The latent period was found to be 2.5 - 3 hours for cell suspensions and monolayers and at the end of that time there was a rapid virus increase. The titre of the culture reached a peak of $10^{6.7}$ - $10^{7.5}$ pfu/ml at 6 - 8 hours after which it remained constant for a period up to 18 hours and then fell gradually.

Virus grown in pig kidney monolayers was inactivated by adsorption on aluminium hydroxide and treatment with 0.05% formalin. The vaccine thus produced protected guinea pigs against challenge with homologous culture virus and stimulated the production of neutralising antibody. Investigations were made into the optimum time of virus harvest. It was found that vaccine made from virus harvested 24 - 42 hours after infection of the monolayers, when the cytopathogenic effect was complete, gave the best protection to guinea pigs on challenge. The size of challenge dose which distinguished between different levels of protection, was found to be 10^4 guinea pig ID_{50}, and a tentative scheme for a potency test was put forward based on the dilution of vaccine protecting 50% of the guinea pigs from generalisation after challenge with $10^4 ID_{50}$ of virus.

Pig kidney monolayer tissue cultures were also used as a means of differential diagnosis of vesicular diseases and for the assay of neutralising antibody.

These findings were discussed in relation to diagnosis, identification and classification of outbreaks of foot-and-mouth disease in the field and to the selection of virus strains for preparation of attenuated and inactivated vaccines. The method of virus titration by the plaque technique was discussed with regard to vaccine production, fundamental virus research, plaque analysis of strains and genetic studies. The importance of virus growth studies and production of high titre virus stocks was emphasised in relation to virus-cell interactions and in relation to provision of suitable material for biophysical, biochemical and biological investigations on the virus.

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