RECOMBINATION BETWEEN TWO
STRAINS OF VACCINIA VIRUS

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STATEMENT

Part of this work has been published (Fenner and Comben, 1958).

The bulk of the work presented in this thesis was performed by the candidate. The following major exceptions are noted.

Section C. The three virus strains were originally described by Fenner (1958). The measurements for Figure 2 were made by Professor F. Fenner.

Section D. Electron microscope particle counts were made by Miss M. Briggs. Table 2. Suspension A was prepared and pock counts made by Professor Fenner.

Section H. Dr. D.O. White collaborated in the early stages of this section.
Grateful acknowledgement is made of the invaluable guidance and help of Professor Frank Fenner.
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SUMMARY

Two strains of vaccinia virus were selected which differed by five separate markers: pock morphology on the chorioallantoic membrane of the chick embryo, the production of haemagglutinin, heat resistance, mouse virulence when inoculated intracerebrally and type of skin lesion produced in the rabbit.

It was shown that the two strains were homogeneous for the five markers, and that the markers were stable on pure culture passage.

Chorioallantoic membranes and HeLa cell cultures were infected with mixtures of the two strains and an analysis was made of the progeny of such infections. One hundred and forty-nine progeny clones were isolated, and of these seventeen differed from both parental strains in their marker characteristics.

It is believed that these arose by genetic recombination.
Intermediates were found for the markers: pock morphology, mouse virulence and type of rabbit skin lesion, whereas haemagglutinin production and heat resistance appeared to be all-or-none characters.

A preliminary analysis was made of single HeLa cell yields and of the proportion of heat resistant recombinants to the total progeny.
Since virus recombination was first demonstrated in the bacteriophage T2 – *E. coli* system, (Hershey, 1946 a, b; Delbrück & Bailey 1946), a great volume of work has appeared on phage recombination and significant contributions have been made to the understanding of the bacterial virus – host cell relationships, and in particular to the understanding of the processes of replication of phage material.

So far only two animal virus systems have been investigated, influenza and herpes simples virus. Of these two, the influenza virus system alone has been extensively studied and inherent difficulties in the system have limited the amount of information obtained.

These systems will be discussed as an introduction to the work to be presented on recombination between strains of vaccinia virus. The discussion will be biased towards the bacteriophage system for two reasons. Firstly, there are more detail similarities between the vaccinia and bacteriophage systems than between vaccinia and its fellow animal viruses. Secondly, it is in the bacteriophage system that the most detailed, coherent conclusions have emerged as to the mechanism of the recombination process; conclusions which will guide future research in other viruses.

**BACTERIOPHAGE RECOMBINATION**

Most of the work to be discussed has been performed on the phage T2 or T4 – *E. coli* system, and other systems will be mentioned briefly in so far as they bear on the general applicability of the results obtained with T2 and T4.
The structure of phages T2 and T4 is well understood. They are sperm shaped particles which can be differentiated into two functionally and chemically dissimilar parts, the nucleic acid core and protein shell (Anderson et al., 1953). All phages so far analysed have been of similar chemical composition to T2 and T4. Following osmotic shock (Anderson, 1950) practically intact "ghost" membranes can be seen which contain almost all of the phage protein, retain the phage antigenicity, and are able to absorb to and kill sensitive bacteria (Herriott, 1951). The phage nucleic acid (DNA) is released into the medium by this procedure (Williams, 1953). Other phages have also been shown to possess a head membrane (Kellenberger and Kellenberger, 1954).

The process of infection is initiated, in the case of T2 and T4 by the tip of the phage tail becoming attached, at first reversibly and later irreversibly to the bacterial cell surface (Puck, Garen and Kline, 1951; Kellenberger and Arber, 1955; Williams and Fraser, 1956). If, at this stage, the bacterium-phage complex is exposed to shearing forces, the majority of the phage protein, but little of the DNA can be recovered from the medium (Hershey and Chase, 1952). This has also been shown with phage T5 (Lanni, 1954). The protein shells which are stripped off the bacteria lack the tip of the tail and are unable to reabsorb. Interruption of the adsorption process in this way does not affect the lytic process, from which it has been inferred that phage DNA alone is essential for subsequent multiplication (Hershey and Chase, 1952; Hershey and Burgi, 1956). This conclusion has been reinforced by tracer studies which show that phage DNA, but, provided that protein on the cell surface is removed, not phage protein is substantially transferred to the progeny phage (Hershey and Chase, 1952; Hershey, 1953; French, 1954). This conclusion is of great importance in that it equates the phage genetic material
with DNA, and simplifies the next stage of the problem, the intracellular replication of the phage.

A major advance in the study of this problem was the discovery that phages T2, T4 and T6 contained hydroxymethyl cytosine in place of the normal base cytosine (Wyatt and Cohen, 1953). This has allowed accurate estimates of the amount of phage DNA in the bacterial cell.

Chemical studies indicate the following sequence of events once phage DNA is established inside the host cell. In general it can be said the phage, lacking enzyme system of its own, takes over the host synthetic mechanisms and directs them to reproduce phage components. Cytological observations support this in that host nuclear material appears to be dispersed soon after infection (Luria & Human, 1950). This complete disruption seems to be confined to T2, T4 and T5. Almost immediately after DNA entry there is substantial synthesis of a protein of unknown functions (Hershey et al, 1954) and of small amounts of RNA (Volkin & Astrachan, 1956). It is possible that these substances are intermediates in DNA replication. In any event inhibition of protein synthesis early in the lytic cycle suppresses phage DNA production (Cohen 1948; Burton, 1955). New phage DNA then appears, and synthesis proceeds uninterrupted until the cell lyses, (Cohen, 1949), raw materials being drawn from phage DNA, degraded host cell DNA and RNA, and from the medium (Stent & Maaloe, 1953; Hershey, Dixon and Chase, 1953; Hershey 1954; Hershey, 1955). This pool has been observed with the electron microscope (Kellenberger, Ryter and Sechaud, 1958), and is composed of fine fibrils clearly separated from the host cytoplasmic granules. DNA synthesis is followed by synthesis of phage protein (Cohen, 1948; Hershey et al, 1954). As the infectious process proceeds, various organised protein structures can be isolated, including solubilised phage units.
(De Mars et al, 1953; De Mars, 1955) and "doughnuts" which resemble phage heads in size and shape (Levinthal and Fisher, 1952; 1953). It is believed that the doughnuts are breakdown products rather than precursors of a mature phage.

The final stage in the history of an individual progeny phage particle is the withdrawal of DNA from the pool and coupling with protein to form a mature phage (Maaloe & Stent, 1952). This process begins about half way through the lytic process, when mature phage can be demonstrated in prematurely lysed cells (Doermann, 1948).

The probable steps in phage maturation are; formation of the DNA material into the correct shape, envelopment with protein, and finally formation of the tail (Kellenberger, 1959). It is assumed that maturation is an irreversible process and that mature phage takes no further part in the activities of the DNA pool.

Phage genetic studies have been made with this background information available and have confirmed and extended the observations sketched above.

A large number of mutants is available for genetic studies in phages T2 and T4. Phenotypically they occur as:

1. Mutants affecting host range (H) (Hershey, 1946)
2. Mutants affecting plaque type; the r or rapid lysing mutants (Hershey, 1946), the m or minute plaque mutants and tu or turbid plaque mutants.

Normal genetic nomenclature has been adopted in that the wild type genotype is indicated by +.
From the point of view of the examination of progeny of mixedly infected cells, these markers are extremely convenient. For instance, Visconti & Delbrück (1953) describe a "direct" method in which it is possible, with one exception, to score, after a single plating, every plaque for h, r, and m. This is achieved by plating the phage onto a double bacterial layer, one component being sensitive to both the h mutant and the wild-type phage, the other to the mutant only. The mutant thus produces clear plaques, and the wild type produces turbid plaques. Using this technique it is difficult to distinguish between r m and r + m plaques, but this can be resolved where necessary by plating initially onto a single bacterial layer and then picking plaques and streaking onto the other bacterium to test for the presence of the h marker. The extreme simplicity of this procedure is apparent.

Although phenotypically the range of mutants is small, a large number of genetic loci are involved (Hershey & Davidson, 1951; Doermann & Hill, 1953). For example, each separate r mutant, when crossed with another r mutant, produces a proportion of wild type progeny, the proportion being characteristic of the mutants involved. Identical mutants breed true. This technique has allowed the construction of a genetic map in T2 and T4 in which all known mutants are observed to lie in 3 linkage groups.

Luria (1951) demonstrated a clonal distribution of r mutants in single cell yields of phage T2, and was able to deduce that phage replication was a geometrical process, i.e. each new vegetative particle could act as a source of new particles.
Genetic studies in phages T2 and T4 were sufficiently advanced in 1953 for Visconti and Delbrück to present a theory which convincingly explained most of the basic facts of phage recombination. The same authors also presented additional experimental work to support various aspects of this theory. This theory had to cover the following observations:

1. Recombinants had been isolated which combined genetic markers from three parents (Hershey and Rotman, 1948; 1949). Also, where the multiplicity of infection was unequal, a two factor cross could give rise to progeny which contained more recombinants than minority parent types (Doermann and Hill, 1953).

2. Crosses between closely linked markers (with a low recombination rate) yield, from individual bacteria, recombinants which are randomly, not clonally distributed (Hershey and Rotman, 1949; Doermann and Hill, 1953).

3. As lysis is delayed, so does the percentage of recombinants approach the theoretical equilibrium of 50% recombinants (Doermann and Hill, 1953; Levinthal and Visconti 1953).

4. Although in the yield from a group of mixedly infected cells the number of opposite type recombinants is roughly equal this does not apply to single cell yields (Hershey and Rotman, 1949).

5. There was evidence that the chances of a second crossover were increased after the first had occurred. In higher organisms the opposite occurs, one crossover lessens the chance of a second in the same region of the chromosome. This phenomenon is referred to as interference and the reverse effect in phage was called
negative interference (Hershey and Rotman 1948, 1949).

Visconti and Delbrück's theory made the following assumptions:

(a) Mating occurs between vegetative DNA particles and the chance of a mating occurring increases as the number of such particles increases. This assumption is in agreement with points 2 and 3 above.

(b) Each particle may mate several times and such matings are random as regards time and partner. This assumption accounts for point 1 above. Any other explanation of these observations would involve the improbable event of simultaneous mating of three particles.

(c) Any DNA particle may be withdrawn, by coating with protein, from the pool at any time and the probability of this event is not influenced by the mating history of the particle. As a result of this process it is easy to visualise that one component of a cross could be removed before further replication, thus accounting for the observation that opposite type recombinants can occur in unequal numbers when small populations are sampled (point 4 above). It also accounts for negative interference in that all observations are made on a mixture of progeny, some of which have mated and some of which have not. When the incidence of double recombinants was calculated on the basis of the number of particles undergoing mating no evidence of interference, positive or negative, could be found.

More detail has been added to this concept of phage recombination by the study of heterozygotes found during recombination.
Hershey and Chase (1951) demonstrated that bacteria mixedly infected with T2r and T2r+ phage gave rise to a small proportion of progeny particles, about 2%, which produced mottled plaques, from which both r and r+ particles could be recovered. Five different r mutants and one h mutant were studied for a variety of lysis times and in all cases the proportion of heterozygotes was the same, indicating that these particles do not reproduce as such.

The structure of heterozygotes was studied in detail by Levinthal (1954). He devised experiments to decide between 2 likely structures.

(a) Where a piece of haploid genetic material attached to the normal genetic structure giving rise to a small diploid region.

(b) Where two pieces of linkage group joined together with a small region of overlap at the function.

Levinthal made a cross involving 3 markers and analysed for the central marker.

(a) Above would normally give rise to a progeny structure which was, in terms of the end markers, parental.

(b) Would be expected to give progeny which were recombinant with respect to the end markers, and this is what was found in these experiments.

Basing his calculation on the Visconti Delbruck model and making the additional assumptions:

1. That the overlap model is correct.
2. That heterozygotes produce at a normal rate in the DNA pool.
3. That the overlap can occur at any point in the genetic structure.
Levinthal was able to show that heterozygotes could account for all the recombinants found. He suggested that heterozygotes could be formed when two linkage structures replicated in close proximity to each other, and, that replication proceeded stepwise along each structure and ceased when the two new structures met and overlapped slightly.

The establishment of this "copy-choice" mechanism for virus recombination has led to much speculation as to the details of replication in the light of the generally accepted structure of DNA (Watson and Crick, 1953; Stent, 1958).

The great attention paid to the replication of phages T2 and T4 has, in a way, resulted in a narrow approach to the problem of virus recombination; however in the wider field of bacterial genetics it has led the way to a greater unity of thought on the problems of bacterial recombination (Wollman, Jacob and Hayes, 1956), transduction, and transformation (Hotchkiss, 1955), in all of which DNA is apparently the active agent.

INFLUENZA RECOMBINATION.

In 1951 Burnet and Lind, following mixed infection of the mouse brain with the influenza virus strains MEL and neuro-WS, isolated a new type of virus, called NM, which was serologically MEL but possessed a new property, neuropathogenicity, which was presumably derived from neuro-WS. Further investigation revealed that other neuro-WS properties were also transferred and that the NM strains exhibited a wide range of virulence (Fraser and Burnet, 1952). To exclude any possibility of selection of neuro-pathogenic strains during mixed infection these experiments were repeated in a "neutral" host and similar NM strains were recovered from the allantoic cavity of the chick embryo (Fraser and Burnet, 1952).
Subsequently a great deal of work was done on the system WSE-MEL, two strains which could be distinguished by six genetic markers.

These markers are stable as judged by limit dilution passage (Burnet, 1951; Isaacs and Edney, 1950). As no method comparable to plaque production in phage is available in the influenza system, all pure clone isolation must be made by a method which involves dilution to a level at which only a small proportion of tubes contain virus, in most cases a single particle. Safety checks must always be employed, and the necessity for using this method greatly reduces the volume of work which can be done in the influenza system. Recombination regularly occurred between two linkage groups. One comprised the serological character of the virus, the resistance of the haemagglutinin to destruction by heating at 60°C, the inhibition or otherwise of indicator virus by sheep mucoid and the mouse lung pathogenicity. The other comprised the effect of heat on the indicator character of the virus and the presence or absence of a haemorrhagic action on the chick embryo. Double infection of a suitable cell system with appropriate recombinants yielded, among the progeny, the original parental types (Lind and Burnet, 1953).

Again, a wide range of virulence was observed in the recombinants. (Lind and Burnet, 1953; Burnet and Lind, 1954).

In this system recombination has been demonstrated between a variety of A strains, and between two B strains (Perry and Burnet, 1953; Perry et al, 1954), but not between an A and a B strain. It has also been observed that the A strain MEL recombines more readily with A strains isolated at about the same time than with more recently occurring strains.
These facts serve to emphasise the close relationship between ability to recombine and antigenic structure.

In addition to these recombinants, which were stable on repeated limit-dilution passage, a number of reports have described the presence of heterozygotes amongst the progeny of mixed infections. The X virus of Hirst and Gotlieb (1953); Gotlieb and Hirst, (1954), obtained after mixed infection with MEL and neuro-WS, was able to neutralise sera specific to both the parent strains. On repeated passage X virus gave rise to X, MEL and neuro-WS fluids, suggesting the presence of both types of genetic determinant in the X virus. Late in the passage series one X fluid was found to breed true. Gotlieb and Hirst concluded from these experiments that influenza heterozygotes were diploid, and the high proportion of heterozygotes found suggested to them that all influenza virus particles may be diploid. Successful passage of heterozygous particles at limit dilution strengthened this view. Further studies (Hirst and Gotlieb, 1955) suggested that heterozygotes were almost or entirely, the sole source of recombinants. However, Lind and Burnet (1957) found heterozygotes to comprise only 12% of progeny from mixed infections in the same systems.

It has not been possible to formulate any convincing and detailed theory of replication or recombination in the influenza virus system. Such a theory would be of great interest as the genetic determinants of influenza virus are apparently carried by R·N·A (Aaga and Perry, 1954; Miller, 1956).

Recombination has also been observed between two herpes simplex strains (Wildy, 1955).
The two herpes strains chosen by Wildy differed in two major respects, the type of pock formed on the chlorioallantoic membrane of the chick embryo and the virulence for mouse and rabbit. He described evidence to suggest that pocks arose from one virus particle, i.e. were clones, and stability of the markers was judged on their reproducible behaviour during a number of single pock isolations. In this case progeny were isolated which possessed the pock morphology of one parent and, in varying degree, the mouse and rabbit pathogenicity of the other.
EXPERIMENTAL

A. ABBREVIATIONS

Chorioallantoic membrane - CAM
Pock-forming unit - PFU
Single pock - SP
Haemagglutinin - HA
Balanced salt solution - BSS

B. FORMULAE

1. HeLa cell growth medium

   Hank's balanced salt solution - 55%
   Lactalbumin hydrolysate (5% solution) - 5%
   Human serum from non-vaccinated individuals - 40%

2. Calcium-magnesium-saline

   NaCl 0.8%
   CaCl$_2$.6H$_2$O 0.006%
   MgCl$_2$.6H$_2$O 0.017%
   H$_3$BO$_3$ 0.12%
   Na$_2$B$_4$O$_7$.10H$_2$O $\text{pH}$7.2 0.005%

3. Gelatin saline

   As above, plus 0.5% gelatin.
C. SELECTION OF STRAINS AND MARKERS

From the 23 strains described by Fenner (1958) two were chosen which could be distinguished by five separate tests. It is apparent that the ability to screen a large number of virus preparations is of importance and emphasis was placed on this feature during selection of the strains.

The two eventually chosen were Vaccinia-Lederle - 7N (described hereafter as V-Led-7N) and Rabbitpox - Utrecht (RP-U). In addition a variant of RP-U, Rabbitpox-Utrecht-White (RP -U-W) will be considered. The origins and passage histories of these strains are as follows:

V-Led-7N (obtained from Dr. B.A. Briody)

Derived from Vaccinia Lederle-original after 7 passages intradermally in rabbits and then 3 passages on the chorioallantoic membrane. Vaccinia Lederle-original was first isolated from human vaccination scabs obtained from the New York City Board of Health in 1909. It has since been subjected to passages in calves, rabbits and humans.
RP-U (obtained from Dr. R. Gispen)

Originally isolated from a spontaneous outbreak of rabbitpox in Utrecht in 1941. Has since been passed once on eggs and twelve times intracerebrally in mice. It strongly resembles a highly virulent strain of neurovaccinia.

RP-U-W

A white pock variant of RP-U (Fenner 1958) arising with a frequency of about $10^{-3}$ (Fenner, personal communication).

The stock cultures used in the experiments to be described were produced on the chorioallantoic membrane of the chick embryo following two successive single pock isolations of the above material.

The tests employed to characterise these strains will now be described.

1. Pock morphology

For the purpose of this test chick embryos were inoculated on the chorioallantoic membrane (CAM) after 11 or 12 days incubation at 38°C.
The method adopted was the alternative method of Beveridge and Burnet (1946) in which the shell membrane, exposed over a small area, is pierced with a needle through 0.05 ml. of inoculum. As the chorion drops, the inoculum spreads over it. Provided that care was taken during drilling and dropping, and that the dropping was done within a few minutes of drilling, non-specific lesions were rare. Inoculation was followed by incubation at \( 35-36 \, ^\circ C \).

When a small number of virus particles are inoculated on to the CAM of the chick embryo, small discrete foci of infection, or pocks, appear after a suitable incubation period. The morphology of the pocks produced by V-Led-7N and RP-U differs markedly. The pock produced by V-Led-7N after 48 hours incubation is approximately 1.3 mm in diameter, white, opaque and raised above the surface of the membrane. It sometimes shows a small area of haemorrhage and necrosis in the centre (Fig. 1 a.) RP-U on the other hand, after 48 hours incubation,
forms a pock approximately 0.9 mm in diameter which is rather transparent and haemorrhagic, with a thin translucent white border. With the exception of the border the pock is not appreciably raised above the surface of the membrane (Fig. 1 b.). The white variant of RP-U, RP-U-W, forms a pock which is similar to the V-Led-7N pock but much smaller, 0.7 mm in diameter (Fig. 1 c.) The three types of pock are readily distinguishable with the naked eye.

FIGURE 1

Histograms showing the distribution of pock sizes for each virus, as measured with an ocular micrometer at a magnification of 5 x are included for comparison. (Fig. 2).

**FIGURE 2**

The size distribution of pocks of V-Led-7N, RP-U and RP-U-W after 48 hours incubation on the CAM.

The nomenclature adopted for V-Led-7N and RP-U pock morphology is as follows:
- The V-Led-7N pock is designated CAM-White (CAM-W)
- The RP-U pock is designated CAM-Red (CAM-R)
2. **Haemagglutinin titration**

Most poxvirus strains produce a haemagglutinating substance distinct from the virus particle. Fowl cells are commonly used for the titration of this haemagglutinin; however only about 50% of fowls yield cells susceptible to its action and preliminary titration is necessary to choose suitable birds.

Haemagglutination (HA) titrations were performed on virus suspensions prepared by grinding and clarifying membranes showing confluent growth, as described in detail on p.28, and were carried out in plastic trays using Takatsy's loop (Takatsy 1955). Dilutions were made in calcium-magnesium saline containing 1% normal rabbit serum to inhibit non-specific lipid agglutination (Stone 1946). After the dilutions were made a standard drop of 4% suspension of susceptible fowl cells was added to each cup, producing a final concentration of 0.5% fowl cells. The tray was then shaken, incubated at 37°C for 20 minutes, shaken again, re-incubated for 40 minutes and read, partial agglutination being taken as the end-point (Nagler, 1942). V-Led-7N and RP-U controls were included in all tests.
Where results are expressed numerically, the reciprocal of the dilution showing partial agglutination is given, where no agglutination is observed the result is expressed as less than the reciprocal of the first dilution.

Using this standard test a V-Led-7N virus suspension containing $10^{6.5}$ PFU agglutinates fowl red cells at a dilution of 1 in 10. With ground confluent membranes, the titres of which varied around $10^{8.3}$ PFU/ml., haemagglutinin titres of 1 in 160 to 1 in 1280 were regularly obtained. RP-U produced no detectable haemagglutinin at all, and no anti-haemagglutinin was produced when rabbits were inoculated with this virus (Fenner 1958). The white variant of RP-U, RP-U-W, also does not produce haemagglutinin.

The nomenclature adopted for the production or otherwise of haemagglutinin is as follows: - The production of haemagglutinin by V-Led-7N is designated Haemagglutinin-high (HA-h). The absence of haemagglutinin production by RP-U is designated Haemagglutinin-nil (HA-0).
3. **Heat Resistance**

All samples were sealed in glass capillaries and totally immersed in a stirred constant temperature water bath operating at 55°C. No allowance was made for the time taken for the suspension to reach the temperature of the bath. After immersion for the allotted time the samples were removed, cooled quickly and titrated in duplicate immediately.

Eggs inoculated with undiluted heated material frequently produced membranes on which counting was difficult, so, as a routine, a $10^{-2}$ dilution was the lowest used.

Suspensions of V-Led-7N virus, prepared by grinding and clarifying infected CAM material, as described on p.28, dropped over 4.5 logs in titre after exposure to heat at 55°C for 40 minutes, whereas similar suspensions of RP-U dropped only 1 log in titre under the same conditions. (Fig.3). RP-U-W shared the relative heat resistance of RP-U.
2. FIGURE 3

The rate of inactivation of V-Led-7N and RP-U in the presence of ground CAM.

As a standard test for the extreme differences in heat resistance existing between V-Led-7N and RP-U a heating time of 40 minutes at 55°C was chosen. After exposure to this time and temperature V-Led-7N survivors never appeared at the 10⁻² dilution which was the lowest used;
for this reason the log drop in titre of V-Led-7N suspensions is shown below as $\geq 4.5$

The resistance to heat of V-Led-7N and RP-U is designated as follows:

The low V-Led-7N resistance is designated Heat resistance-low (HR-1).

The high RP-U resistance is designated Heat resistance-high (HR-h).

Suspensions of virus prepared from the CAM by the Genetron extraction process (Gessler et al, 1956), as well as suspensions prepared from single pocks and washed HeLa cells, showed a much greater loss of titre under these heating conditions. The Genetron process, which involves high speed homogenisation of infected membranes with buffer and Genetron, achieves a high degree of separation of viral (aqueous phase) and non-viral (organic phase) material. The low heat resistance of these suspensions was presumably due to lack of protective host material. Rather than re-standardise the test for suspensions from different sources, a saline extract of uninfected CAM was added in a 1:1 ratio to these suspensions. Their virus loss on heating was then identical with that of normal CAM preparations.
4. **Type of Rabbit Skin Lesion**

Rabbits were bred in the University Animal Breeding Establishment.

4-6 month old rabbits were inoculated intradermally with 0.1 ml. of virus suspension diluted to contain $10^5$ PFU's. Up to 30 separate virus suspensions could be inoculated into the clipped back, and each suspension was inoculated into at least 2 rabbits during each test, as an occasional rabbit showed a greatly reduced response. Controls of V-Led-7N and RP-U were always included.

Six days after inoculation of $10^5$ PFU of V-Led-7N virus intradermally into the clipped rabbit back, a small, red, nodular lesion was observed. With the same dose of RP-U virus a large flat, spreading, purple-centred lesion was observed. RP-U-W gave rise to a lesion of the same type as V-Led-7N virus.

The nomenclature adopted for the different types of lesion is as follows:
The V-Led-7N type of lesion is designated Rabbit skin-Nodule (RS-N).

The RP-U type of lesion is designated Rabbit skin-Purple centre (RS-PC).

5. **Mouse Inoculation**

Mice were bred in the University Animal Breeding Establishment from stock originating from the Walter and Eliza Hall Institute, Melbourne.

5-7 week old mice were inoculated intracerebrally under ether-chloroform anaesthetic, with 0.03 ml. of a suitably diluted virus suspension. As a standard test the inoculum was adjusted to give 10 PFU's in 0.03 ml. V-Led-7N and RP-U controls were included in each test. Deaths were recorded daily; mice dead on the first day (about 2%) were scored as non-specific deaths, and deaths thereafter were regarded as being due to virus.

Experiments were terminated at 21 days and all living mice were classified as survivors.
Following inoculation of V-Led-7N virus 96-98% of mice survived, whereas inoculation with the same dose of RP-U killed 100% of mice with a mean death time of $4.6 \pm 0.4$ days. RP-U-W shared the low mouse virulence of V-Led-7N.

The nomenclature adopted for the difference in virulence between the two viruses is as follows:

The low V-Led-7N virulence is designated Mouse virulence-low (MV-1).

The high RP-U virulence is designated Mouse virulence-high (MV-h).

D. THE BEHAVIOUR OF A NUMBER OF CLONES ISOLATED FROM THE PARENT STRAINS

The homogeneity of the markers was then investigated. If variations in the characteristics of the viruses were to be attributed to genetic recombination it was first necessary to show that the parent virus population behaved uniformly and that its
characteristics did not alter on pure culture passage.

Thirty three V-Led-7N and nineteen RP-U single pock isolations were therefore made as detailed below and, after various passaging procedures, submitted to the full range of tests already described. Results of these tests are included in Table 1.

**Isolation of Single Pocks**

The material from which the single pock (SP) is to be isolated is titrated accurately and the figure obtained is used to calculate a dilution at which, theoretically, a proportion of eggs receives 1 PFU. A number of eggs is then inoculated with this dilution; on the average it is necessary to allow about 4-6 eggs for each SP required. After two days incubation at 35-36 °C the eggs are flamed and opened aseptically, care being taken to ensure that no shell fragments fall on the membrane, and the membranes observed. The
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<th>Large module (N11)</th>
<th>Viable time (mean sur)</th>
<th>Rate of mortality</th>
<th>Size of testes</th>
<th>Number of VTU (on CAM)</th>
<th>Number of Pockets on CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-0</td>
<td>0.0 ± 0.33</td>
<td></td>
<td>0.0 ± 6</td>
<td>100%</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-1</td>
<td>0.0 ± 0.33</td>
<td></td>
<td>0.0 ± 6</td>
<td>100%</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA-H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA-N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The table presents data from Penner (1958) on the biological properties and virological characteristics of strain RP-W-™, including vital time, mean survival, rate of mortality, size of testes, and number of viable tumor units (VTU) on CAM.
procedure then followed depends on the initial naked eye observation of the membrane in situ, and the various alternatives will be considered separately. The major complication is that the RP-U pock is difficult to see with the membrane in place, although the V-Led-7N pock is very readily observed.

1. More than 1 pock seen: the membrane is discarded.

2. One pock seen: this is usually the V-Led-7N type: the pock is removed aseptically with sterile forceps and scissors and placed in a sterile glass grinder. The remainder of the membrane is then removed and examined on a glass slab under more favourable lighting conditions. The pock obtained is discarded if another pock is found, or if the membrane exhibits any blemish capable of concealing another pock.

3. No pock seen: the membrane is removed aseptically, placed on a sterile glass slab and examined. If only one pock is found and
the membrane is free from blemish, the pock is removed aseptically to a glass grinder. The majority of RP-U type pocks was isolated in this way.

**Preparation of Virus Suspensions from SP's**

The pock, together with as small a section of the surrounding CAM as possible, was removed aseptically as detailed above and placed immediately into a small sterile glass grinder. After a short preliminary grind the material was frozen at -20°C and vigorously ground from the frozen state without abrasives. The ground material was finally suspended in 1 ml. of gelatin saline and stored at -60°C.

The amount of virus obtained from a single pock was insufficient for some of the marker tests and therefore it was necessary to produce a more highly concentrated virus suspension.

Membranes which exhibited confluent growth of virus were prepared by the inoculation of approximately 10⁴ or more PFU's. In practice
0.05 ml. of undiluted single pock suspension was inoculated on to the CAM and the membrane was harvested after 2 days at 35-36°C. The confluent membrane was removed aseptically, cooled in a sterile petri dish in the refrigerator, and placed in a porcelain mortar which had been chilled to -20°C. The membrane quickly froze and was ground until thawed. The partially ground material was then re-frozen and ground once more. At this stage gelatin saline was added, the suspension was clarified by centrifugation and the supernate was ampouled and stored in a mechanical deep freeze at -60°C. An ampoule was later removed, thawed and titrated. Little or no deterioration of these stock suspensions was observed over a period of more than 12 months. This material was used for all marker tests.

Evidence that SP's represent pure clones of virus

It is believed that suspensions formed in this manner from SP's represent pure clones of the parent virus, i.e. they are derived from single virus particles. Evidence that this is so may be obtained by two methods.
1. **Comparison of pock counts and particle counts**

Were a 1:1 relationship found it would follow that each particle produced a pock and that each pock was derived from a single virus particle.

Pock counts and particle counts were made with three different preparations.

**Suspension A** was prepared by grinding from the frozen state, without abrasive, confluent CAM's which had been inoculated 48 hours before reaping. The resulting suspensions were clarified at 750 G for 10 minutes.

**Suspensions B and C** were prepared according to the method described by Gessler et al (1956).

Four confluent membranes were homogenised with 5 ml. of genetron (trifluorotrichlorethane) and 10 ml. of McIlvaine's buffer for 10 minutes, in a high speed blender, following which the homogenate was centrifuged at 750G for 10 minutes. The relatively clear central
layer which was retained proved to be a potent source of relatively pure virus.

Particle counts were made in the following manner. A known concentration of polystyrene latex spheres (1.171/\text{in diameter}) was standardised by weighing, mixed with the virus suspension, and sprayed in distilled water on to a collodion film. After dialysis for 3 hours in distilled water about 200 virus particles (contained in 15-20 droplets) were counted at a magnification of 2000. The results are included in Table 2.

Each suspension yielded easily countable droplets, however suspensions B and C, prepared by the "genetron" extraction process were seen to be in a much purer state. In addition the virus particles from these two suspensions were very well dispersed, no clumps being seen, whereas with suspension A, prepared by grinding, about 5 clumps, of 2 or 3 particles, were seen per 200 units counted.

Two methods were used to determine the pock count of the suspensions. In all cases
the pock count was made immediately the suspension was prepared.

**Method I.**
The method of Westwood et al (1957) had been shown in preliminary experiments to give consistently higher counts with the two strains employed.

In this method inoculation is delayed for some hours after the membrane is dropped, and the egg is candled prior to inoculation to detect any haemorrhage on the membrane. 12 eggs were used per dilution.

Counts obtained by this method are listed under Method I in Table 2.

**Method II**
The alternative method of Beveridge and Burnet (1946) has already been described. In this case 12 eggs were used per dilution.

Counts obtained by this method are listed under Method II in Table 2.
### TABLE 2.
Comparison of Particle and Pock Counts with V-Led-7N and RP-U.

<table>
<thead>
<tr>
<th>Strain of virus</th>
<th>Suspension no.</th>
<th>Particle count (per ml.)</th>
<th>Pock count (per ml)</th>
<th>Ratio particle count; pock count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Method I</td>
<td>Method II</td>
</tr>
<tr>
<td>V-Led-7N</td>
<td>A</td>
<td>$10^9.7$</td>
<td>$10^9.0$</td>
<td>5:1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>$10^9.9$</td>
<td>$10^9.2$</td>
<td>5:1</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>$10^9.9$</td>
<td>$10^9.3$</td>
<td>4:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^9.08$</td>
<td>7:1</td>
</tr>
<tr>
<td>RP-U</td>
<td>A</td>
<td>$10^{10.2}$</td>
<td>$10^9.5$</td>
<td>5:1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>$10^9.8$</td>
<td>$10^8.8$</td>
<td>10:1</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>$10^{10.0}$</td>
<td>$10^9.04$</td>
<td>9:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^8.7$</td>
<td>20:1</td>
</tr>
</tbody>
</table>

Comparison of the results obtained shows that Method I gives counts twice as high as the standard method. The best ratios of pock count to particle count obtained using Method I were 1:5 for RP-U and 1:4 for V-Led-7N.

2. Determination of any dissociation of the infective unit on dilution.

Since no 1:1 ratio between particle count and pock count could be demonstrated, tests were made to determine whether the infective unit could be dissociated by dilution.
A suspension of each virus was first titrated and appropriate dilutions, calculated to contain from \( \frac{1}{4} \) to 100 pock-producing particles, were each inoculated on to the CAM's of 12 chick embryos. The method of inoculation was different to that normally employed in that the membranes were first dropped using sterile gelatin saline and were subsequently inoculated with 0.1 ml. of the virus dilution, rocked to distribute the fluid, and incubated as usual. It was thought that this procedure would eliminate any error caused by variable amounts of suspension remaining on the inside of the shell membrane and at the site of puncture.

The proportional relationship between dilution and pock count is shown in Fig. 4.

From these two experiments the following conclusions may be drawn:

1. Each pock is initiated by a single infective unit.

2. The infective unit does not dissociate on dilution.
3. Almost all the virus particles occur as single isolated particles.

4. In most cases the infective unit is identical with a single virus particle, and single pocks may be regarded as clones originating from a single virus particle.

**FIGURE 4.**

The proportional relation between virus concentration and the number of pocks of two stock suspensions of V-Led-7N (two types of cross) and of two stock suspensions of RP-U (open and solid circles). (The values for corresponding relative virus concentrations have been chosen arbitrarily, so as to bring the points representing the highest virus concentration (circled) on to the same line.)
E. **BEHAVIOUR OF MIXTURES**

Although the purification techniques employed were considered to be reliable it was decided to investigate the behaviour of artificial mixtures of V-Led-7N and RP-U when submitted to the various characterisation tests.

Consequently a series of artificial mixtures were prepared and tested fully for pock type, haemagglutinin production, heat resistance, mouse virulence and type of rabbit skin lesion.

The results obtained from the investigation of each marker will be detailed separately.

1. **Pock type**

The two different types of pock were readily seen on membranes except when V-Led-7N pocks were present in large enough numbers to obscure the less obvious RP-U pocks. This did not occur on membranes which would normally be counted in routine titrations, i.e., on membranes containing less than about 75 V-Led-7N pocks. On the other hand a single white pock, because of its opacity, was obvious on membranes containing several hundred RP-U pocks. The presence of one pock type did not affect the expected count of the other.
Unfortunately the existence of the RP-U-W variant made it impossible to definitely identify, without further testing, such a pock as V-Led-7N. However, this fact would obviously be of great value in detecting possible V-Led-7N contamination of a predominantly RP-U suspension.

2. **Haemagglutination**

The presence of RP-U had no effect on the normal V-Led-7N haemagglutination as shown in Table 3.

**TABLE 3.**

Haemagglutination tests carried out with artificial mixtures of V-Led-7N and RP-U.

<table>
<thead>
<tr>
<th>Mixture composed of: V-Led-7N</th>
<th>Ratio</th>
<th>HA titre (log10)</th>
<th>Ratio</th>
<th>PFU V-Led-7N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RP-U</td>
<td>V-Led-7N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Nil</td>
<td>8.5</td>
<td>-</td>
<td>3.1</td>
</tr>
<tr>
<td>8.5</td>
<td>6.5</td>
<td>10</td>
<td>100:1</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>7.5</td>
<td>8.5</td>
<td>10:1</td>
<td>10</td>
</tr>
<tr>
<td>8.5</td>
<td>10</td>
<td>5</td>
<td>1:1</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>10</td>
<td>1:10</td>
<td>10</td>
</tr>
<tr>
<td>7.5</td>
<td>10</td>
<td>8.5</td>
<td>1:100</td>
<td>10</td>
</tr>
<tr>
<td>6.5</td>
<td>10</td>
<td>8.5</td>
<td>-</td>
<td>Nil</td>
</tr>
</tbody>
</table>
3. Heat resistance

As shown in Table 4 the extent of inactivation of each virus after 40 minutes at 55°C is unaffected by the presence of the other type. The usual difficulty was experienced in attempting to titrate the small amount of V-Led-7N remaining.

An additional mixture which contained $8.5 \times 10^5$ PFU of V-Led-7N and $10^5$ PFU of RP-U was included in this test. After heating it was titrated at $10^{-2}$, the lowest dilution routinely employed in the heat resistance test. RP-U virus was readily detected in the heated mixture. Thus a very low level of RP-U contamination of a predominantly V-Led-7N suspension is detectable.

4. Mouse virulence and type of rabbit skin lesion

As shown in Table 5 the RP-U component dominated in both these tests.
### TABLE 4.

Heat resistance tests carried out with artificial mixtures of V-Led-7N and RP-U.

<table>
<thead>
<tr>
<th>Mixture composed of:</th>
<th>V-Led-7N Ratio RP-U</th>
<th>Heat resistance Log drop in titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-Led-7N RP-U</td>
<td>V-Led-7N RP-U</td>
</tr>
<tr>
<td>8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Nil</td>
<td>5.5</td>
</tr>
<tr>
<td>8.5</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>10</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>10</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Nil</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>V-Led-7N Ratio RP-U</th>
<th>Heat resistance Log drop in titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-Led-7N RP-U</td>
<td>V-Led-7N RP-U</td>
</tr>
<tr>
<td>3000:1</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>100:1</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>10:1</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>1:1</td>
<td>10</td>
<td>4.5</td>
</tr>
<tr>
<td>1:10</td>
<td>10</td>
<td>3.5a</td>
</tr>
<tr>
<td>1:100</td>
<td>10</td>
<td>2.5a</td>
</tr>
<tr>
<td>2.5a</td>
<td>10</td>
<td>1.0</td>
</tr>
</tbody>
</table>

- The high concentration of RP-U pocks interfered with recognition of V-Led-7N pocks at higher concentrations of the heated mixture.

### TABLE 5.

Mouse virulence and rabbit skin lesion tests carried out with artificial mixtures of V-Led-7N and RP-U.

<table>
<thead>
<tr>
<th>Mixture composed of:</th>
<th>Mouse virulence</th>
<th>Rabbit skin lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-Led-7N</td>
<td>Ratio V-Led-7N</td>
<td>Mortality rate</td>
</tr>
<tr>
<td></td>
<td>RP-U</td>
<td></td>
</tr>
<tr>
<td>10^5</td>
<td>10^5</td>
<td>1:1</td>
</tr>
<tr>
<td>10^5</td>
<td>10^4</td>
<td>10:1</td>
</tr>
<tr>
<td>10^5</td>
<td>10^3</td>
<td>100:1</td>
</tr>
</tbody>
</table>
The results of these experiments may be briefly summarised as follows: (Table 6)

**TABLE 6.**

Summary of the results of tests with artificial mixtures of V-Led-7N and RP-U

<table>
<thead>
<tr>
<th>Test employed</th>
<th>Pock type</th>
<th>HA</th>
<th>Heat resistance</th>
<th>Mouse virulence</th>
<th>Rabbit skin lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant component</td>
<td>Independent</td>
<td>V-Led-7N</td>
<td>Independent</td>
<td>RP-U</td>
<td>RP-U</td>
</tr>
</tbody>
</table>

F. **ISOLATION OF RECOMBINANTS**

Recombinants were isolated from both the CAM of the chick embryo and cultured HeLa cells. In all cases the basic method was the same and involved inoculation with both strains of virus in such a way as to produce mixed infection of as great a proportion of cells as possible within a given area. Time was allowed for at
least one cycle of growth in the infected cells which were then ground to release progeny virus, individual clones of which were submitted to the tests described in Section C. Each technique employed, and the results obtained from it, will be treated separately.

1. Inoculation of the CAM with high titre suspensions of V-Led-7N and RP-U.

The initial experiments involved simultaneous inoculation of the CAM of an 11 day chick embryo with doses of each virus theoretically large enough to multiply infect the great majority of cells in the dropped area. High titre suspensions of V-Led-7N and RP-U, containing about 10 \(^9\) PFU/ml., were prepared as described in Appendix 1.

In a preliminary experiment described in Appendix 2, it was established that new virus, presumably largely formed in the first cycle of infection, was produced in some quantity 12 hours after massive infection of the CAM, so this time was chosen for harvesting the membranes.
Two 11 day chick embryos were inoculated on the CAM with 0.25 ml of each of the virus stocks, each egg receiving 10 PFU of each virus. After incubation at 36 °C for 12 hours, the membranes were removed aseptically, washed in 4 changes of sterile gelatin saline, ground from the frozen state, clarified and stored at -60 °C. This virus suspension was referred to as "recombinant material".

The isolation of pure clones from virus suspensions is fully described in Section D but the special application to recombinant material will be described briefly here.

A sample of recombinant material was thawed and titrated and a large number of eggs was then inoculated with a dilution of the recombinant material sufficient to give SP's on a proportion of the membranes. With suitable precautions against the possibility of contamination, the single pocks were aseptically removed from the membrane, ground from the frozen state in small glass grinders and suspended in 1 ml of gelatin saline. This virus suspension was referred to
as "SP material", and was used, undiluted, to inoculate two more eggs. The virus suspension obtained from these confluent membranes was referred to as "stock material", and was tested fully for pock type, HA production, heat resistance, mouse virulence and type of rabbit skin lesion.

The results of this type of experiment are summarised in Table 7. The single presumed recombinant from these experiments is included in Table 12, designated as R1-1.

TABLE 7

Results of Experiment 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Virus Dose</th>
<th>Time of reaping</th>
<th>No. SP's Tested</th>
<th>No. Recs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-Led-7N</td>
<td>RP-U</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10 8.4 10 8.4</td>
<td>12 hrs.</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>10 10 10 8.4 8.4</td>
<td>12 hrs.</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

2. Inoculation of the CAM with V-Led-7N and RP-U suspensions of higher titre:

In another short series of experiments the conditions were varied to the extent of using a rather larger inoculum of V-Led-7N and RP-U.
Virus used in these experiments was obtained by scraping infected rabbit skin, and was sometimes concentrated by centrifugation followed by resuspension in a small volume of gelatin saline. In comparison with the first experiments the virus content of the inoculum was raised by a factor of 2-5. The results of these experiments are summarised in Table 8. The single presumed recombinant obtained is included in Table 12, designated as R1-2.

**TABLE 8.**

**Results of Experiment 2.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Virus Dose</th>
<th>Time of reaping</th>
<th>No. SP's Tested</th>
<th>No. Recs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-Led-7N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP-U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>8.7</td>
<td>12 hrs.</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>8.7</td>
<td>12 hrs.</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>9.2</td>
<td>12 hrs.</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>9.1</td>
<td>12 hrs.</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. **Inoculation of the CAM followed by more than one multiplication cycle.**

Comparison of the results of the first two types of experiment clearly indicated that no gain in the efficiency of recombinant production could be expected if the virus inoculum was further increased. It seemed likely that the relative slowness and inefficiency of virus absorption could considerably reduce the effective concentration of virus in the CAM cells, and so, in a third type of experiment, the mixedly infected CAM was incubated for 24 hours before it was removed from the egg, thus allowing more than one cycle of infection and multiplication. It was thought that this would be a more efficient way of producing a high concentration of virus in the membrane. In all other respects the technique was the same as before.

As indicated in Table 9, a better ratio of recombinants to SP's tested was observed. The presumed recombinants from this experiment are included in Table 12, designated as R2-1,2,3,4.
TABLE 9.

Results of Experiment 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Virus Dose</th>
<th>Time of Reaping</th>
<th>No. SP's Tested</th>
<th>No. Recs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8.4 V-Led-7N 8.4 RP-U</td>
<td>24 hrs.</td>
<td>31</td>
<td>4</td>
</tr>
</tbody>
</table>

4. **Overlapping pocks**

It seemed likely from the results of Experiment 3 that a further increase in the time during which the two virus strains grew together on the CAM would again raise the proportion of recombinants in the progeny. However, the massive inoculation technique was approaching its limit, in that a further increase in incubation time resulted in the death of a high proportion of embryos, and the technique of harvesting overlapping pocks was therefore adopted.

In this method each membrane was inoculated with a mixture containing about 20–40 particles of each virus type. After incubation for 48 hours at 36 °C, examination of the membrane
usually revealed one or more areas where the two different pock types overlapped. After aseptic removal of the membrane to a sterile glass slab it was possible to remove part of the overlapping area with a sharp scalpel. Care was taken not to include any of the parent pocks which lay adjacent to this area, as this would dilute recombinants with parent types. As a rule 2-5 overlapping areas were pooled in a small glass grinder and thereafter treated in the same way as membrane material in the previous experiments.

As indicated in Table 10, a much higher proportion of recombinants was observed. The presumed recombinants from this experiment are included in Table 12, designated as R3-1,2,3,4,5,6.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Time of reaping</th>
<th>No. SP's Tested</th>
<th>No. Recs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>48 hrs.</td>
<td>21</td>
<td>6</td>
</tr>
</tbody>
</table>

TABLE 10
Results of Experiment 4
5. **Isolation of Recombinants from HeLa cell culture**

Monolayers of HeLa cells which had grown for 4-5 days were washed several times with BSS prior to inoculation with the virus mixture. At this stage each bottle contained $2.4 \times 10^9$, $1.8 \times 10^9$ cells. 10 PFU of V-Led-7N and 10 PFU of RP-U were introduced into the tissue culture bottles in 1.5 ml. of growth medium; this fluid remained in contact with the cells during a further eight hours incubation while absorption was allowed to occur. The fluid was then removed, the cell layer washed several times with BSS and fresh growth medium introduced. After a further 10 hours incubation, making a total of 18 hours, the medium was removed, BSS was added, and the cells removed from the glass by pipetting. The cells were washed by centrifugation in several changes of BSS and the final deposit, together with a small amount of BSS, was transferred to a small sterile glass grinder and ground from the frozen state.

The suspension obtained was treated in the same manner as were those obtained from mixed
infection of the CAM, i.e. SP's were obtained and stock suspension made from them and tested.

The results of this experiment are summarised in Table 11. The presumed recombinants are included in Table 12, designated as $R^4 - 1,2,3,4,5$.

### TABLE 11.

Results of Experiment 5.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Virus dose</th>
<th>Time of reaping</th>
<th>No. SP's</th>
<th>No. Recs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-Led-7N</td>
<td>RP-U</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9.1</td>
<td>8.8</td>
<td>18 hrs.</td>
<td>16</td>
</tr>
</tbody>
</table>
### TABLE 12.

Properties of Recombinants Obtained on Mixed Infection of CAM or HeLa Cells with V-Led-7N and RP-Uα.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAM</td>
</tr>
<tr>
<td>V-Led-7N</td>
<td>W</td>
</tr>
<tr>
<td>R1-1</td>
<td>W</td>
</tr>
<tr>
<td>R1-2</td>
<td>W</td>
</tr>
<tr>
<td>R2-1</td>
<td>W</td>
</tr>
<tr>
<td>R2-2</td>
<td>W</td>
</tr>
<tr>
<td>R2-3</td>
<td>W</td>
</tr>
<tr>
<td>R3-1</td>
<td>W</td>
</tr>
<tr>
<td>R3-2</td>
<td>W</td>
</tr>
<tr>
<td>R4-1</td>
<td>W</td>
</tr>
<tr>
<td>R4-2</td>
<td>W</td>
</tr>
<tr>
<td>R4-3</td>
<td>W</td>
</tr>
<tr>
<td>R4-4</td>
<td>W</td>
</tr>
<tr>
<td>R2-4</td>
<td>W</td>
</tr>
<tr>
<td>R3-3</td>
<td>W</td>
</tr>
<tr>
<td>R3-4</td>
<td>unstable</td>
</tr>
<tr>
<td>R3-5</td>
<td>W</td>
</tr>
<tr>
<td>R3-6</td>
<td>W</td>
</tr>
<tr>
<td>R4-5</td>
<td>W</td>
</tr>
<tr>
<td>RP-U</td>
<td>R</td>
</tr>
</tbody>
</table>
a **underlining** indicates properties in which the recombinant clones differ from the V-Led-7N parent.

b **int.** mouse virulence intermediate between that of parental strains (percentage mortality after standard test shown in brackets)

c **int.** (*P.C.*) rabbit skin lesion indurated with purple center but smaller than lesion produced by RP-U.

d **non**-homogeneous and unstable.

---

**G. HOMOGENEITY AND STABILITY OF RECOMBINANTS**

When tests of the progeny of virus particles derived from recombination experiments disclosed a difference from the parental types the following procedure was used.

The original SP was removed from the -60°C store and, after a preliminary titration, was inoculated on to a sufficient number of eggs to produce 10 or more SP's. 10 SP's so obtained were passaged once to produce a high titre suspension and tested fully.

Where these 10 suspensions did not differ among themselves or from the original SP in the characters tested, the original SP was assumed
to be homogeneous although the sample taken was necessarily small.

The eleven recombinants in the first part of Table 12 fell into this group.

A further series of nine tests was done on each homogeneous recombinant; the method used in deriving the material for test is shown in Figure 5.

**FIGURE 5.**

The procedure followed in testing homogeneity and stability of presumed recombinants.

![Diagram](image)

The solid circle denotes a SP; the open circle denotes a stock suspension derived from it. An enclosed T indicates that the stock suspension was fully tested.
Where these nine tests showed no divergence, in the characters tested, from the original SP the recombinant was assumed to be stable.

The same eleven recombinants (Table 12) also fell into this group.

It will be seen that the original recombinant virus particle has undergone 10 cycles of growth at the point where the last three tests were made and, in addition to the homogeneity test described above which involved the isolation of 10 SP's, two smaller tests, involving 3 SP's each, were made for homogeneity after 3 and 7 cycles of growth.

The eleven recombinants which were homogeneous and stable exhibited the following differences from V-Led-7N.

3 (R1-1, R3-1, R4-4) produced no detectable haemagglutinin.

3 (R2-2, R3-2, R4-3) possessed a heat resistance which fell into the range characteristic of RP-U.
1 (R⁴-1) combined both the above characters.  
1 (R¹-2) possessed a high heat resistance,  
produced no haemagglutinin, and also  
killed 70% of mice when 10 PFU's  
were inoculated intracerebrally.  
1 (R²-1) produced no detectable haemagg-  
lutinin and also killed 50% of mice.  
1 (R²-3) killed 90% of inoculated mice and  
also produced an intermediate type  
lesion on the rabbit skin. This  
lesion was similar in appearance to  
that produced by RP-U, but was much  
smaller and lacked the spreading  
character of the RP-U lesion.  
1 (R⁴-2) possessed a high heat resistance and  
also produced an intermediate type  
lesion on the rabbit skin.  

It will be noted that both R¹-2 and R⁴-1  
produce no detectable HA and possess a heat  
resistance in the range characteristic of RP-U.  
These two characters, in conjunction with a  
white pock on the CAM, suggest the variant of
RP-U, RP-U-W. The two strains differ from RP-U-W in the following respects.

The virus content of the pock of R1-2 (5.5 (10 PFU) was higher than that of RP-U-W (4.3 PFU, Fenner, 1958), although the pocks were of the same size. The mouse virulence (70%) was considerably greater.

The pock size of R4-1 was the same as that of V-Led-7N, i.e. it was considerably larger than that of RP-U-W. The virus content of the pock of R4-1 was 5.4 PFU.

Few strains of RP-U-W have been studied and it is possible that strains will be found that are similar in character to the two strains R1-2 and R4-1, which will be provisionally classified as recombinants.

The last six recombinants in Table 12 were not homogeneous in one character as judged by the isolation of 10 SP's and, in addition, the passaging tests in every case revealed instability in the same character.
The characteristics of these strains are summarised in Table 13.

**TABLE 13.**

The results of tests on six recombinants which were neither homogeneous nor stable in one character.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Stable characters</th>
<th>Result of Homogeneity test (10 SPs)</th>
<th>Result of Stability tests</th>
</tr>
</thead>
</table>
| R2-4        | CAM-W            | 2 HA-h                             | h
|             | HR-h             |                                    | h—o—o
|             | MV-1             |                                    | o—o—o
|             | RS-N             |                                    |
| R3-3        | CAM-W            | 7 HA-h                             | h
|             | HR-h             |                                    | h
|             | MV-1             |                                    | h
|             | RS-int.          |                                    | o—o
| R3-4        | HA-0             |                                    | W (a)
|             | HR-1             |                                    | R
|             | MV-1             |                                    | int.
|             | RS-N             |                                    | W
|             |                  |                                    | R
|             |                  |                                    | int.
| R3-5        | CAM-W            | 5 HR-h                             | h—h
|             | HA-h             |                                    | 1—1
|             | MU-1             |                                    | 1—1
|             | RS-int.          |                                    |
**TABLE 13 (continued)**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Stable characters</th>
<th>Result of Homogeneity test (10 SP's)</th>
<th>Result of Stability tests</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R3-6</strong></td>
<td>CAM-W</td>
<td>8 HR-h</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>HA-0</td>
<td></td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>MV-1</td>
<td>2 HR-1</td>
<td>l—l</td>
</tr>
<tr>
<td></td>
<td>RS-N</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>R4-5</strong></td>
<td>CAM-W</td>
<td>1 HA-h</td>
<td>o</td>
</tr>
<tr>
<td></td>
<td>HR-1</td>
<td></td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>MV-int.</td>
<td>9 HA-0</td>
<td>o—O</td>
</tr>
<tr>
<td></td>
<td>RS-N</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Egg-to-egg variation made these results difficult to interpret. (See text).

It should be noted that in each of the six unstable recombinants described above another character was also found to differ from the parental V-Led-7N. In every case this character was homogeneous and stable as judged by the standard testing procedure.
The combinations observed were:

Absence of HA production (unstable) with high heat resistance (stable), with high heat resistance and intermediate type rabbit skin lesion (both stable) and with intermediate mouse virulence (stable);

high heat resistance (unstable) with absence of HA production (stable) and with intermediate type rabbit skin lesion (stable);

unstable pock type with absence of HA production (stable).

The unstable pock morphology displayed by R3-4 was difficult to interpret for two reasons. Firstly, the classification into white, red and intermediate pocks was extremely arbitrary; a continuous range was seen between the extremes of white and red and, also, the extreme white pocks and red pocks were not quite "normal" as judged by the standard of appearance of the parental V-Led-7N and RP-U. The haemorrhagic centre of the white pocks was more pronounced than with V-Led-7N, as were the borders of the red pocks when compared with RP-U.
Secondly, great variation in the appearance of the pocks occurred when the same suspension was inoculated on to several eggs.

It was not uncommon for consecutive eggs, inoculated with the same material, to be scored 90% white, 10% intermediate and 50% intermediate, 50% red.

A PRELIMINARY STUDY OF THE YIELD OF SINGLE HeLa CELLS MIXEDLY INFECTED WITH RP-U AND V-LED-7N

4-5 day old HeLa cell cultures, with a cell count of $2 - 4 \times 10^5$ were washed several times with BSS and inoculated with a mixture containing $10^{9.1}$ PFU V-LED-7N and $10^{8.8}$ PFU RP-U in 1.5 ml. of growth medium. After 8 hours incubation this fluid was removed, the cell layer was again washed several times with BSS, fresh growth medium was introduced and incubation continued for a further 10 hours. At the end of the 18 hour incubation period the growth medium was removed, the cells were shaken off the glass, trypsinised (Syverton et al., 1954) and washed several times in BSS with light centrifugation. The cells were then diluted in BSS so that one cell appeared in a proportion of standard drops taken. Each drop was placed on a sterile slide and examined microscopically. Where a single, apparently intact
cell appeared in a drop, it was crushed under a cover slip, suitably diluted, and the entire volume of fluid was distributed over 4 CAM's.

Every pock formed was examined in an abbreviated way. Considering the preliminary nature of these experiments, it was not considered essential to isolate SP's, thus allowing considerable economy of time and materials. Each pock was cut from the membrane regardless of the presence of other pocks and suspensions were prepared in the usual way. The number of pocks/membrane was low and it was not necessary to reject any pock because of overlapping.

Mouse virulence and rabbit skin lesion tests were omitted as a safeguard against any error due to possible slight contamination when several pocks were isolated from the same membrane.

As previously established (Section E) the presence of a slight degree of contamination does not affect the pock type, haemagglutinin and heat resistance tests; however, the mouse virulence and type of rabbit skin lesion tests would be seriously affected by RP-U contamination. Heavy contamination can be detected
in the pock type and heat resistance tests if the contaminant gives rise to a pock different to that under study. No such contamination was observed with the recombinants (all CAM-W) isolated from single HeLa cells.

Of nineteen cells examined, recombinants for these three characters were isolated from nine. Five cells contained both types of parental virus but no recombinants and five contained either one type of parental virus or no virus at all (Table 14).

Table 15 sets out in more detail the number of pocks and the characters of the recombinants isolated from these cells.

**TABLE 14**

<table>
<thead>
<tr>
<th>Types of pocks isolated from single cells.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cells examined</td>
<td>19</td>
</tr>
<tr>
<td>No. cells containing recombinants and both parent types</td>
<td>9</td>
</tr>
<tr>
<td>No. cells containing both parent types</td>
<td>5</td>
</tr>
<tr>
<td>No. cells containing V-Led-7N</td>
<td>1</td>
</tr>
<tr>
<td>No. cells containing RP-U.</td>
<td>1</td>
</tr>
<tr>
<td>No. cells containing no demonstrable virus</td>
<td>3</td>
</tr>
</tbody>
</table>
Two subsidiary experiments were carried out to check whether the virus isolated from a particular HeLa cell was in fact entirely produced by that cell.
The first experiment was designed to test the efficiency of the washing process employed, i.e. does virus, originally present free in the growth medium, appear in the fluid associated with the single cell, and be wrongly included in the yield from that cell.

One bottle of HeLa cells was inoculated with $10^9.1$ PFU V-Led-7N, another with $10^8.8$ PFU RP-U. The usual medium replacement and washing steps were carried out and after 18 hours incubation the cells were removed from the glass, trypsinised, and the two cell suspensions were mixed. Immediately the mixed suspension was washed three times by centrifugation with BSS. Finally the cells were resuspended in BSS and single cells were isolated and crushed. Material from the crushed cells was inoculated on to 1 CAM.

Of 22 single cells examined 2 contained no virus, 12 contained RP -U virus only, 7 contained V-Led=7N virus only and 1 cell produced 1 RP-U pock as well as 11 V-Led-7N pocks.

It would seem, therefore, that accidental contamination during the washing process occurs to a very slight degree only.
When, however, the separate cultures were trypsinised and mixed after fourteen hours incubation (their treatment up to this point being identical with the previous experiment) and the cells spun lightly and incubated for a further four hours before washing and crushing, clear indication of considerable transfer of virus from cell to cell was obtained.

When the contents of twelve cells were examined both viruses were present in seven cases. Table 16 sets out the results of this experiment.

**TABLE 16**

Types of pocks isolated from single cells after prolonged contact.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. cells</th>
<th>Mean No. V-Led-7N pocks</th>
<th>Mean No. RP-U pocks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>2.5</td>
<td>16</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>
It is apparent that Group A cells containing V-Led-7N virus were contaminated with RP-U virus and that in Group B the opposite occurred. There was no contamination of Group C cells (containing RP-U virus) with V-Led-7N although contamination with RP-U from other cells might have occurred. This might also have happened in Groups A and B. The cells in Group D may originally have contained no virus, in which case contamination by V-Led-7N has occurred. The figure of 7 out of 12 cells contaminated is therefore a minimum estimate.

It is probable that virus is leaking from some or all cells to the surface of others, and that this contaminating virus is not removed by the washing procedure described above.

I. THE PROPORTION OF RECOMBINANT VIRUS TO TOTAL VIRUS APPEARING IN HeLa CELL CULTURES AFTER VARIOUS INCUBATION TIMES.

The extreme difference in heat resistance which exists between V-Led-7N and RP-U allows
an effective screening test for CAM-W, HR-h recombinants.

As previously stated no V-Led-7N pocks appeared when a suspension, prepared by grinding confluent CAM's, was heated at 55°C for 40 minutes and then inoculated, at an appropriate dilution, on to the CAM. When a mixture of RP-U and V-Led-7N was treated in the same way, again no V-Led-7N pocks appeared.

When an artificial mixture was prepared in the ratio 10 RP-U: 10 V-Led-7N: 1 CAM-W, HR-h recombinant, heating at 55°C for 40 minutes and subsequent titration resulted in a ratio of 10 CAM-R pocks to 1 CAM-W pock. 16 CAM-W pocks which appeared in the titrations were tested fully for heat resistance. In each case their heat resistance fell into the range characteristic of RP-U.

In the following experiment the assumption has been made that every CAM-W pock which appears after heating, at the dilutions employed, is a recombinant of the type CAM-W,
Several 4-5 day old HeLa cell cultures were inoculated as described previously with a mixture of $10^9 \cdot 1$ PFU V-Led-7N and $10^8 \cdot 8$ PFU RP-U in 1.5 ml growth medium. The growth medium was removed and the cells were washed after eight hours incubation. With the exception of the eight hour samples, fresh growth medium was introduced and the cells incubated for an appropriate further period.

After 8, 12, 18, 24 and 36 hours incubation the cells were removed from the glass, washed three times in BSS and ground from the frozen state. An equal quantity of a saline extract of CAM was added to each suspension. They were then heated at 55°C for forty minutes and titrated at a $10^{-2}$ dilution. Results are shown in Table 17.
TABLE 17

The ratio of CAM-W, HR-h recombinants to CAM-R pocks following mixed infection of HeLa cells with subsequent heating.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>8 hrs.</th>
<th>12 hrs.</th>
<th>18 hrs.</th>
<th>24 hrs.</th>
<th>36 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAM-W pocks</td>
<td>3</td>
<td>9</td>
<td>30</td>
<td>31</td>
<td>22</td>
</tr>
<tr>
<td>CAM-R pocks</td>
<td>32</td>
<td>91</td>
<td>232</td>
<td>302</td>
<td>203</td>
</tr>
<tr>
<td>Approx. ratio</td>
<td>1/11</td>
<td>1/10</td>
<td>1/8</td>
<td>1/10</td>
<td>1/9</td>
</tr>
</tbody>
</table>

The ratio CAM-W:CAM-R, which is taken as an index of the proportion of CAM-W, HR-h recombinants in the progeny of the mixed infection, is relatively constant over the incubation time range 8-36 hours.

This range includes a period when very little progeny virus was recoverable: presumably very little progeny virus had been produced by the cell at this stage.
DISCUSSION

A total of one hundred and forty-nine clones obtained from mixed infection of the chorioallantoic membrane of the chick embryo and HeLa cells were examined in detail.

Of these about one third formed red pocks on the membrane, and in every case they were indistinguishable, on the tests employed, from the parental RP-U strain.

The remainder (with one exception, an unstable pock) formed white pocks similar to those produced by the other parental strain V-Led-7N. Of these, seventeen differed from V-Led-7N in at least one character which, within the limits of the samples taken, was now permanently incorporated in the make-up of the virus. Additionally, six of these seventeen strains were heterogeneous for a further character.

There seems little doubt that these strains were produced by a process of genetic recombination.
Although vaccinia virus is larger and morphologically more complex than phage or influenza and, also, forms inclusion bodies during its multiplication cycle (Smadel and Hoagland, 1942) there seems no reason to suppose that in mixedly infected cells the physical exchange necessary for recombination might not be possible. There is good evidence to show that vaccinia undergoes a true eclipse phase (Briody and Stannard, 1951; Crawford and Sanders, 1952; Anderson, 1954) during which it might be presumed that the genetic material would be more "exposed". Electron micrographs of thin sections of vaccinia infected cells show the emergence of virus particles, together with what are believed to be various incomplete and intermediate forms, from a homogeneous electron dense inclusion body (Wyckoff, 1951; Banfield et al, 1951; Gaylord and Melnick, 1953, a, b). It seems possible that contact between the genetic material of two infecting vaccinia strains could take place either before the formation of these matrices or that the apparent localisation of the inclusion body is not in fact so marked in the living cell (Himmelweit, 1938).
Recombination is a relatively common event as judged by these experiments. When all the mixed infection experiments are included, seventeen recombinants were isolated from one hundred and forty-nine clones tested; however under the most favourable conditions six out of twenty-one clones tested showed evidence of recombination.

Within the limitations of the rather crude technique used in the single cell experiments, it seems probable that recombinants are formed in the majority of cells which receive virus particles of both types.

Putting technical difficulties aside, it would appear that the vaccina virus - host cell system is basically similar to the T phage - E - coli system. In both cases related strains are available which are capable of mixedly infecting cells, and which possess well defined genetic markers.

The markers employed in this study, with the exception of mouse virulence and rabbit skin lesion, are reasonably convenient to handle and, in the case of heat selection could be used as a valuable selection technique. A wide variety of strains is available (Fenner, 1958) and pairs of strains other than those used in the preceding
experiments could be chosen to fulfil special requirements.

In both systems, also, methods are available for isolating clones of virus with reasonable certainty, although in this respect the technical differences are greatest.

A further point of similarity is the common occurrence of DNA in both viruses. However no evidence exists to eliminate substances other than DNA from complicity in vaccina reproduction and recombination processes.

Although it will probably not be possible to eliminate all extraneous material as neatly as has been done with phage, there seems no reason why experiments should not be devised to pin-point DNA as the material responsible for vaccina replication. It is possible that some DNA base difference, analogous to hydroxymethyl cytosine in phage may be discovered, or that the DNA of virus may differ in base ratio to that of the cell. Alternatively it may be possible to follow the fate of virus containing labelled DNA as has been done so successfully in phage.
Since this work was completed, Gemmell and Cairns (1959) and Gemmell and Fenner (1960) have extended the studies on pock type mutants in R P - U. Several different mutations to white pock (R P - U - W) have been described and have been shown to occupy different loci by back crossing experiments. This work is completely analogous to the work which produced the phage T2 and T4 genetic maps, but has been hampered by egg to egg variation as described in this thesis. For this reason no recombination frequencies can be calculated. This variation is unfortunate, as it is with this marker that the technical simplicity of phage is most nearly approached. Gemmell and Fenner (1960) estimate that there may be up to 1000 genetic loci controlling the R P - U to R P - U - W mutation.

It would seem probable that mouse virulence and rabbit skin lesion, which have given rise to a variety of intermediates in this study, are also controlled at a number of loci, but no estimate of their number can be made from the results obtained. No white pock recombinant showed virulence equal to that of RP-U, nor did any lesion produced on the rabbits' skin by a white pock strain equal in size that produce by RP-U. The
finding of intermediates is consistent with previous investigations into the virulence of viruses (Fraser and Burnet, 1952; Burnet and Lind, 1954; Sabin, 1955; Fenner and Marshall, 1957). Both these markers are extremely unwieldy to work with and estimates of recombination frequency would no doubt be confused by animal to animal variation and by the selection of more virulent strains during animal passage.

Two of the markers studied, heat resistance and haemagglutinin production, appeared to be all-or-none characters. Due to the extreme differences which existed between the parental strains in these two respects, any intermediates would have been readily detected, but none were found.

This suggests that these two markers are controlled by a single locus, but study of crosses between a number of strains would be necessary to prove this.

What conclusion can be drawn from the results obtained as to the behaviour of vaccina virus during replication; in particular what analogies can be drawn with the phage system?
From the observation that recombination does occur, it is possible to infer that an intimate mixing of the genetic material of the invading strains takes place in the host cell, analogous to the DNA pool in the phage system.

In addition, it was observed that recombination appeared among the earliest infectious progeny but, as far as can be judged from the limited data, the recombination rate does not seem to increase as more units become available for mating, as has been shown with phage. However, Fenner (1959) states that the proportion of recombinant clones increases to a maximum at 24 hours in the HeLa cells. Any lack of agreement may reflect differences in the time or method of maturation, or in the spatial relationship of the particles rather than a basic difference between the vaccinia and phage systems.

Several clones were isolated which gave rise to a variety of progeny. The behaviour of these clones was somewhat different to that of phage heterozygotes in that, with one exception, they continued to segregate on repeated passage. In this respect the behaviour seemed more analogous with the X virus of Hirst and Gotlieb (1953)
Not enough information is available to speculate as to the genetic structure of these strains; for example whether they are diploid or not. Nor can any conclusions be drawn as to the relationship between heterozygosis and recombination. Such a relationship can certainly not be eliminated on the basis of the results presented.

No information can be obtained from the results presented as to the existence of repeated mating. There seems no reason why three parent crosses should not yield definite information on this point, provided care was taken to ensure that a single cycle of infection only was studied. The phage experiment where, with uneven multiplicities of infection, more recombinants than minority parent types were found, would be difficult to repeat with vaccina as too little is known about the process of infection, interference, and exclusion to make any estimate of the effective multiplicity.

Again too few single cell yields have been analysed to determine the ratio of opposite type recombinants to each other.

Two factors hamper the study of recombination in vaccinia and, indeed, in any animal virus system. Firstly, too little is known about the basic chemistry.
of animal virus replication and the interchange of chemical and genetic data and, in fact, the merging of the two disciplines, which is becoming apparent in the phage system, is not yet possible. Secondly, in the vaccinia system, isolation of clones of virus and examination for markers is a cumbersome procedure when compared to the convenient phage technique.

Any comparison between vaccinia and phage which can be made and verified will therefore be of importance, as it obviously will be impossible for some time to build up an independent theory of vaccinia replication to the refined stage which has been reached in the phage system. However, if it can be shown that vaccinia virus behaves like phage in these observable ways it will be legitimate to borrow further from the phage theory.
1. **Preparation of high titre virus suspensions**

Groups of 11 day eggs were inoculated with $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ PFU of V-Led-7N virus and 4 eggs in each group were harvested after incubation at 36°C for 24, 36, 48, and 60 hours. A virus suspension was prepared from these membranes and titrated, using 4 eggs per dilution. The experiment was repeated with the same concentrations of RP-U virus.

Results are shown in Fig. 7.

**FIGURE 7.** Increase in virus content of membranes inoculated with a series of concentrations of V-Led-7N (crosses) and RP-U (circles) and harvested after 24-60 hours incubation.
For the purpose of preparing high titre virus stock it was necessary to consider one additional factor, namely the proportion of dead embryos at each point on the curves. With an inoculum of 10 PFU of V-Led-7N incubated for 48 hours the death rate was negligible, whereas with RP-U under the same conditions it was over 80%. However at 36 hours with an inoculum of 10 PFU of RP-U the death rate was less than 20%. The following conditions were used routinely:

V-Led-7N: An inoculum of 10 PFU harvested at 48 hours.

RP-U: An inoculum of 10 PFU harvested at 36 hours.

2. Determination of 1st cycle time

CAM's of thirty-six 11 day chick embryos were dropped and were then inoculated with approximately 10 PFU of V-Led-7N contained in 0.25 ml of gelatin saline. At two-hourly intervals from 2-16 hours three membranes
were harvested aseptically, washed in five changes of sterile gelatin saline, pooled, ground, and clarified. The same procedure was followed using RP-U as inoculum. All samples were titrated using 4 eggs per dilution.

Results are shown in Fig. 6.

**FIGURE 6.** Increase in virus content of membranes massively inoculated with V-Led-7N (crosses) and RP-U (circles).
It is apparent that a considerable increase in virus content of the membrane can be demonstrated after about 10 hours. It is realised that, due to irregularity of absorption and possible irregularity of rates of multiplication, the yield at this point may not be exclusively first cycle virus, indeed a proportion is undoubtedly unaltered inoculum. However it is likely that the bulk of the virus detected is freshly produced progeny.
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