INTRACELLULAR INTERACTIONS OF POLIOVIRUSES: INTERFERENCE and MULTIPLICITY REACTIVATION

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John W. Drake

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ABSTRACT

This study concerns interactions among polioviruses infecting the same cell, and can be divided into three parts. The first part consists of the detailed study of certain conditions of infection of HeLa cells by poliovirus. The second is concerned with the interfering activity of virus, either live or inactivated, with the multiplication of superinfecting live virus, either homotypic or heterotypic. It was found that live virus interferes with heterologous virus (homologous not tested), while UV-killed virus lacks interfering ability. It was also found that the virus released from cells infected by two heterotypic polioviruses, when interference does not occur, contains particles which are neutralized by both specific antisera. This phenomenon may be the result of a "phenotypic mixing" mechanism similar to that observed with phages.

The third part of the study deals with multiplicity reactivation among homotypic polioviruses inactivated by UV irradiation. When killed viruses are adsorbed to cells at multiplicities greater than one, more cells release live virus than can be accounted for on the basis of the input of UV-surviving viruses. This multiplicity reactivation is a function of the UV'd particles, not of non-viral agents in the lysate, heat-killed viruses, originally uninfectious particles, nor anomalies in adsorption. The fraction of cells releasing virus increases with multiplicities up to 40-80, when saturation appears to set in. At high doses the fraction of yielders decreases with the UV dose to the virus at a rate equal to that at which the virus itself is killed. The parameter n, representing the number of segments within a virus which can interact with segments from other particles, is calculated, and its significance is discussed.

ABBREVIATIONS AND SYMBOLS

A -- fraction of adsorbed virus.

D -- dilution factor.

DNA -- desoxyribonucleic acid.

I -- normalized fraction of infected cells.

LA -- lactalbumin hydrolysate medium.

LY -- LA with yeast extract.

m -- multiplicity (average viruses per cell).

MR -- multiplicity reactivation.

MW -- molecular weight.

n -- number of hypothetical units in viral genome.

NDV -- Newcastle disease virus.

PBS -- phosphate-buffered saline.

PFU -- plaque-forming units.

PM -- phenotypic mixing.

r -- lethal hits (average per virus).

RDE -- receptor-destroying enzyme.

RNA -- ribonucleic acid.

S -- virus fraction surviving after irradiation, determined on monkey kidney monolayer.

TD -- titer discrepancy.

UV -- ultraviolet light.

V* -- irradiated virus titer determined on monkey kidney monolayers.

V_c -- virus titer determined on suspended S3 cells.

V_p -- virus titer determined on monkey kidney monolayers.

TABLE OF CONTENTS

INTRODUCTION

The adaptation of tissue culture techniques for the plaque assay of poliomyelitis virus by Dulbecco and Vogt (1,2) has made possible new and more exacting studies of animal virus systems. The new plating methods, the better ways available for handling single cells in isolation, and increased knowledge of the chemistry of animal virus systems have permitted a beginning in the quantitative investigation of the genetic behavior of animal viruses and their mode of association with their cellular hosts. The work to be presented here will deal primarily with two aspects of poliovirus interaction with a host cell: interference, or the ability of a virus infecting a cell to prevent the multiplication of superinfecting virus; and multiplicity reactivation, the ability of several viruses killed by ultraviolet irradiation and simultaneously infecting a single cell, to interact in some manner so as to produce viral progeny.

A. The position of poliovirus among animal viruses.

Polioviruses exist in three distinct types (I, II and III). These types are differentiated by their strikingly complete lack of antigenic relationship (60). They are grouped together because of the characteristic pathology they produce in their normal host, the primate central nervous system. More specifically, polioviruses preferentially attack the anterior horn of the spinal cord, with concomitant cell destruction. They also appear to resemble each other in

size and chemical simplicity (see below). In view of their lack of antigenic relatedness, one may ask if their common grouping is justified, especially in the absence of experimental studies demonstrating a genetic relationship. In answer to this question, one might surmise that strong selective pressures would exist against viruses with a related pathology which also possessed a related antigenic make-up: antibodies formed against one type during an infection would cross-react with another type in a subsequent infection. It thus seems quite likely that in the course of time, various strains differing slightly in antigenic make-up would separate still further, and such a process may have operated to give rise to the poliovirus situation.

Poliovirus is revealed in the electron microscope to be a spherical particle with a diameter of 27 $\pm$ 2 mμ (3,4), and as such is representative of the smallest group of animal viruses measured to date. This group also includes the Coxsackie viruses, with reported diameters around 37 mμ (5) (probably too large), Theiler's virus of diameter 28 mμ (6), and encephalomyelitis virus (7). By contrast, it is much smaller than members of the Myxovirus group (viruses of mumps, influenza, Newcastle disease, fowl plague) whose diameters approximate 85 mμ (8). Schaffer and Schwerdt (9) have shown that type II poliovirus forms tetragonal crystals with pyramids at both ends. Their calculation of the particle mass from Stoke's law was 1-1.5 X 10^{-17} gm (3), and calculations based on determinations of plaque-forming

units (PFU)/gm and particles/PFU yielded 2.0×10^{-17} gm (9). These figures correspond to a molecular weight (MW) of about 9 million; calculations from partial specific volume (0.64-0.69) lead to an estimate of 5.3-7.3 million MW (3). All three poliovirus types appear to have the same size and mass.

Electron microscope particle counts have given particle/PFU ratios for poliovirus up to 1000 (3), but this high value probably represents inactivation during the preparation. Ratios of the order of 30 were obtained by using virus harvested from monolayers infected at large (10-50) multiplicities, at the peak of infection, and quickly purified and assayed on human amnion cells (10). The relatively high ratios suggest low efficiencies of infection in tissue culture, a considerable lability of the viral infectivity, or the formation of many imperfect particles. Thus the ratio depends upon the type of cells used to assay the virus: amnion cultures are reported to support a plating efficiency some three times higher than monkey kidney cultures (11).

Corresponding to its small size, poliovirus exhibits considerable chemical simplicity. Nucleic acid analyses (3) show that about 24% of the type II particle consists of ribonucleic acid (RNA), and less than 1% is desoxyribonucleic acid (DNA). No lipid nor non-RNA phosphorus has been reported. The virus is completely resistant to the action of RNAase and DNAase at 37° and 43°C (12). A reasonable model for the virus at the present time, and one consistent with the more detailed knowledge of other viruses, is that of a

small, roughly spherical particle consisting of an outer protein coat and an inner nucleic acid core.

The virus differs chemically and structurally in several ways from members of the Myxovirus group, the other most extensively studied group of animal viruses. The enzymatic activity of Newcastle disease virus (NDV), reportedly responsible for the penetration of the virus into the cell (13), has no parallel in poliovirus. Furthermore, infectious particles of influenza virus are separable by ether treatment (14) into several chemically and biologically distinct substances--complement-fixing soluble antigen (nucleoprotein) (15), hemagglutinating antigen, and a large amount of phospholipid (about 34% of the viral mass) (16,17)--which have no counterparts in poliovirus.

The large MW of the Myxovirus group (e.g., 370 million for influenza) (18) apparently does not set them off from poliovirus in their RNA content. Influenza consists of 0.78% RNA (19), or about 2.9 million MW; this compares with about 2.2 million MW for poliovirus RNA (3,9). Only 3000-5000 base pairs can be formed from this RNA if arranged in a structure homologous to the Watson-Crick DNA model, and according to current guesses these might be expected to specify about 1000-1700 amino acids in a polypeptide. These amounts of nucleic acid are very small compared to the values for coliphages T2 (20) and T4 (20,21) of around 120-160 million MW of DNA per particle (ca. 40-50% of the particle mass).

Schaffer has reported the complete absence of DNA from purified poliovirus (22), but there is still some doubt about residual DNA in influenza virus. Ada and Perry (19) reported no DNA (0.05 ± 0.04%), but using more sensitive assays Miller found about 0.1% DNA (23). This is 3.7 million MW per ID50 (10 particles in his preparations), or 370,000 MW per particle if evenly distributed, and corresponds to about 600 base pairs.

A variety of reports tell of differences in base ratios among nucleic acids of related strains of plant viruses, but little information is available about animal viruses. Ada and Perry (24) compared five A and three B strains of influenza virus, and found that the adenine+uracil/guanine+cytosine ratios were constant within the A or B strains (1.22-1.28 and 1.38-1.43 respectively), but showed definite differences between A and B strains. Schaffer (22) compared MEF-1 (Type II) and Mahoney (Type I) polioviruses and found equal adenine (25%) and guanine (30%) contents. Uracil content was up in the Mahoney virus (25%) compared to the MEF-1 (20%), and cytosine was correspondingly down.

B. Host cells for poliovirus.

The extensive adaptability to many animal and cellular hosts is well known for many animal viruses. The usual host for all three poliovirus types is the primate central nervous system, particularly the anterior horn of the spinal cord. They also grow in other primate cells, such as monkey

kidney cells, the human HeLa and KB cell, and others. The Lansing strain of type II virus has been adapted to the cotton rat (3), and the MEF-1 strain (type II) has even been adapted to chick embryos (25). The adaptation in these cases often required many serial passages, and presumably selected for variant virus particles. Type I and II polioviruses are very difficult to adapt to growth in non-primate animals.

Kibrick (26) has reported differences in susceptibility of various human and monkey tissues to the same poliovirus strain. A further degree of host specificity is demonstrated in the studies of Dulbecco and Vogt on the HeLa (S3) cells maintained in this laboratory (27): about 5% of the cells were some ten times more resistant to infection even though they adsorbed virus at about the same rate. Type I virus adapted to monkey kidney culture and showing reduced plating efficiency on monkey testis has been adapted to the latter cell type, giving rise to strains with a higher plating efficiency on testis (28).

C. Course of tissue culture infection by poliovirus.

The quantitative aspects of virus release have been studied by Lwoff et al. (12) in isolated cells, and by Dulbecco and Vogt (29) in mass cultures (one-step growth curves). The characteristic growth curve has a latent period of $5\frac{1}{2}$-$6\frac{1}{2}$ hours for isolated cells. Similar results were obtained with HeLa cells. About three hours of exponential

release follow in mass cultures, during which the monkey kidney cells release an average of 150-200 PFU. Similar growth curves are characteristic of other animal viruses (e.g., Western Equine Encephalitis (30), latent period $2\frac{1}{2}$-$3\frac{1}{2}$ hours).

Studies of isolated cells show that the one-step growth curve may be considered to result from cells which release virus over short periods, but whose latent periods extend over quite variable times and whose yields also show considerable variation. The period of greatest release is quite brief for each cell, nearly all of the virus being released in less than an hour.

A correlation between cytological changes (phase microscopy) and viral release in the same cell was described by Lwoff et al. (12). The cell begins to round up about one hour before the first virus release, granular material concentrating centrally around the nucleus and leaving a hyaline peripheral zone. During the period of virus release the hyaline zone becomes extensively vacuolated and shows an irregular outline; it finally disintegrates entirely. This suggests that the virus is expelled from the cells more gradually than is the case with bacteriophages.

Cytological studies of infected monkey kidney and S3 cells were carried out by Tenenbaum (31). Cells were observed in visible light after Feulgen or acriflavin staining, and in UV after acriflavin staining. Preparations were treated with RNAase and DNAase to determine the nature of

the observed substances. Infected cells on cover slips show some rounding up after about 4-5 hours, and the nucleus appears to swell. Its green fluorescence (DNA) becomes intense, and the cytoplasm concurrently becomes a more intense red (RNA). The nucleus then appears to rupture and its DNA content to pass into the cytoplasm, often in a long, spiral arm. The cytoplasm becomes loaded with heavily-staining RNA. From this time on the red cytoplasmic material forms granules and is extruded from the cell. As infection proceeds the cell becomes wrinkled, rounds up, and detaches from the cover slip. Similar observations (32) were made with S3 "giants" produced by X-irradiation (33).

The changes in the staining intensities of cellular DNA and RNA suggest profound alterations in their quantities and/or states, but whether any role for DNA in poliovirus multiplication is being observed here is a question for the future.

That the above course of infection is not universal is indicated by the report of Dunnebacke (34). Infected cultures of human amnion cells first showed a disappearance of the nucleolus (hematoxylin staining) after 8-10 hours, corresponding to the initiation of virus release. During the period of maximum release (ca. 19 hours), large spherical nodules form on the cell surface.

D. Interference.

Some understanding of the extent to which interference may occur is a prerequisite for the study of many intracel-

lular features of a virus-cell system. In particular, one must be able to assure the successful penetration of several viruses into a cell if any kind of viral interactions on a genetic level are to be studied. In the case of NDV (35) and bacteriophages (36), the block to the multiplication of superinfecting virus is set up within a matter of minutes after the attachment of the first virus particle. Following the initial changes suffered by the bacterial cell wall, which are completed within about 5 minutes, superinfecting phage have a very low probability of infecting the cell (37). One is able to circumvent interference by obtaining synchrony of the earliest stages of development in such systems by adsorption after starvation, cyanide or cold treatments (38); returning the cells to normal medium then initiates the activity of the genetic material of several viruses at once. With polioviruses infecting monkey kidney cells, however, interference is not overcome by cold or cyanide (39).

The development of our understanding of interference has been adequately reviewed by Baluda (35), and it is only necessary here to reiterate some of the conclusions. Interference systems can be divided into two kinds, those involving a pair of closely related viruses, and those involving genetically more distinct viruses. In the case of closely related bacteriophages such as T2, 4 and 6, the related superinfecting phages attach and are broken down, but do not multiply. Here the "penetration hypothesis" holds that as a result of the first infections, a change rapidly occurs on the cell surface

which results in the failure of the viral genetic material of superinfecting phages to successfully penetrate and establish itself intracellularily. This effect is achieved even if the interfering virus is but a protein "ghost" lacking in DNA. The breakdown of superinfecting phages may be related to the rapid mobilization of a DNAase system within the infected cell. It was originally felt that the "depressor effect" (sharp reduction in the yield of the established interfering phage) argued against this interpretation, that the superinfecting virus must indeed become partially established in the cell to compete with the first virus. However, the depressor effect may result from cell leakage, the release from the infected cell of many small-MW components, from the first few minutes after infection until the impermeability of the membrane is again established (40). The leakage effect increases with increasing multiplicity, so that several superinfecting viruses would more drastically deplete the cell of nutrients important to phage multiplication.

Dulbecco (41) studied exclusion by UV-killed phages of the T series, finding for the T-even phages that the irradiated phage could interfere with a related phage as efficiently as live phage. When both interfering and superinfecting phages were irradiated, that fraction of the multiplicity reactivation (see Section F) due to interaction between early and late phages fell off in the same manner as superinfecting phage was excluded.

The penetration hypothesis cannot apply to cases involving unrelated phages, however. The prophage lambda, already present within the cell, is in some cases still excluded from multiplication by superinfecting T5 (42). Furthermore, in the exclusion of T1 or T3 by T2 the DNA of the superinfecting virus injects but is not broken down, its multiplication being blocked at some stage after penetration (43).

Little quantitative information is available about interference in animal virus systems, with the one exception described below. In general, live virus or virus whose infectivity has been destroyed (heat, formaldehyde, UV) excludes either homologous or heterologous virus; exceptions are UV-killed fowl plague virus (44) and poliovirus (See Section C of Experiments and Results). The work of Baluda (35) demonstrated that a single particle of UV-killed NDV is capable of interfering with live homologous virus in chick lung cell cultures. This interference is completed within a very brief time (0.1-0.5 minutes), and is maintained upwards of 60 hours or more. There was no depressor effect in this system: addition of UV-killed virus shortly after infection by live virus had no detectable effect on the fraction of yielders or the burst size. The interference is clearly a surface effect: homologous live virus attaches to the cell and is inactivated, as is shown by the fact that from cells broken open by freezing and thawing and grinding, no live virus is recovered; and antiserum can completely remove the

interference up to 28 minutes after the attachment of the killed virus, even though live virus is known to penetrate within a few minutes (13). The rate at which interference becomes established depends upon the multiplicity of the UV-killed virus. The interference is, however, never completely effective: about half of the cells can be superinfected by very large amounts of live virus, and Baluda's calculations indicate that this process can be interpreted on the assumption that about 4% of the surface of these cells has remained receptive to attack by live virus. Since heated virus loses both interfering and enzymatic activities together, it may be that the interfering activity of NDV is expressed through its enzymatic properties.

Interference studies involving poliovirus have dealt with analysis of the yields of mass cultures and have not penetrated to the question of individual cell yields. Ledinko and Melnick (45) studied interference among all three types of poliovirus in monkey testis roller tube cultures. Infected cultures were ground in alumina and the centrifuged supernatants were titered after antiserum treatment to determine relative amounts of each viral type. In general, any polio type was capable of interfering with the production of an heterologous type, if conditions were sufficient to insure that the first infecting virus became established (and presumably infected a majority of the cells) before the superinfecting virus infected many cells. Viruses of the Coxsackie group caused at most a partial interference with poliovirus

(type I), and were themselves not subject to interference by type I virus. Finally, no homologous interference in polio systems was observed: type I virus killed by formalin, or by electron or UV irradiation, was not capable of interfering with live type I virus, and similarly for type II virus.

Another instance of a possible interference with poliovirus is the report of Chanock (46), who showed that in the case of NDV infection of monkey kidney cells, which produced cytopathogenic effects but very little virus, the surviving cells were at least temporarily resistant to all three types of poliovirus. In view of the fact that the fraction of cells unaffected by NDV was about 50%, and that monkey kidney cultures often contain about 50% of cells which are not immediately infected with poliovirus but which do become infected after 18-30 hours, the possibility exists that the NDV merely selected for cells physiologically unable to support any kind of viral infection at a given moment.

The studies of this report will develop some facets of intertypic interference with live poliovirus, and will show a complete lack of interference by UV-killed virus. It has also been shown by Dulbecco and Vogt (27) that strong interference occurs between genetic markers within type I viruses. Coinfection of monkey cells by h^r and h^s (heat resistant and heat sensitive respectively) led to heavy reduction of h^r genotype yields, even when the h^r type was adsorbed 30 minutes earlier. These results show

that interference with live polioviruses can be a function of genotype as well as of sequence of infection.

E. Phenotypic mixing.

The process wherein a cell infected by two virus strains yields progeny virus in which the phenotype of the particles does not correspond to the genotype, was discovered by Novick and Szilard (47) in studies involving serological markers of bacteriophages. It was named phenotypic mixing (PM) by Hershey et al. (48) and has since been shown to occur quite commonly for certain types of genetic markers (e.g., host range markers (49) in phage lambda). An analysis by Streisinger (50) of host range and serological specificities of coliphages T2 and T4 showed that mixedly infected bacteria release progeny viruses whose genotypes showed no correlation with their phenotypes for either or both of these characters and whose phenotypes themselves may be mixed. PM is usually attributed to the random association of protein coat material with genetic material from pools within the cell.

Studies on PM with animal viruses so far have been limited to serological specificities, and the "titer discrepancy" (TD) has been adopted as the yardstick (also called "anomalous neutralization"). A suspension to be tested for "mixed" particles is titered in the presence of antiserum against each of the original viral serotypes, and without antiserum. If the titers in sera are T_1 and T_2

and the titer without serum T, the TD is defined as

$$\frac{T - (T_1 + T_2)}{T} \quad ;$$

in other words, the fraction of the viral particles inactivated by both sera. The interpretation of a positive TD must rest on further information. In particular, one must determine whether the mixed particles remain mixed on further passage at low multiplicities (in which case a genetic mechanism is implicated), or whether they are unstable at any except quite high multiplicities (implying phenotypic mixing). It should be added that, in view of the data now available on control mixtures, a TD of less than 0.3 may not be significant. It is also important that antiserum containing anti-cell components not be carried over in low dilution into the assay system, since anti-cell sera can strongly inhibit the course of viral infections (51); in the work discussed below, studies on control mixtures rule out this possibility.

The earliest reports on the serological makeup of viruses from mixed infections are those of Burnet and Lind (52, 53). Using influenza strains, they found the TD to be very dependent upon the pH in the serum-inactivation tube, rising from 0.5 at pH 6, through 0.88 at pH 7, to 0.95 at pH 9. The mixed serotypes were unstable after two or three passages, even at high virus concentrations. The authors' interpretation hypothesized diploid particles which could segregate parental types, but PM could also have accounted

for the data. Despite the striking pH dependence, controls to rule out clumping were not performed. Similar results were reported by Fraser (54).

In the course of analyzing the yields of mixed infections with influenza viruses, Hirst, Gotlieb and Granoff (55, 56, 57) encountered a variety of particle types. In this case the TD was measured by the hemagglutination inhibition technique, rather than by neutralization. "Combination" forms (maximum TD of 0.84) were obtained in the allantois with concentrated inocula of several pairs of type A strains, and these could be maintained only with concentrated inoculations without reverting to the faster-growing parental type. After repeated serial passages, particles were obtained which could transmit mixed serotypes even at limiting dilutions. These results were later extended to mixed A and B strains (58). Unfortunately no data have appeared to eliminate the possibility of clumping, which is especially possible in the presence of filamentous forms of the virus; discussions in some of the papers merely hint that UV and ultracentrifuge studies eliminate aggregation.

Sprunt et al. (59) have reported the production of TD's up to 0.6 following mixed infection by types I and II polioviruses. The very large scatter of the titration data, however, and the lack of experimental details in the paper make it difficult to judge whether PM has occurred, as well as whether stable, genetically recombined virus

may have been produced. Instances in which high TD's were maintained or in which both parentals were given off in limiting dilution assays were reported without detail.*

Despite the uncertainties attaching to each of these observations, it does seem possible that PM occurs in animal virus systems. If this is true, then we may say that an hypothetical pool of protein exists, from which coat materials are withdrawn randomly to be arranged with RNA core material from another pool. Furthermore, the existence of this kind of interaction would suggest that other (e.g., genetic) viral interactions should be looked for following co-infection of a cell by two or more polioviruses.

F. Multiplicity reactivation.

The ability of bacteriophages killed by ultraviolet irradiation (UV) to initiate infection when multiply infect-

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* Studies of the serological classification of polio strains isolated from human sources have shown cases in which antigenic crossing seems to occur. Normally there is a complete lack of cross reaction between the three types of polioviruses, but Melnick (60) found that among 70 strains which were monotypic as tested by neutralizing antisera, a variety of cross reactions were revealed by the complement fixation test. Of 41 type I strains, 28 reacted with type II serum and two were tritypic. Similar results were obtained with type II and III strains. Wenner et al. (61) corroborated these findings. It is unlikely that the sera employed contained cross-reactive components, since many virus strains showed only one serotype. However, none of the strains were purified by plaque techniques, and some may have contained small amounts of heterologous genotypes; the extent of cross reactivity may then have been broadened by PM.

ing a cell was first reported by Luria in 1947 (62). He found that multiplicity reactivation (MR) will occur with phages T2, 4, 5 and 6, and that the T-even phages (T2, 4, 6) are capable of reactivating each other.

The first model for MR (62) held that each phage consisted of n units of equal UV sensitivity, that the phages on entering the cell dissociated into these units, and that the probability of the cell then producing live phage equaled the chance that at least one copy of each of the n units was present in an undamaged state within the cell. It was assumed that if such a full quorum of genetic material existed, the recombination leading to multiplying phage proceeded with an efficiency close to one. Analysis of the model predicted that the probability for a cell to release virus approached $m^n e^{-r}$ for large UV doses, where r is the UV dose to the whole phage in "hits" (defined as the dose giving e^{-1} survival), m is the average multiplicity of virus, and n is the number of units per phage. The back extrapolate of the exponential part of the experimental survival curve to r = 0 permitted an estimation of n. However, it was clear that systematic deviations towards a reduced probability to yield occurred as the multiplicity increased; this was attributed to inefficiencies in the reactivation process when many phage genomes were present.

Luria and Dulbecco examined the quantitative aspects of MR much more closely in a subsequent study (63). They showed that for UV doses up to 25 hits, the ability of the

phage to attach to susceptible cells is not impaired, and further demonstrated that the agent responsible for reactivation is indeed the phage itself. No reactivation was induced by a supernatant from an uninfected bacterial culture or by killed lysates from genetically heterologous phage (T1, 5 and 7 on T2-infected cells). Only T-even phages could reactivate T-even phages, and the MR of a given lysate did not vary after prolonged standing or purification by centrifugation. They also showed that the bacterium must adsorb more than one phage in order for MR to occur.

A more exact theoretical formulation of the probability for a cell to support reactivation was derived from the original model by adding the assumption that the multiplicity k and the hits r per phage or n-unit are Poisson distributed. The probability for the ith n-unit to survive irradiation is $e^{-r/n}$, and the probability for that unit to be killed is $1 - e^{-r/n}$. If k phages attack a cell, then the chance that all k of the ith units be killed is $(1 - e^{-r/n})^k$, so that the probability of at least one surviving is $1 - (1 - e^{-r/n})^k$. The chance that a complete complement of n units survives is therefore $(1 - (1 - e^{-r/n})^k)^n$. However, since the number k of viruses per cell is Poisson distributed, the probability of reactivation must be summed over each class of bacteria infected with k viruses; and since at least two such viruses must infect a cell, the result must be normalized to the fraction of multiple

infected cells $(1 - e^{-m} - me^{-m})$:

$$w = \frac{\sum_{k=2}^{\infty} \frac{m^k e^{-m}}{k!} \left(1 - (1 - e^{-r/n})^k\right)^n}{1 - (m + 1)e^{-m}}, \qquad (1)$$

where m is the average multiplicity. Thus the Luria-Dulbecco expression represents the multiple infected yielders divided by the multiple infected cells.

As r becomes large this function approaches the slope characteristic of phage survival without MR (low multiplicities) (e.g., log w = K - 0.4343r, where K is a constant depending on m and n).

Experimental values were obtained by dividing the observed fraction of yielders by $(1 - (m+1)e^{-m})$. Curves of log w against r (for selected values of m and n) and against m (for selected values of r and n) were laboriously calculated and compared with the experimentally obtained values. The scatter of experimental points around these plots was fairly great, but average best-fits could be determined by inspection. It was estimated that n was about 25 for T2, about 15 for T4, and about 5 for T1 (minimal values), in agreement with the decreasing UV sensitivities of the three viruses. However, serious deviations towards smaller values of w appeared for the larger multiplicities, and towards larger values of w for high UV doses. The former was again interpreted as inefficiency of reactivation.

The possibility remained that deviations in the UV dose dependence of w arose from a non-Poisson distribution of the phages among the bacteria. Because it may be assumed that a

cell adsorbs virus proportionally to its surface area, measurements of bacterial size were made. Deviations from the Poisson virus distribution were estimated not to be enough to account for the observed deviations. It was furthermore found that lysis-from-without and the limitations on the number of live phages that can grow together in a cell, could not conspire to give the deviations (63).

Several attempts have been made to explain deviations from the simple Luria-Dulbecco model. Dulbecco (64) repeated the experiments, adsorbing phage T2 in the dark to starved cells. This permits all the phage to adsorb, but infection does not proceed until the cells are diluted into broth, and thus exclusion at large multiplicities is prevented. Since the UV-killed phages can kill host cells, the average multiplicity was determined from the fraction of surviving cells. Furthermore, in parallel experiments photoreactivation was permitted, thus eliminating the photoreactivable damages and thereby possibly narrowing the spectrum of UV damage types and simplifying the system. Under these conditions the final slope of the dose dependence of w deviated strongly upwards from the expectation, either with or without photoreactivation. For a large phage such as T2, furthermore, the expectation is that linearity will not be reached until survivals down to 10^{-7} are reached, whereas Dulbecco found that linearity was reached much earlier. The back extrapolates also gave values of n much smaller than those obtained from estimates

based on log w-vs-m curves. At this point Dulbecco decided that the simple genetic recombination model was unsatisfactory.

Calculations were later made by Cairns and Watson (65) to see whether the cell size distribution reported by Dulbecco was indeed insufficient to explain the deviations. For a low multiplicity (0.5) the resultant curves fitted the data very well, lying well above the Luria-Dulbecco curve, but at a multiplicity of 4 the points fell into an intermediate position between the curves of Cairns-Watson and Luria-Dulbecco. They pointed out that the sample size (764) used by Dulbecco did not exclude large cells which could become important at multicomplex survivals of 10^{-6} to 10^{-7}.

An attempt to free E. coli cultures of large cells was carried out by Harm (66) in order to narrow the size distribution of the cells and thus perhaps bring the slope closer to the expected value. Cultures were passed through a fritted-glass filter, thus reducing the normal 2-5% of "snakes" (filamentous cells) to less than 10^{-5}. However, MR experiments with T4 using such filtered cultures gave results identical with those of Dulbecco (0.4 slope of theory), and an important role for giant cells should therefore be discounted. Furthermore, the typhi-phage of Bernstein (67) (see below), which gives MR in close agreement with Luria-Dulbecco theory, was used on cells with a size distribution close to that reported by Dulbecco. This

suggests that the deviation in T2 is not a function of aberrantly large cells. It should be pointed out that for the small n determined for this phage, these deviations would be small, as seen in the earlier observation (63) that the deviations diminish in the same order as the value of n. Therefore, the evidence now indicates, especially in the survival range 10^{-2} to 10^{-5}, that deviations in cell size and concomitant increased probabilities to adsorb virus, cannot account for the disagreement between theory and fact.

A theoretical study by Barricelli (68) may throw some light on the problem. He used a model in which the phage genome consisted of a large number (much greater than 25) of small genes totaling 89.5% of the genome, and a few large units totaling 10.5% of the genome. Thus the chance that all of the representatives of a small gene will be killed is minute compared to the chance that all of one of the large units die. For large UV doses, his model predicts a survival given by

$$\log w = n \log m - 0.4343 \left(\lambda r + m(1-\lambda) L^{m-1} r^{m}\right) \qquad (2)$$

where n is the number of <u>large</u> units (3 in this case), λ is the fraction of the genome in the large units (10.5%), L is the average length of a UV hit (0.009 of the genome, or 0.0034 with photoreactivation), and r, w and m are as before. With judicious choice of the many parameters, the data of Dulbecco (64) fit this expression very well, and indeed it is not surprising that there might be such large

units. Regions similar to the rII cistrons in T4 (69), which appear to have a critical function which must act before the formation of the vegetative pool in some hosts, could be such units, although these regions themselves could not since they affect only plaque size and not the presence or absence of plaques on E. coli B. A locus similar to that determining UV sensitivity in T2 and T4 (70), which does not grossly affect the amount of DNA in the phages, might also represent a type of large unit.

Probably the most basic question at present about the mechanism of MR is whether it is brought about by genetic recombination. One aspect of this problem is the fate of the irradiated genetic material: is it replicated to any extent before reactivation, and if so are the damaged portions included in mature phage? The results of Doermann, Chase and Stahl (71) demonstrated that damaged genetic material was not included in many mature phage. They examined the ability of a stock obtained from MR to contribute markers to the progeny virus when adsorbed to cells along with live phage. The live phage served as a carrier which could "rescue" (see below) markers from a phage which might contain damaged genetic material and thus be unable to multiply by itself. They found that in single bursts, if one of the MR-stock markers was present, all were, and therefore not many phages could have existed with a damaged marker. They also found that the plaque-forming and the bacterial-killing titer were equal, and concluded that the

MR stock did not contain many particles which were not genetically complete. Furthermore, since the burst size from cells supporting MR was not reduced even after massive UV doses to the virus, it seems unlikely that damaged material is replicated very extensively.

Some further experiments of Doermann, Chase and Stahl may throw light on genetic events in MR (71). When a cell receives one UV-inactivated phage plus one or more live phages, the progeny virus may contain markers from the UV'd phage (cross-reactivation or rescue). They discovered that unlinked markers were rescued independently of each other, and that the probability for a marker to be rescued decreased exponentially with the UV dose. Linked markers, on the other hand, simultaneously failed of rescue at a slower rate than the sum of the rates for the markers singly, and when one marker was not rescued, a linked marker suffered a reduced burst size. These results agree with the authors' hypothesis that rescue is the result of genetic recombination incorporating material from a damaged genome into an unirradiated genome. If, therefore, MR proceeds by means of a mechanism of mutual rescue among damaged genomes, a genetic mechanism seems likely.

Harm (72) has studied genetic events with phage killed by X-irradiation. He found that intracellular irradiation of phage T4 increased recombination in E. coli B/r by about a factor of 5 under conditions of MR, which further serves to link MR and recombination.

MR has been reported for several other UV-killed phages. Klebsiella pneumoniae B supports a phage Kp (73) which shows MR. Jacob reported that the temperate phage lambda growing in E. coli K12S does not undergo MR, but that virulent mutants of this phage do (74). MR with Vi-II type A phage of Salmonella typhi type A has been studied in some detail by Bernstein (67). This phage, which has a UV sensitivity 10 times smaller than T2, behaves in excellent agreement with the Luria-Dulbecco model: the data fit dose curves through 6 log cycles with an n value of 4. Luria and Dulbecco (63) found a weak MR in T1, corresponding to an n value less than 5. Stent (75) reported weak MR for phage P22, but none for P8. Coliphages T3 and T7 apparently do not give much MR, or if they do it is obscured by other features of the system (63, 75).

Bacteriophages may undergo MR after inactivation by any of several types of high-energy irradiations. Watson (76) reported that phages T2, 4 and 6 killed by hard X-irradiation are capable of weak MR. Assuming that only those dead phage which retained the ability to kill the bacterial host were involved in the reactivation, Watson calculated that the number of multi-complexes releasing virus reaches a maximum at a multiplicity of about 2. He attributed this apparent saturation to suppression by the great majority of phages unable to kill the bacterial cells. Weigle and Bertani (77) showed that T2 killed by hard X-rays or gamma

rays showed a weak MR when old cells (E. coli) were used, and that at least for gamma rays this was increased 10-fold with young cells. This difference may not have reflected differences in the efficiency of the reactivating mechanism itself, but rather the increased susceptibility of older cells to lysis-from-without. They failed to detect MR after inactivation by soft X-rays unless the phage-cell complexes were irradiated, in which case good MR resulted. A weaker MR could also be obtained with extracellularily X-rayed phage on irradiated cells.

Harm (66, 72) found that the reactivation behavior of T4 also depended on whether soft X-irradiation was applied intra- (IXR) or extracellularily (OXR). The former case gave MR as efficient as that following UV, the latter very weak MR. He found that the final slope for T4 MR was about 25% that of the free phage, similar to Dulbecco's finding with UV'd T2 (64), and suggested (in a manner formally the same as Barricelli, above) that about 75% of the damages were nearly always reactivated (small units) and 25% never reactivated (large units).

It has not been possible to obtain MR in bacteriophages following P32 "suicide" (killing by disintegration of incorporated P32) (78).

A report by Mutsaars (79) dealing with phage N growing in E. coli W indicated that a mixture of formalized and UV killed phage gave 10-30 times more infective centers (yielders) than the sum of the titers obtained by titering

the two stocks independently at the partial multiplicities they had in the mixture. He claimed a weak MR with either UV'd or formalized phage alone, but the data are confusing on this point. The reactivation seemed to be polarized; that is, with a given UV dose to one suspension of phage and a variable amount of formalin killing to another, about a constant number of plaques appeared; whereas phage killed by a given formalin dose were reactivated by UV'd phage in a manner strikingly dependent on the UV dose. It was reported that UV-killed T2 was also capable of reactivating formalized T2, 4 and 6. This would be difficult to reconcile with Hershey's finding that formalin-killed phage is largely incapable of injecting its DNA (80).

Luria and Dulbecco failed to detect MR in phages T2 and T6 inactivated by nitrogen mustard (63).

Little information is available concerning biochemical events in MR systems. Luria and Dulbecco reported that the latent period is somewhat extended, 26 minutes instead of 21 for T2 and 30 instead of 25 for T4 (63). In view of the fact that a great many UV quanta--about 10^4 (63)--are absorbed for each lethal quantum, one may expect that non-lethal damages occur which could slow down phage multiplication. Cohen reported that the beginning of DNA synthesis in T2 MR is delayed about 25 minutes--the latent period in this case was considerably longer than 26 minutes, perhaps because of differences in growth medium and temperature--but intracellular phage appear within 1-2 minutes after

phage DNA, although normally DNA synthesis begins very quickly (1-5 min.) and mature phage do not appear for about 10 minutes (81). This strongly suggests that a considerable part of the reactivation process must occur before the vegetative pool of phage DNA begins to form, but that other processes occurring in the first 10 minutes of infection by normal phage (such as synthesis of phage protein) can proceed. Purine and pyrimidine synthesis in T2 MR begins immediately upon infection (81).

In summary, then, we may say that phage MR systems fall into two classes with respect to their agreement with the Luria-Dulbecco model: those which exhibit a dose dependence slope equal to that of the free virus, and those which do not. In the latter case one may explain the deviations by saying that part of the genome is efficiently reactivated and part is not. With some radiations (high-energy ionizing radiations) it is necessary to irradiate the phage intracellularly. Although not critically established, genetic recombination as the basis for MR is implicated both by experiments and by the applicability of the Luria-Dulbecco model itself.

We now turn to the question of MR in animal viruses, which is of particular importance in those viruses whose genetic material appears to be RNA. MR has been claimed to occur in influenza virus growing in the chick egg. Henle and Liu (82) irradiated the PR8 and Lee strains (A and B strains respectively) to survivals of 10^{-2} as measured by

end-point titration, and compared infection by such stocks with infection by unirradiated virus diluted 10^{-2}. They found that the lag period before hemagglutinin appeared in the membranes and allantoic fluid was 4 to 5 hours shorter with the UV'd inoculum than with stock diluted to the same level of live virus; such a shortening of the lag period occurs also if the multiplicity of infection of live virus is increased. The interpretation of their findings as MR is subject to the objection that the hundred-fold excess of killed virus in the UV'd stock could block loss by reinfection of the first virus released in the egg. Nevertheless, Henle and Liu felt that the phenomenon is due to MR. They treated membrane suspensions with receptor-destroying enzyme (RDE), thus hoping to prevent differences in readsorption, and found that hemagglutinin still appeared earlier with UV'd virus. However, no data or methods were given to support this statement, and one cannot know if their treatment was indeed adequate.

Growth curves on eggs infected with UV'd or with diluted virus also showed a shortening of the lag period for infectious virus release with the irradiated virus, while virus in the membranes appeared with the same lag period in both cases. This appears to contradict the data obtained with hemagglutinin determinations. The "reactivation" was of viral origin, in that "membrane suspensions" (not defined) did not reactivate the virus in vitro. The authors were able to eliminate the selection of fast-growing mutants

as causing the shortened lag period. A similar reactivation was achieved with heat-killed virus (3-4 hours shorter lag period). Since heat-killed bacteriophages do not show MR, more doubt is cast upon the validity of their interpretation.

This problem was further investigated by Cairns (83), using the MEL strain of influenza virus. Virus heated to 56° for 30 minutes was apparently nearly completely killed: no hemagglutinin increase was produced in eggs in a period of 12 hours. When, however, a small amount of active virus was mixed with a heat-killed stock, a great shortening of the lag period was observed compared with infection with the same amount of live virus in the absence of UV-killed virus, again suggesting that the effect was due to a block to reinfection. This facilitation for virus release into egg fluids was mimicked by RDE (3-fold decrease in lag period, as compared with a 5-fold decrease with heated virus), but RDE had no effect on the appearance of membrane virus.

An enhancement of the yield of virus from chick eggs infected with live or heat-killed influenza viruses in the presence of cortisone has been reported by Kilbourne (84). In both eggs and mice, the final yield of Lee and PR8 strains is increased about 10-fold, even though the survival of mice is enhanced if cortisone is administered at the peak of viral invasion rather than before infection. Infection by virus inactivated by exposure to 24° for 12 days, either

followed or not by active virus, gave an increased yield (50X) following cortisone treatment; this held both for infective titer and hemagglutinin. Similar to the findings of Henle and Liu, much higher titers were obtained with killed virus plus cortisone, than with live virus diluted to the same level of infectivity. Membrane virus paralleled released virus, implying that the stimulation was not an artifact due to receptor-destroying action of the killed virus. Prior incubation of a mixture of cortisone and heat-killed virus was not sufficient to cause an increased yield. Kilbourne favored an MR interpretation of his results. However, a more likely mechanism would be that the cortisone induced a change in the cell resulting in the failure of the killed virus to interfere with infection by live virus, thus opening to infection many cells which would otherwise have remained resistant. Since the total yield was affected, and not the lag period as in the study of Henle and Liu, it seems unlikely that an early block to reinfection was responsible for the results.

MATERIALS AND METHODS

A. Solutions and media.

Bottled Puritas distilled water was used in all cases. Phosphate-buffered saline (PBS) and trypsin solution were prepared according to Dulbecco and Vogt (2).

Lactalbumin hydrolysate with yeast extract (LY): (A) NaCl, 7.175 gm; KCl, 0.4 gm; $CaCl_2$, 0.2 gm; $MgSO_4 \cdot 7H_2O$, 0.2 gm; $NaH_2PO_4 \cdot H_2O$, 0.175 gm; dextrose, 4.5 gm; streptomycin, 0.1 gm; penicillin, 500,000 units; stock solution of antimycotic (n-butyl-p-hydroxybenzonate, 0.02%), 1 ml; water, 400 ml; (B) lactalbumin hydrolysate (Nutritional Biochemicals Corp.), 5 gm; $NaHCO_3$, 2.2 gm; phenol red (1% solution), 1.5 ml; water, 250 ml; (C) yeast extract (Difco), 1 gm; water, 50 ml. Autoclave (C). Heat (B) slightly to clear and add 0.75 ml N/10 NaOH. Mix all three and bring to 1000 ml with water. Flush with CO_2 until slightly acid and sterilize by filtration.

Double-strength lactalbumin hydrolysate (2XLA(5/4)): same as LY except yeast extract and antimycotic omitted, 2.0 gm bovine albumin added to (A), 2.6 gm sodium bicarbonate instead of 2.2 gm used in (B), and double amounts of all other ingredients used.

Double-strength agar: (A) washed (2) Difco agar, 3.6 gm; water, 100 ml; (B) neutral red, 0.010 gm; water, 100 ml. Dissolve (A) in 95-100° water bath, heat (B) and add to (A).

Agar was prepared for use by melting double-strength agar, cooling to 44°, and adding an equal volume of 2XLA(5/4).

Versenate: .02% disodium versenate in Ca- and Mg-free PBS.

Horse, ox, human and monkey sera were centrifuged free of cells, sterilized by filtration and stored frozen.

B. Virus stocks.

Poliovirus type I stocks (Brunhilde strain) were prepared by infecting monolayer cultures of monkey kidney cells at multiplicities of 3-20, adding LY plus 10% monkey serum free of viricidal activity, allowing nearly complete cell destruction (24-48 hours) to occur, centrifuging the lysates free of cell debris, and freezing. Since freezing and thawing result in an appreciable drop in plaque titer, only singly frozen stocks were used in most experiments. Type II (Yale-SK) and type III (Leon) stocks were similarly prepared. Type I stocks usually contained 1.5-4 X 10^8 PFU/ml, and type II and III stocks about 2 X 10^7/ml.

Concentrated type II and type III stocks could be prepared by repeated application of a lysate to fresh monolayers, giving up to 2-4 X 10^8 ml in four cycles. Concentrated type I stocks were prepared by centrifuging stocks for 15 minutes at 10,000 rpm in the Spinco Preparative Ultracentrifuge in the #40 head, decanting the supernatant and recentrifuging it for two hours at 40,000 rpm. The glassy pellet was broken up in 1 ml or less of LY plus 10%

monkey serum, and allowed to resuspend overnight; the stock was briefly centrifuged at low speed and then frozen or used immediately. Titers of about 1-4 x 10^9 could be prepared in this manner, and the total recovery was about 50-80%.

Anomalous titers often developed in stocks kept for more than about a week at 4°, being either higher or lower than expected. Slight precipitates often formed. Lowered titers could be partially restored by incubating at 37° for 30 minutes. Because of this apparent clumping effect, this method of storage was abandoned.

C. Cells.

Monkey kidney monolayers were prepared according to Dulbecco and Vogt (2) except that they were grown under LA with 6% Ox serum. Plates and bottles were used on the sixth day to prepare stocks or titer virus. Four- or five-day plates were used for monkey kidney cell suspensions.

Most experiments involving infection of cell suspensions, including all MR experiments, were performed with the S3 sub-strain of the HeLa strain (85). S3 cells are also hosts for NDV, and were originally obtained from the laboratory of Dr. T. T. Puck. The cells were grown in bottles in LY containing 15% human and 15% horse serum; 0.275% bicarbonate was used for petri dish cultures, which were kept in the CO_2 incubator. S3 cells for experiments

were taken from 3-5 day petri dishes. In transfers or infection experiments, plates or bottles were washed once in versenate, and then kept in versenate for about 30 minutes. On gentle pipetting, suspensions containing almost exclusively single cells were obtained; for infection experiments, appropriate volumes of cell suspensions were centrifuged at low speed, sedimenting the cells but leaving nearly all the cell debris behind. This insures against loss of virus by adsorption to cell debris.

D. Antisera.

Powerful antisera (k values up to 400/min) against all three poliovirus types were kindly supplied by Dr. Marguerite Vogt (rabbit and monkey sera) and by the National Foundation for Infantile Paralysis (monkey sera). No cross reactivity resided in these sera at concentrations well above those used in the experiments. Normal rabbit and monkey sera free of viricidal activity were used at equivalent dilutions as controls. Poliovirus treated with antiserum usually shows a variable residual "serum-resistant" fraction of 5×10^{-3}--5×10^{-5}.

E. Irradiation.

The undiluted virus suspensions were placed in petri dishes in layers less than 5 mm thick at a distance of about 15 inches from two partially-shielded Westinghouse germicidal lamps (window area ca 10 x 10 cm), and agitated

intermittently during irradiation. Despite the UV absorption of undiluted medium, the fraction of survivors decreased exponentially with no perceptible shoulder, indicating that the lethal damages were randomly distributed among the viruses. This result agrees with the earlier report of Dulbecco and Vogt that polioviruses are killed in a one-hit manner (29).

In some instances stocks were thawed for irradiation and then re-frozen for storage. In these cases, some loss of titer resulted from the freeze-thaw process, and unirradiated samples were not similarly thawed and re-frozen. To determine the "original titer" of such irradiated preparations the log of titer as a function of dose was back-extrapolated linearly for the zero-dose using least squares. That this is a reliable method is indicated by the fact that such preparations behaved in an identical manner to stocks irradiated before the first freezing. UV irradiation of the medium, followed by the addition of live virus, did not affect the live virus titer.

F. General experimental conditions.

Nearly all the experiments involving infection of suspended cells were performed as follows. Single-cell suspensions were prepared as above and counted in a Neubauer counting chamber. Generally well over 100 cells were counted, so that the standard error of such counts was 10% or less. A volume containing 3.0×10^6 cells was

centrifuged. The pellet was then resuspended in adsorption tubes in 0.40 ml of virus suspension, and maintained for one hour at 37° with frequent agitation. The pH of the adsorption tubes was held between 6.9 and 7.6 by placing them in the CO_2 incubator when necessary, and then stoppering the tubes. After adsorption, the suspension was centrifuged and the supernatant titered to determine adsorption. Usually between 80% and 95% of the virus was adsorbed. After removing the remaining supernatant, excess virus was removed and clumps formed in the adsorption tube were dispersed by suspending the cells for about 10 minutes in 5 ml of warm versenate, and recentrifuging. The pellet was taken up in 1.5 ml of versenate and 0.5 ml was removed to a separate tube, counted in a Neubauer counting chamber, and versenate added to give a suspension containing 2×10^5 cells/ml. The suspension was then diluted in versenate in tubes prepared previously, and plated. It was not necessary to titer the dilution tube supernatants, since unadsorbed virus in dilution tubes composed less than 5% of the plaque count.

Infected cells were plated as follows. An underlay of 1.0 ml agar was put on monkey kidney monolayers. This gives a more level surface resulting in equal distribution of plaques, and permits the rapid plating of infected cells. Aliquots of infected cells were added to 2.5 ml agar tubes and immediately poured onto the plates. No further overlay

was used. Plates were usually counted after three days.

It was discovered that free virus could be adsorbed onto monkey kidney monolayers directly from the agar overlay with an efficiency equal to or slightly greater than the alternative method of adsorption from a small volume of PBS directly on the cells, and the following plating techniques were employed. Monolayers were washed once in about 3 ml of warm PBS and the fluid well removed; 0.1 to 0.4 ml of virus suspension was added to 2.5 ml agar tubes at 44° and these were poured onto the plates immediately. After about half an hour an additional 2.5 ml of agar overlay was added. Plates were counted after three to four days.

G. Notes on alternative experimental conditions.

The conditions for handling and infecting cells described above were arrived at after examining many factors. The following is a summary of alternate conditions examined.

Suspension of cells by trypsin gives excessive clumps with monkey kidney cells. Dispersion by trypsin is satisfactory with S3 cultures, but much more cell debris appears than with versenate. The fraction of plaque-forming cells is the same after brief versenate or trypsin treatment. It does not drop during prolonged tenure in versenate (up to an hour or more), but does decrease in trypsin.

S3 and monkey kidney cells are sensitive to strong pipetting; about 5% lose their infectability after each up-

down passage through a 5 ml pipette with a narrow hole, although repeated gentle pipetting through a 1-2 mm hole does not thus affect them. These cells are resistant to several minutes of centrifugation in the International Clinical Centrifuge at about 1000 rpm.

The fraction of infected cells which form plaques (about 50% under the above standard conditions) is not appreciably affected by raising the multiplicity from 4 to 100, nor by infecting on the plate rather than in suspension (although larger volumes of virus suspension are required on the plate). The plaque-forming fraction is, however, a function of the adsorption time in adsorption tubes, being 20-30% after 30 minutes and reaching 80-90% after 75 to 120 minutes, even though not appreciably more uninfected cells adsorb live virus after a short time. This suggests that some early step of infection occurs more rapidly in some cells than in others and is irreversibly blocked by the treatment of the cells between adsorption and plating. Results after more than 60 minutes of adsorption are quite variable, since in some cases plaque-forming cells later decline in numbers.

Adsorption was sometimes carried out in paraffin-lined tubes to avoid cells sticking to the tube walls. However, an equal or slightly smaller fraction of cells could be infected in paraffin tubes compared to glass tubes. Poliovirus does not adsorb either to glass or paraffin tubes, nor is it heat inactivated, during one hour at 37°. Further-

more, both free virus and infected cells can stand in agar tubes at 44° for several minutes without detectable inactivation.

Cells should be washed and diluted out in versenate rather than in calcium- and magnesium-free PBS. Tenure in the latter causes a loss in numbers of cells due to sticking to the glass. Washed infected cells can remain for at least 30 minutes in versenate without ill effects. A single versenate wash (5 ml on 3 million cells) is sufficient to lower free virus to less than 5% of infected cells at input multiplicities of 50 or less.

Virus thawed and then passed through another freeze-thaw cycle, either in dry ice--acetone or slowly in the freezer, irreversibly loses about 30-40% of its titer.

EXPERIMENTS AND RESULTS

Unless otherwise indicated, experiments were performed with type I virus and S3 cells.

A. Determination of virus titer.

In MR experiments it is important to have a well-defined value for the virus titer. The titer V_c of an unirradiated stock was obtained as follows. In each experiment, virus was diluted by D to give a low multiplicity (ca. 0.02) in one of the adsorption tubes (3 million cells, 0.4 ml. virus), and the fraction of plaque-forming cells was determined. This fraction was divided by the fraction of plaque-forming cells at high multiplicities. Together with the measured adsorption A (from plaque titers before and after infection), the titer V_c of the virus on S3 cells in suspension was determined from

$$\frac{V_c \times 4 \times 10^{-1} \times A}{D \times 3 \times 10^{6}} = I, \qquad (3)$$

where I is the normalized fraction of infected cells. This value was usually 2 to 4 times greater than V_p, the titer of the virus as determined by plaque assay on monkey kidney monolayers.

The virus survival S after irradiation was measured by dividing the titer V* after irradiation by the titer V_p of the original stock. In most cases S was so small that the virus titer of an irradiated stock could not be determined

on suspended S3 cells, after diluting the stock so that multiple infection could not occur. Therefore, the titer of the live virus in a UV'd stock was determined by multiplying the V_c titer of the unirradiated stock by the survival S as measured on monkey kidney monolayers. Errors from the differences in V_c and V_p do not occur here, because S is determined from the ratio of titers on monkey kidney monolayers. In fact, values of S for a given irradiated stock were constant even when the efficiencies of plating on monkey kidney monolayers varied from one preparation to another. In a few experiments involving small UV doses, the irradiated suspension was titered at low multiplicities, and V_c for the survivors agreed with the value as determined by the above method.

A variety of factors such as the presence of cell clumps in the adsorption tubes and of a large excess of "originally uninfective particles" in a virus stock (see MR controls) might have a depressing effect on infection, giving different values of V_c at different virus dilutions. To examine this possibility, the fractions of infected cells were determined over several serial 10-fold dilutions of virus. At multiplicities below about 0.1, the dilution factor D should be inversely related to the normalized fraction I of infected cells. The results of two such experiments are presented in Figure 1, where it is seen that the fraction of infected cells falls off with slope 1 at increasing dilutions.

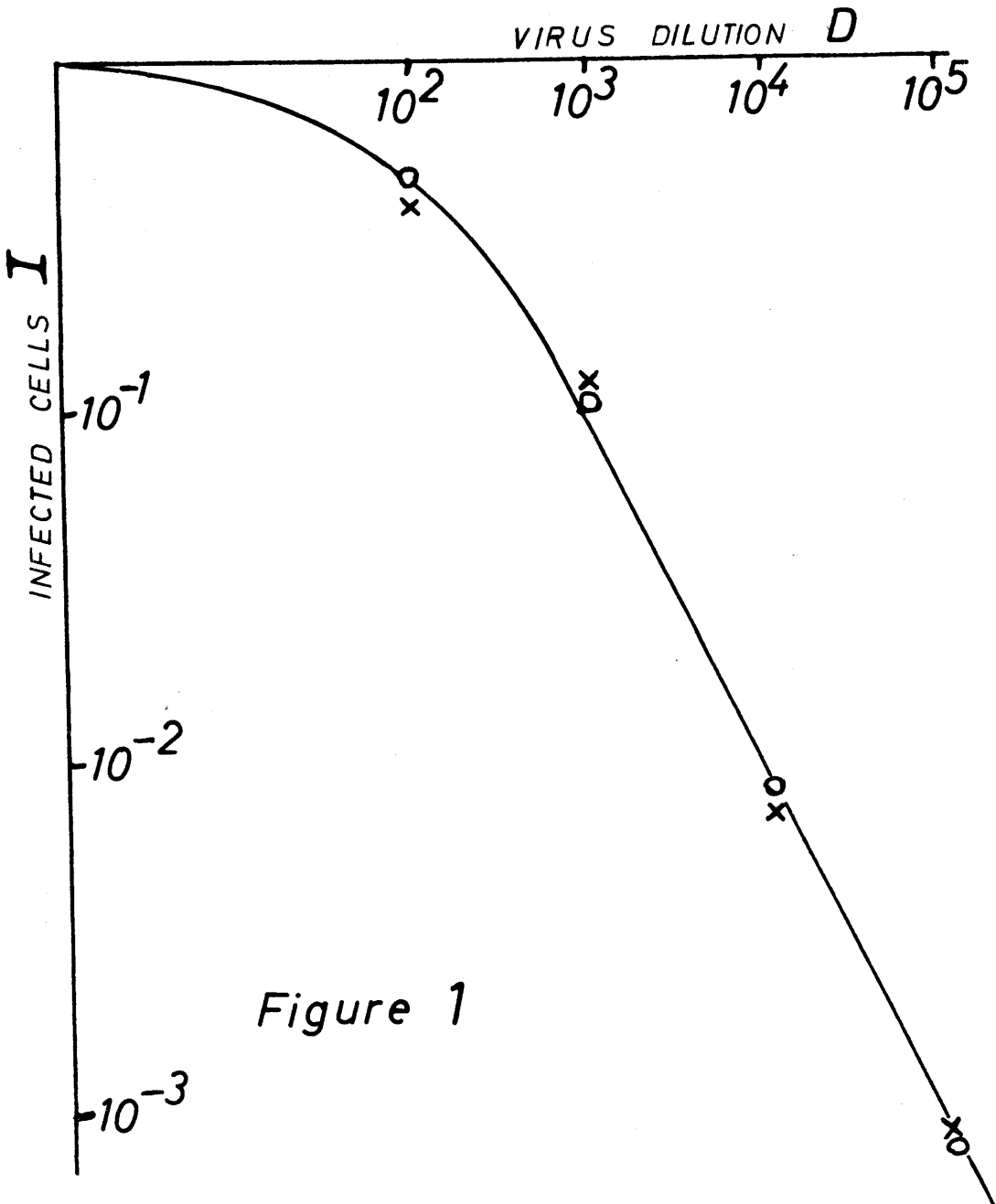


Figure 1

Figure 1. The normalized fraction I of infected cells is plotted against serial 10-fold dilutions D of virus. The linear part of the curve has unit slope.

The question arose in MR experiments whether UV-killed virus was adsorbed differently from live virus. To examine this point, the serum-blocking power of killed virus was measured and the disappearance of serum-blocking power by adsorption to S3 cells was followed. One series of antiserum tubes was set up containing 0.4 ml of anti-type I serum (1/1500) and 0.1 ml of various dilutions of virus (V_c = 8 x 10^8 ml) irradiated to a survival of about 4 x 10^{-4}. A second series contained antiserum and 0.1 ml of the supernatant from adsorption tubes. The adsorption tubes contained 0.4 ml killed virus suspension and 3 x 10^6 cells (standard conditions of infection), and had been incubated for one hour at 37°. After allowing two hours for the virus in the antiserum tubes to adsorb as much antiserum as possible (sufficient time as determined in earlier experiments), 0.1 ml of undiluted live virus was added to each tube and incubation was continued for another two hours. At that time the contents of the antiserum tubes were diluted for titration. The first series of antiserum tubes served as a calibration of serum-blocking power for known amounts of killed virus, and the second gave the corresponding values for residual killed virus remaining in the adsorption tubes. Residual virus values obtained from the antiserum tubes were compared with the adsorption as determined directly from adsorption tubes (plaque titers), either with killed or live virus. The results appear in Figure 2 and Table I. Column 4 in Table I shows that the PFU titers of the live virus in

Figure 2. Serum blocking by UV-inactivated virus. Antiserum diluted 1/1500 was equilibrated with UV'd virus of various concentrations V_k, and the remaining serum titer measured by its ability to inactivate live virus. The virus survival V is relative to survival in tubes with no UV'd virus. A least squares treatment of the data from two experiments (circles and squares) gave $V = 4.48 V_k + 1.06$ (straight line).

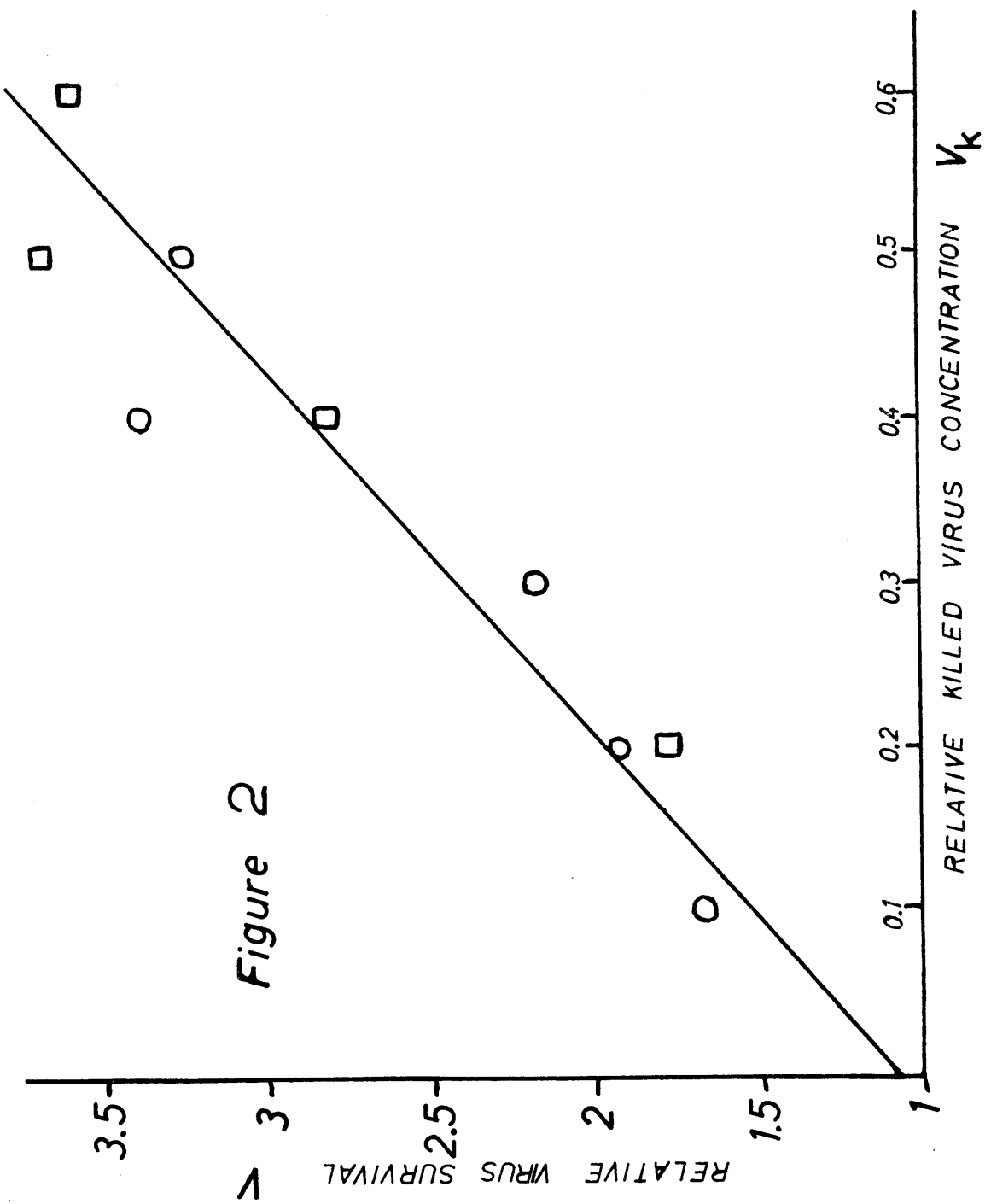

RELATIVE VIRUS SURVIVAL
λ
3.5
3
2.5
2
1.5
1
0.1
0.2
0.3
0.4
0.5
0.6
RELATIVE KILLED VIRUS CONCENTRATION V_k

Figure 2

Table I

Adsorption of Irradiated Virus

Exp. No.	Adsorption Tube	Adsorption Tube Virus	Adsorption in Adsorption Tubes: By PFU Ratios	Relative Virus Survival[a]	By Serum Blocking[b]
1	1	live	0.70		
	2		0.76		
	3		0.73		
	Ave.		0.73		
	4	killed	0.77	2.19	0.75
	5		0.78	3.42	0.47
	6		0.72	3.44	0.47
	Ave.		0.76		0.56
2	1	live	0.79		
	2		0.76		
	3		0.88		
	Ave.		0.81		
	4	killed	0.75	2.50	0.68
	5		0.79	2.79	0.61
	6		0.71	3.00	0.57
	Ave		0.75		0.62
	Ave.		0.76		0.59

[a] Survival of live virus in antiserum tubes compared to tubes receiving no UV'd virus.

[b] Determined from Figure 2. The relative survival V of virus in antiserum tubes was used to determine the relative killed virus concentration V_k from the graph. This value of V_k is the amount of killed virus remaining in the adsorption tubes relative to the input, so that the adsorption is $1 - V_k$.

irradiated and unirradiated suspensions are identically adsorbed. Using the least squares equation from Figure 2 and the relative virus survival data for antiserum tube virus (column 5), the adsorption of the serum-blocking particles was calculated (column 6). The killed virus was clearly adsorbed to nearly the same extent (78%) as the live virus.

B. Infection of cells.

It is of cardinal importance for the determination of accurate multiplicities to know whether a virus has the same probability of infecting a cell at all multiplicities. If the multiplicity as determined from V_c and the dilution D is m_{ads}, and the probability for a virus disappearing from the adsorption tube to infect a cell is p at higher multiplicities, then the effective multiplicity will be pm_{ads}. It was previously determined that virus is not adsorbed to the walls of the infection tube to a detectable extent, and that heat inactivation at 37° over the course of the infection time (one hour) is very small. On the assumption that the virus is Poisson distributed among the cells, then, the fraction of uninfected cells will be given by $(1 - I) = e^{-pm_{ads}}$. In experiments in which various dilutions of virus were adsorbed to cells under standard conditions, the fraction I of plaque-forming cells was determined (normalized to the fraction of cells forming plaques at large multiplicities). A plot of log (1 - I)

against m_{ads} appears in Figure 3, from which the apparent value of p was calculated to be about 0.7 (e.g., log (.516) = -.4343p).

The assumption that the virus particles are Poisson distributed among the cells presupposes that all cells have the same probability to adsorb a particle. However, a cell suspension is composed of cells of varying sizes. In order to test whether variations in cell size could account for the fact that the measured value of p obtained above is less than unity, the size distribution of suspended S3 cells was determined with an ocular micrometer. The following distribution (diameter in arbitrary units versus frequency) was obtained: $2\frac{1}{2}$ units, 0.009; 3, 0.078; $3\frac{1}{2}$, 0.129; 4, 0.302; $4\frac{1}{2}$, 0.198; 5, 0.164; $5\frac{1}{2}$, 0.069; 6, 0.035; $6\frac{1}{2}$, 0.017. This distribution is only roughly Gaussian, with mean diameter 4.32 units and σ = 1.31 units. Assuming that the probability for a virus to adsorb is proportional to the cell surface area, the expected fraction of uninfected cells as a function of adsorption multiplicity was calculated using the above distribution. The resulting curve (Figure 1) was closer to the experimental points. This indicates that about half of the difference between the observed value of p and the expected value, unity, can be accounted for by the size distribution of the S3 cells, and further suggests that all of the difference may be a result of a distribution of probabilities for cells to adsorb a virus particle.

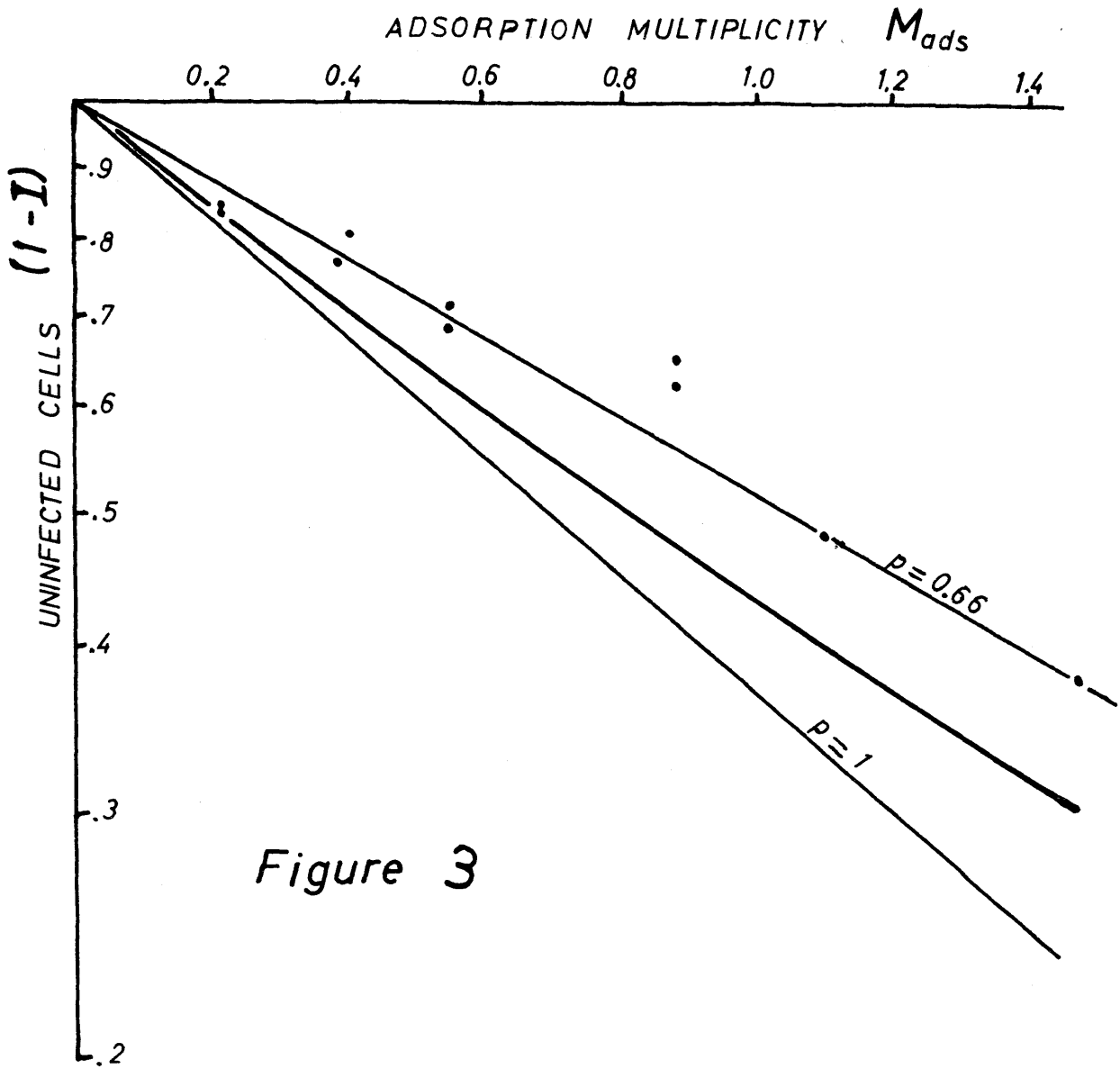


Figure 3

Figure 3. Infection of S3 cells. The fraction I of infected cells was determined for adsorption multiplicities m_{ads}. The slope of the uppermost straight line corresponds to a probability of 0.66 for a virus particle disappearing from the supernatant to infect a cell. The middle curve is the expected value of (1 - I) taking into account the distribution of cell sizes. The bottom line is $e^{-m_{ads}}$.

C. Interference by UV-inactivated virus.

In a variety of experiments a large multiplicity (10-30) of UV-inactivated virus ($S = 10^{-4}$--10^{-3}) of one type was adsorbed to cells and, after a variable time, replaced by live virus of the same or a heterologous type (multiplicity = 10-20). Controls in which cells were treated identically except that they did not receive live virus demonstrated that the UV'd virus preparation never infected (either by UV survivors or MR) more than 1% of the number of cells infected by the challenge dose of live virus. The number of cells releasing virus was compared with the number of yielders in tubes which received live virus only. In these experiments the fraction of yielders was <u>not</u> normalized to the fraction of cells forming plaques at large multiplicities of live virus. The data from several such experiments are reported in Table II. Table III is a check-list of all such experiments conducted. It is clear that there was no detectable interference in any of the cell--dead virus--live virus combinations tested within the time allowed between infections.

D. Interference by live virus.

Several experiments were conducted using live virus as an interfering agent against an heterotypic virus. Cells were infected with one virus at large multiplicities (10-40) for 20-40 minutes, the virus removed, and an excess of a second heterotypic virus added with or without an interval

Table II

Parallel infection tubes were set up, one with UV-killed virus and the other with LY. After various times the cells were centrifuged down and resuspended in live virus. "Total plaques" refers to equal dilutions within each experiment.

Exp. No.	Cell Type	Interfering Virus Type	Ads. Time, Min.	Superinfecting Virus Type	Ads. Time, Min.	Total Plaques
1	monkey kidney	I	30	I	30	41
			60		30	53
			90		30	57
			120		30	53
		--	--		30	55
		--	--		120	38
2	monkey kidney S3	III	40	III	40	43
		--	--		40	41
		III	40		40	27
		--	--		40	24
3	monkey kidney	II	20	I	20	51
		--	--		20	48
		II	20	III	20	48
		--	--		20	46
4	human amnion	I	30	II	30	78
		--	--		30	65
		I	30	III	30	80
		--	--		30	71

Table III

Non-Interference by UV-inactivated Poliovirus

No depression of the fraction of cells yielding the superinfecting virus type was observed in any case. Cell Types: MK, monkey kidney; S3, NDV-sensitive HeLa sub-strain; AV3 and F1, human amnion strains.

Interfering Virus Type (UV-inactivated)	Superinfecting Live Virus Type		
	I	II	III
I	MK, S3, AV3	MK, F1	MK, F1
II	MK	MK	MK
III	MK	MK	MK, S3

for the first virus to become further established. These cells were then washed and plated under three conditions: with antiserum against the interfering virus; with antiserum against the superinfecting virus; and without antiserum. Appropriate controls indicated that the antiserum concentrations employed were sufficient to prevent the appearance of 99.9% of the plaques from cells infected by homologous virus.

The results of representative experiments are given in Table IV, and Table V summarizes the results of all experiments using live virus as an interfering agent. In these experiments the fractions of yielders were not normalized to the fraction of cells forming plaques at large multiplicities of live virus.

Live virus appears to act as an interfering agent, but the possibility that phenotypic mixing (see next Section) may have complicated the results must be considered. If a fraction of cells were to release both virus types, but the virus from these cells were to be completely phenotypically mixed, then this virus could be killed by the heterologous serum, giving rise to an apparent reduction in the numbers of cells releasing a given virus type. Two facts argue against this process being an important contribution to the observed interference. First, the extent of phenotypic mixing under similar conditions was at most about 40% of the virus particles; and second, the observed interference increased as the time interval between the two infections

Table IV

Parallel infection tubes were set up, an experimental one with an excess of interfering virus and a control with LY. Monkey kidney cells were used in these three experiments. After various times the cells were centrifuged and resuspended in an excess of live virus of another type. (In Exp. 3, line 3 the cells were further incubated 30 minutes in LY before the superinfecting virus was added.) Numbers in parentheses after the per cents of plaque-forming cells are the actual plaque counts. "% Interference" refers to the reduction in the fraction of cells releasing the superinfecting virus type in the experimental tube compared with the yielders in the control tube (e.g., Exp. 3, first line shows 29% type I yielders after interference by type III, and fourth line shows 89.5% type I yielders without interference; hence 1 - 0.29/0.895 = 68%). "% Depression" refers to the reduction in the total fraction of yielders among cells infected with both viruses, compared to the fraction of cells releasing the superinfecting virus type in the absence of interference (e.g., Exp. 3, second line, 1 - 0.51/0.895 = 43%).

Exp. No.	Interfering Virus Type	Ads. Time, Min.	Superinfecting Virus Type	Ads. Time, Min.	Per cent of Cells Forming Plaques in: No Serum	Anti-I	Anti-II	Anti-III	% Interference	% Depression
1	I	20	II	30	106(31)	11(26)			87	-22
	--	--		30	84(42)	90(45)				
2	II	40	I	40	19(32)		17.5(20)		68	65
	--	--		40	55(69)					
	II	40	III	40	22.5(44)		5(22)		90	54
	--	--		40	49(59)					
3	III	30	I	30	28(41)	13(15)		29(43)	68	64
		60		30	51(64)	24(21)		15(22)	83	43
		30, LY30		30	48(60)	18(16)		32(44)	64	46
	--	--		30	89.5(116)	0.1(0)				
	--	--	III	60	50(62)			0.1(0)		

Table V

Interference by Live Poliovirus

Experiments were done with monkey kidney cells. % Interference (%I) is the reduction in cells yielding the superinfecting virus type, caused by pre-infection with interfering type; % Depression (%D) is the reduction of total yielders in interference tubes compared with the yielders of the second virus type without interference. These figures depend considerably upon the adsorption time of the first virus, so that the values in the table are approximate.

Interfering Virus Type	Superinfecting Virus Type					
	I		II		III	
	%I	%D	%I	%D	%I	%D
I	--	--	87	-22	--	--
II	68	65	--	--	90	54
III	64-83	43-64	53	--	--	--

increased, whereas the largest measured phenotypic mixing occurred after simultaneous infection. It therefore seems unlikely that phenotypic mixing was an important complicating factor in these experiments.

Two points can be inferred from the data on interference. First, no viral type seems to be a more powerful interfering agent than another, and interference is never complete, ranging between 64% and 90% for various combinations of virus types and adsorption times. This may mean that some cells are not susceptible to the first virus, but are to the second; or that some cells are capable of releasing both virus types; or that the second virus may actually displace the first by interference.

Second, there is a depression in the total fraction of yielders. Usually only about half as many cells yielded any virus at all when successively infected with two different virus types, as yield when infected with one only. This depression is not obviously correlated with interference. It is quite different from the "depressor effect" in bacteriophage, in which the average burst size is decreased by about half but the fraction of yielders is unaffected (36). The depression might arise from a process in which each virus blocks some vital step in the reproduction of the other, giving rise to what we may call co-interference. The depression is not a function of phenotypic mixing or other serum action, since it is measured in the absence of all sera.

E. Phenotypic mixing.

The suggestion that cells infected with two poliovirus types may release antigenically mixed virus particles (see Introduction) prompted an investigation of mass lysates from such cell populations. Monkey kidney cells and S3 "giants" induced by X-irradiation (85) were infected on petri dishes with mixtures of polioviruses at large multiplicities. After adsorption the cells were washed several times, overlaid with LY plus 16% monkey serum, and allowed to release virus for 24 hours. The mass lysates thus obtained were titered after incubation with antisera against each of the input types, and without antiserum. The results are presented in Table VI, where "titer discrepancies" (TD) (see Section E of the Introduction) are calculated. Control mixtures (lines 1 and 2) showed insignificant TD's. Positive TD's were produced in most cases in mass lysates; the moderately large negative value in line 4 throws some doubt on the results, but the actual plaque counts in this case are low, and the TD may well be in error. It is not surprising that the S3 giants more often produced positive TD's: their large surface probably permits more rapid virus adsorption, and thus minimizes interference. Giants singly distributed in droplets under paraffin oil often produced huge amounts of virus. Among 15 yielders titered, 3 produced about 5000 PFU, 2 about 7000, and one produced 15,000! These yields were determined on monkey kidney monolayers, and may actually have been several times larger (see Section A of Experiments

Table VI

Production of Titer Discrepancies After Mixed Heterotypic Poliovirus Infection

Input Virus	Cells	Titer Without Serum[a]	Titer[a] in Antiserum Against:[b] I	II	III	TD
I-II	None	2.7 (68)	0.17 (42)	2.4 (59)		4%
I-II		1.3 (33)	0.16 (40)	1.2 (29)		-4%
II-III	monkey kidney	1.64 (143)		0.45 (99)	0.52 (118)	39%
I-III		1.6 (32)	0.10 (39)		1.8 (73)	-19%
I-II		1.16 (74)	0.17 (118)	1.09 (89)		-9%
II-III	S3 giants	0.14 (29)		0.01 (17)	0.08 (100)	36%
I-III		2.6 (13)	0.1 (1)		1.4 (14)	42%
I-II		4.4 (22)	0.1 (1)	2.9 (131)		32%

[a] Titers are x 10^8 for controls and monkey kidney cells, x 10^6 for S3 giants.

[b] Figures in parentheses are numbers of plaques counted.

and Results).

The possible role of virus clumping in the formation of positive TD's, and of unclumping in the production of negative ones, has not been investigated. Various conditions of incubation and inactivation, as well as the nature of UV killing of such lysates, should be investigated. The manner in which TD formation depends upon input multiplicity and time of harvesting should also be looked into. Until more information is available about this system it is not safe to conclude that these TD's do indeed reflect the presence of a fraction of single virus particles susceptible to neutralization by two different antisera.

F. Multiplicity reactivation.

In a large number of experiments with UV-killed virus and S3 cells a consistently greater number of infective centers arose than could be accounted for by input residual live virus. This was true also of monkey kidney cells, and to a lesser extent of AV_3 (human amnion) cells. The number of such cells was always small, however, being generally only 2 to 5 times that expected from live virus. It was therefore necessary to rule out a variety of factors which might conspire to give the excess, before safely concluding that MR was in fact occurring. These controls will be taken up first.

Controls

1. Photoreactivation. Large multiplicities of virus

killed to various levels were adsorbed to cells in dim yellow light, conditions known not to induce photoreactivation in other systems (86). Aliquots of the cell suspensions were then exposed to the amount of light usually encountered under standard operating conditions (about 30 minutes at about 15 inches from a 15 watt Westinghouse daylight fluorescent bulb), or to very intense light known to induce maximum photoreactivation in other systems (86) (one hour at 6 inches from two 40 watt Sylvania daylight fluorescent bulbs). In both cases the white light source was fluorescent bulbs. The results appear in Table VII-A. The effect of light on unadsorbed virus stocks was also followed over the course of a week in refrigerated stocks; see Table VII-B. No photoreactivation was detected in either case, thus paralleling Baluda's finding that photoreactivation does not occur in NDV (35).

2. Effect of heat-killed virus. The conditions under which poliovirus stocks are prepared and used may lead to limited heat inactivation. It was therefore necessary to look for any effects of heat-killed particles on infection under standard conditions with live and UV-killed viruses at high and low multiplicities, with and without an excess of heat-killed virus. Heat-killed virus was prepared by holding stocks grown in low-cysteine medium (cysteine causing phenotypic heat-resistance (28)) at 50° for 20 minutes. The results appear in Table VIII. Where the virus survival in the heated stock was high enough to approach the input

Table VII

A. Photoreactivation of cell-virus complexes

Virus Survival	Light to Complexes	Total Plaques	Fraction of Infected Cells	
			Observed	Expected[a]
5×10^{-4} ($m = 30$)	dim yellow	125	0.030	0.015
	standard conditions	110	0.027	0.015
4×10^{-4} ($m = 60$)	dim yellow	79	0.088	0.024
	intense white	76	0.085	0.024

[a] From residual live virus; excess of observed over expected due to MR.

B. Stability of Irradiated Virus

Virus	Storage (see text)	Plaque Count[a]	
		Original	After One Week
Live	4°, white light	111	111
UV'd	4°, white light	59	61
UV'd	4°, dark	59	66

[a] Live virus dilution factor 2.5×10^6; UV'd virus dilution factor 1.25×10^3.

Table VIII

Cell	Approximate Virus Multiplicity: Live Virus	UV'd Virus	Heated Virus	Total Plaques	Normalized Yielders	
					Obs.	Calc.
AV3	10			47	1	
	10		12	39	0.84	
	0.026[a]	10		19	0.22[b]	
	0.026[a]	10	12	22	0.25[b]	
S3	10			91	1	1
	10		10	98	1.1	1
	0.05[a]	20		168	0.19[b]	--
	0.07[a]	20	7	214	0.24[b]	0.22[d]
	0.12			211	0.12	--
	0.13[c]		7	192	0.11	0.13
	10			96	1	1
	10		10	84	0.9	1
	0.05[a]	20		113	0.24[b]	--
	0.07[a]	20	7	128	0.27[b]	0.26[d]
	0.15			143	0.15	--
	0.17[c]		7	167	0.17	0.17

[a] Survivors in UV'd or heated virus.

[b] Excess over live multiplicity due to MR.

[c] Live virus plus survivors in heated virus.

[d] Sum of observed in previous line plus survivors in heated virus.

of the live unheated virus, the correction due to this factor is included in the table ("Calc."). In no case did the presence of heat-killed virus raise the fraction of cells releasing virus; there may occasionally have been a slight depression in the fraction of yielders.

3. Effect of other non-infectious particles. Poliovirus stocks grown in other laboratories have generally shown particle/PFU ratios of about 30 (see Introduction). The rate of heat inactivation during the preparation and purification of stocks is too low to account for this ratio. It may represent either low plating efficiencies, killing by agents other than heat, or originally uninfectious particles. The last category could conceivably consist of particles lacking a full complement of RNA, or carrying lethal mutations. It was necessary to investigate the role of non-infectious particles (NIP) and plating efficiencies in MR. Serial dilutions of virus, it will be remembered from Section A, at sufficient dilutions gave fractions of infected cells proportional to the number of infectious particles, so that an effect of even a fairly large number of NIP on live virus seems unlikely: such particles would be diluted out in these experiments, and the probability that a cell is infected with one live virus plus one or more NIP eventually becomes negligible even if the NIP are in great excess in the original stock. However, possible effects of NIP might be destroyed by UV at a lesser rate than live virus is killed (due, for instance, to a lesser

RNA content); this possibility can be tested by adding UV-killed virus to diluted live virus, and to virus exposed to a smaller UV dose. It was also necessary to check the effect of the unirradiated NIP in a diluted live virus suspension on a UV'd suspension. Results of these experiments are presented in Tables IX and X, from which one may conclude that NIP do not increase the infectivity of live or UV'd virus, and perhaps may depress it.

4. Effect of cell lysates. It is important to establish that the MR is indeed a function of the virus particle and not of non-viral components produced either in infected or uninfected cell lysates. Since virus purified by differential centrifugation gave the same amount of MR as original stocks, any agent in the lysates mimicking or facilitating the action of virus would have to sediment with the virus. To examine the action of lysates from uninfected cells, cells were infected under conditions allowing MR, with and without the addition of lysates prepared either by freezing and thawing cells, or by killing cells with UV. Another control series was run testing the addition of heterologous virus stocks which had first been neutralized by the addition of an excess of non-cross-reacting antiserum. These experiments are presented in Tables XI and XII, where it is seen that the titers of live or UV'd virus preparations are not increased by these additions, but may be slightly depressed.

Table IX

Viruses irradiated to survivals S were adsorbed to cells and the normalized fractions of yielders I were determined. "V_c in V*" is the survival times the multiplicity, and is less than I because of MR.

Approximate Multiplicity				
V* (S = 1.7×10^{-4})	V* (S = 10^{-6})	V_c in V*	Total Plaques	Fraction of Yielders I
47		.008	30	.026
47	23	.008	16	.014

Table X

Irradiated and/or unirradiated viruses were adsorbed to cells and the normalized fractions of yielders I were determined. "V_c in V*" is the survival times the multiplicity, and may be less than I because of MR.

Approximate Multiplicity				Fraction of Yielders	
V	V*	V_c in V*	Total Plaques	I	Expected
.18			66	.18	
	41	.015	52	.025	
.18	41	.015	111	.22	.21[a]
.18	14	.005	84	.17	.18[b]
.018			87	.018	
.018	20	.007	133	.043	.030[c]
.018	7	.002	57	.014	.021[d]
.017			171	.017	
.017	7	.0045	223	.022	.025[e]
.0094			32	.0094	
	25	.008	30	.026	
.0094	25	.008	13	.015	.035[f]
.0094	23	.0002	27	.008	.010[g]

[a] Sum of I in line 1 and line 2.

[b] Sum of I in line 1 and I/3 from line 2 (multiplicity of UV'd virus being a third that of line 2).

[c] Sum of I in line 5 and I/2 from line 2.

[d] Sum of I in line 5 and I/7 from line 2.

[e] Sum of I in line 8 and I/3 from line 2.

[f] Sum of I in line 10 and line 11.

[g] Sum of I in line 10 and I/40 in line 11.

Table XI

Effects of Non-Viral Cell Lysates

Approximate Multiplicity					
V	V*	V_c in V*	Lysate Added[a]	Total Plaques	Fraction of Yielders I
4.7			none	32	1
4.7			C*	41	.96
4.7			CFT	33	.77
.0094			none	32	.0094
.0094			C*	30	.0088
.0094			CFT	22	.0064
	25	.0079	none	30	.026
	25	.0079	C*	19	.017
	25	.0079	CFT	24	.028

[a] Lysates were prepared from plates containing approximately 10 million cells. C*: lysate from plate given 10 minutes UV (see Methods), then overlayed for two days with 10 ml LY medium containing 10% monkey serum. CFT: lysate from plate frozen and thawed twice (medium as in C*). The lysates were centrifuged to clear them of cell debris and stored frozen before use.

Table XII

Effects of Antiserum-Inactivated Heterotypic Lysate

Approximate Multiplicity						Total	Fraction of Yielders	
V[a]	V*[a]	V_c in V*[a,b]	V-Ab[c]	V_c in V-Ab[c]	Het. Type[c]	Plaques	I	Expected
25						122	1	
25			55	.07	II	162	1.33	
	20	.097				171	.21	
	20	.097	55	.07	II	218	.27	.28[d]
38						102	1	
38			62		II	89	.87	
38			108		III	88	.85	
.039						158	.039	
.039			72	.012	II	136	.038	.051[e]
	77	.010				43	.032	
	77	.010	41	.022	II	68	.057	.054[f]
.008						64	.008	
.008			10-20	.0026	III	34	.007	.010[g]
	12	.007				47	.014	
	12	.007	10-20	.0026	III	60	.023	.017[h]

[a] Type I virus.

[b] Determined from product of multiplicity and survival S.

[c] Heterotypic virus inactivated by specific antiserum; "V_c in V-Ab" determined from product of multiplicity and survival.

[d] Sum of I in line 3 and V_c in V-Ab in line 4.

[e] Sum of I in line 8 and V_c in V-Ab in line 9.

[f] Sum of I in line 10 and V_c in V-Ab in line 11.

[g] Sum of I in line 12 and V_c in V-Ab in line 13.

[h] Sum of I in line 14 and V_c in V-Ab in line 15.

5. Rate of plaque appearance. Although unlikely, it is conceivable that there exists a fraction of virus particles which normally would appear as plaques long after the count on a plate has leveled off (87). If such hypothetical particles exist, their time of appearance might be foreshortened so as to place them within the 'normal' plaque count, either by the direct action of the irradiation or by interaction with other irradiated particles in the virus suspension. The rate of plaque appearance was followed for cells infected with live and irradiated virus, and the fraction of the final count was plotted against time (Figure 4). There is a lag of about one hour between the curves for S3 cells supporting MR as compared with cells infected by live virus. No difference exists between UV-survivors and unirradiated virus plated directly on monkey kidney cells. Furthermore, even after 8 days no late plaques appeared on plates seeded with S3 cells infected with normal or irradiated virus, or with free live or UV'd virus.

6. Lack of MR with heated virus. Henle and Liu (80) reported that heat-killed as well as irradiated NDV exhibited 'MR' in chick eggs. In addition to the probable role of a block to readsorption of the early yield (see Section F of Introduction), the lack of MR with heat-killed phage made it unlikely that their observations were truly MR. In order to determine whether poliovirus killed by

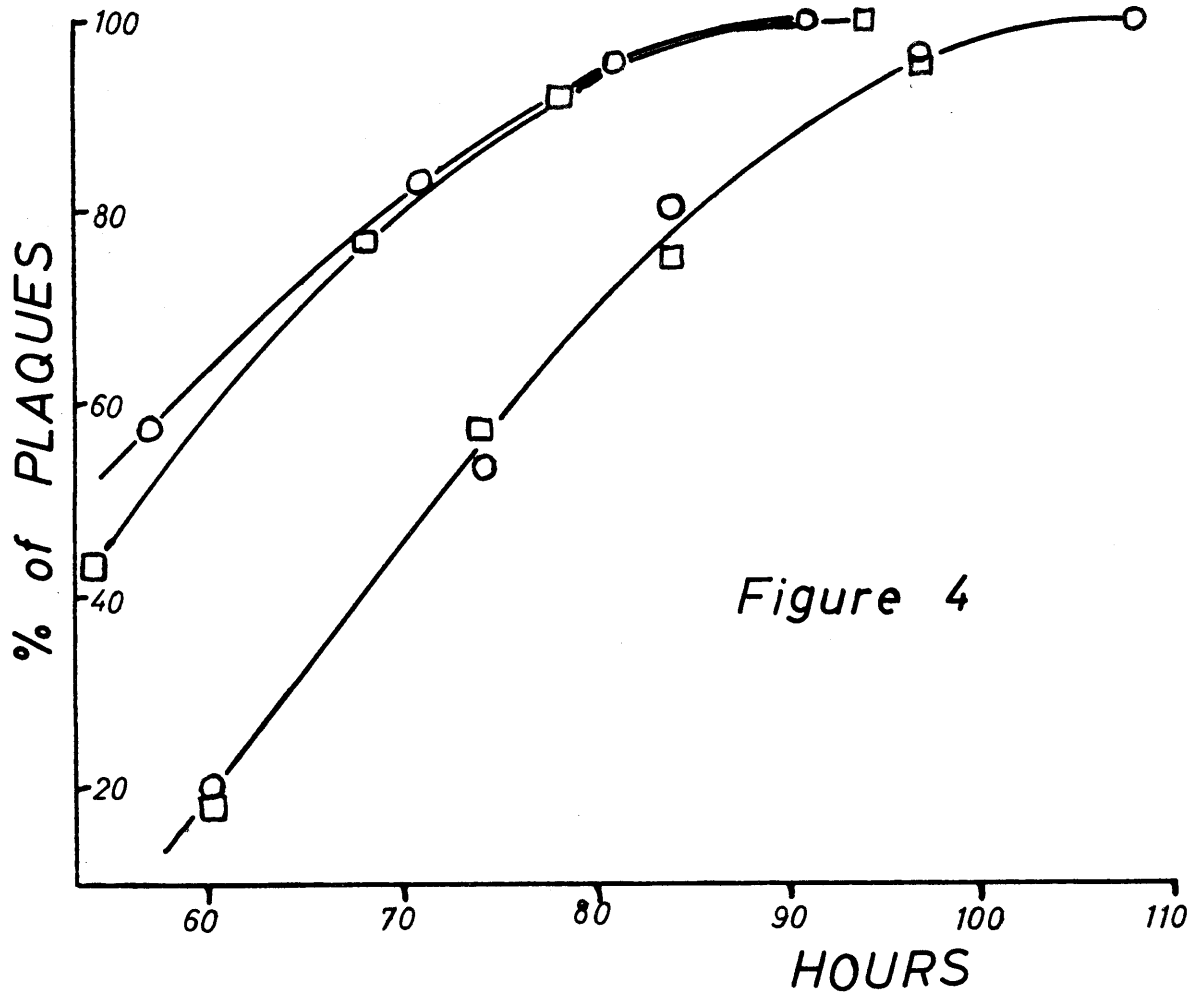


Figure 4

Figure 4. Fraction of plaques appearing is plotted against time. The upper two curves refer to plates seeded with infected S3 cells, the bottom curve to plates seeded with free virus. Circles, live virus; squares, irradiated virus. 69% of the S3 cells in the uppermost curve released virus because of MR. The 100% point for free virus is the same for UV'd and unirradiated virus.

heat could give MR, cells were infected with large multiplicities of killed virus and the fraction of yielders was compared with that following infection at low multiplicities. No excess of yielders was observed (Table XIII).

Table XIII

Virus	Approximate Multiplicity Dead	Live	Total Plaques	Fraction of Yielders I
Type I heated 20 min. at 60°	68	.06	ca. 320	.06
	0.7	.0006	ca. 256	.0006
	68	.06	44	.064
	0.7	.0006	43	.00063

Multiplicity Reactivation

Standard conditions for these experiments have been described in Materials and Methods. A proportion of yielders I is determined for each infection tube; this value has been normalized by dividing the actual plaque titer by that of a control at high multiplicity of live virus. A measure of the amount of MR is that of Luria and Dulbecco (63): the function w is defined as the fraction of multiply infected yielders divided by the fraction of multiply infected cells. If the average multiplicity of killed particles is m, and these are randomly distributed among the cells (see Discussion), then the fraction of cells receiving less than two killed particles is $e^{-m} + me^{-m}$; thus the fraction of multiply infected cells is $1 - (m + 1)e^{-m}$. (In all cases m was large enough so that I could be taken as the normalized fraction of multiply infected yielders). Therefore the function calculated from the experimental data is

$$w = \frac{I}{1 - (m+1)e^{-m}} \quad . \qquad (4)$$

The determination of m depends upon V_c (see Section A), the titer of the virus under the conditions prevailing in the MR tubes. In tubes containing 3 million cells in 0.4 ml virus diluted by a factor D,

$$m = \frac{V_c \times 0.4 \times A}{D \times 3 \times 10^6} \quad , \qquad (5)$$

where A is the fraction of adsorbed virus. In this case V_c refers to the original, unirradiated stock.

For convenience in comparing different UV'd stocks the dose r is given in hits, one hit being the dose killing a virus suspension to e^{-1} (37%) survival.

1. UV-inactivation of type I poliovirus. Figure 5 shows the inactivation of virus as a function of UV dose in minutes. The surviving fraction S, determined from titers on monkey kidney monolayers, shows a regular one-hit dependence on the UV dose through five log cycles, in agreement with the earlier report of Dulbecco and Vogt (29).

2. Multiplicity dependence. In the absence of MR, and at survivals sufficiently low so that cells are rarely infected by more than one live virus particle, the fraction of infected cells should be proportional to the multiplicity ($I = km$). The most simple demonstration of MR, then, is to compare I/m with m. Any rise in I/m with increasing multiplicity would be a demonstration of MR. The results of several experiments are thus plotted in Figure 6, where it is clear that I/m increases as the multiplicity jumps from single to multiple infection. As the multiplicity increases further, I/m tends to pass through a maximum and a minimum, but the data are not sufficiently accurate to establish this fluctuation as a regular event.

The data from a representative experiment appear in Table XIV, together with the calculations of w and m outlined above. The results of several experiments in which the

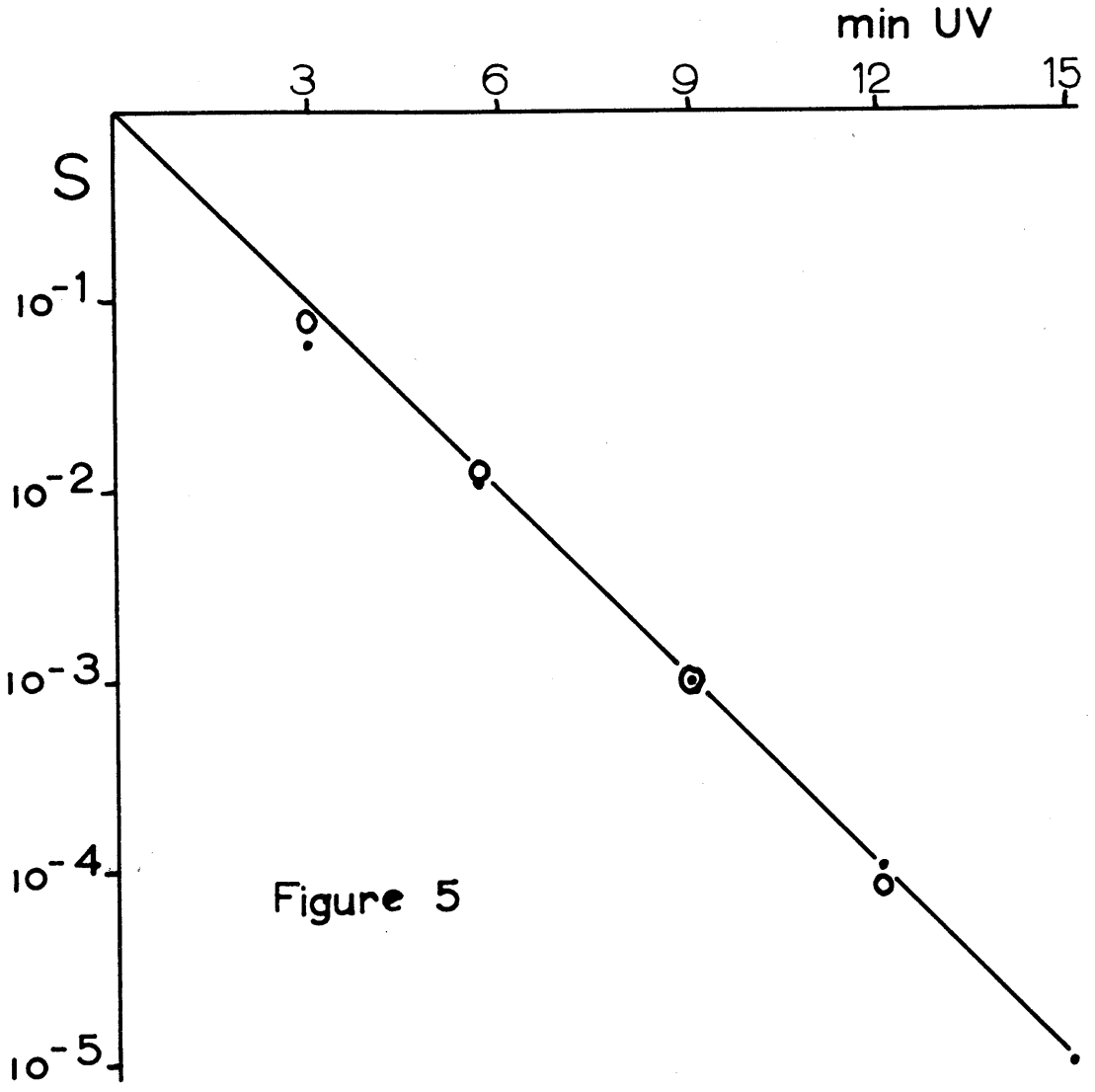


Figure 5. Survival S of type I poliovirus plotted against UV dose in minutes. S is determined from virus titers on monkey kidney monolayers.

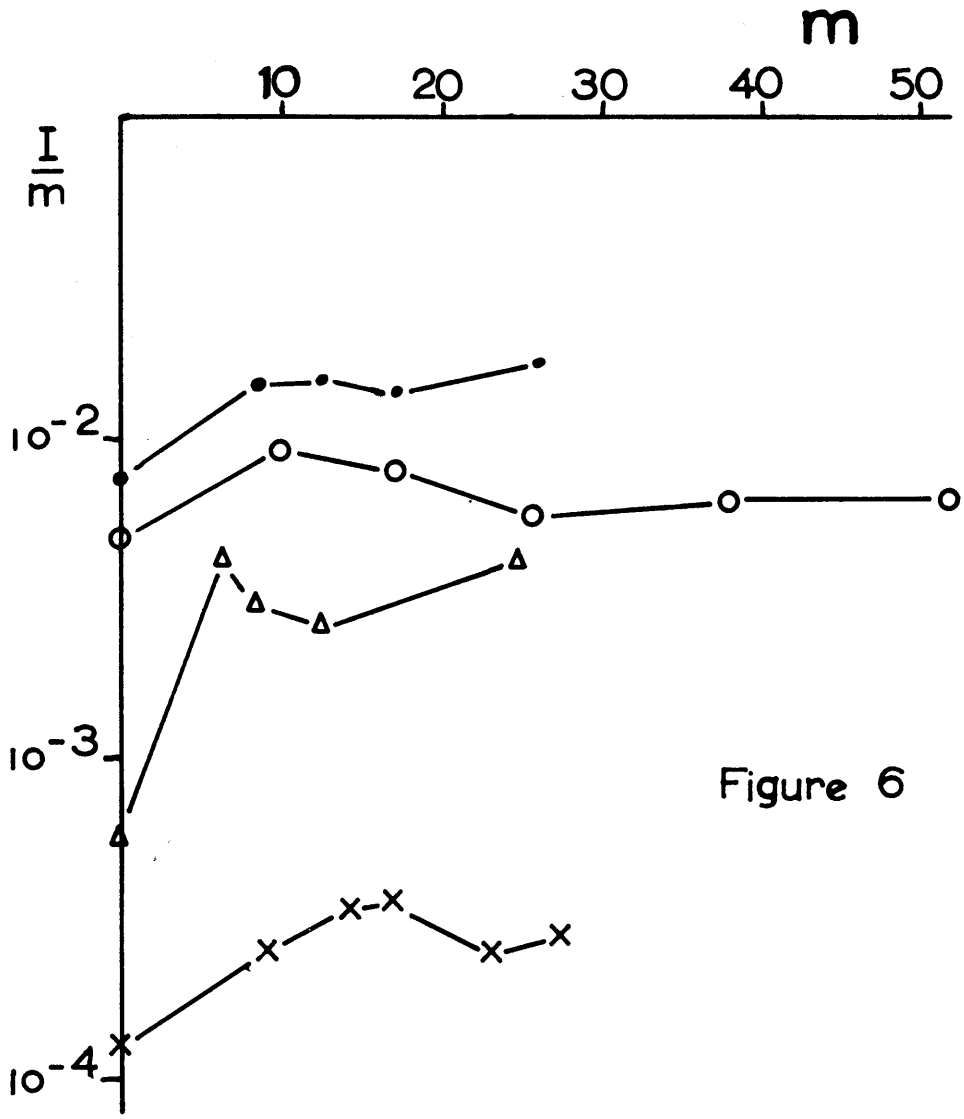


Figure 6

Figure 6. Multiplicity reactivation with type I poliovirus. I is the normalized fraction of yielders, m is the multiplicity. The points at zero multiplicity are the virus titers at very low multiplicity. Dots, zero-multiplicity m = .09, S = 9.6 x 10^{-3} on monkey kidney monolayers (MKM), S = 8.6 x 10^{-3} on suspended S3 cells. Circles, zero-multiplicity m = .013, S = 2.3 x 10^{-3} (MKM). Triangles, zero-multiplicity m = .013, S = 5.3 x 10^{-4} (MKM). Crosses, zero-multiplicity m = .014, S = 8.9 x 10^{-5} (MKM).

Table XIV

Dilution Factor	m[a]	I[b] or w[c]	Plaques Counted
1	25.2	0.42	195
1.5	16.8	0.25	146
2	12.6	0.20	185
3	8.4	0.126	196
280	.089	(I = .00077)	270

[a] Titers and multiplicity calculated from equations 3 and 5 using 0.4 ml virus suspension and 3 million cells (70.7% of virus adsorbed):

$$\frac{V_c \times 0.4 \times 0.707}{2 \times 10^3 \times 3 \times 10^6} = 1.26 \times 10^{-2}, \quad V_c = 2.67 \times 10^8$$

$$m = \frac{2.67 \times 10^8 \times 0.4 \times 0.707}{D \times 3 \times 10^6} = \frac{25.2}{D}$$

$$\frac{V_c^* \times 0.4 \times 0.707}{280 \times 3 \times 10^6} = 7.7 \times 10^{-4}, \quad V_c^* = 2.29 \times 10^6$$

$$S(V_c^*/V_c) = 8.6 \times 10^{-3} \quad (4.7 \text{ hits})$$

[b] I = normalized fraction of infected cells (59% infected at live virus multiplicity of about 13). Survival $(V_p^*/V_p) = 9.6 \times 10^{-3}$ (4.6 hits).

[c] I = w for these values of m.

multiplicity was varied while holding the UV dose constant are summarized in Figure 7. The intercepts of the dose dependence curves (see next section) indicated that the parameter n in the Luria-Dulbecco expression for MR is small here, lying between 1 and 2. For comparison with theory, therefore, curves for appropriate values of n and r have been included in Figure 7.

Examination of Figure 7 shows that w tends to level off sooner than do the theoretical curves as m becomes large. At multiplicities of about 40-80 a form of saturation appears to set in. This indicates that the efficiency of the system is to some extent a function of multiplicity, the Luria-Dulbecco model being inadequate for large m.

3. Dose dependence. The data and calculations from a representative experiment appear in Table XV. The results of all experiments in which the dose was varied while holding m constant are summarized in Table XVI and Figure 8.

Table XVI shows that the function w(r) decreases with a slope indistinguishable from the theoretical expectation of -0.4343 r. The average slope (mean of experiments 1-5) is within 1% of the slope of the virus assayed at low multiplicities. Equation 1 in its asymptotic form for large r was used to calculate the parameter n (see Table XVI), which was found to average 1.6 with some scatter. See the Discussion on the significance of this parameter.

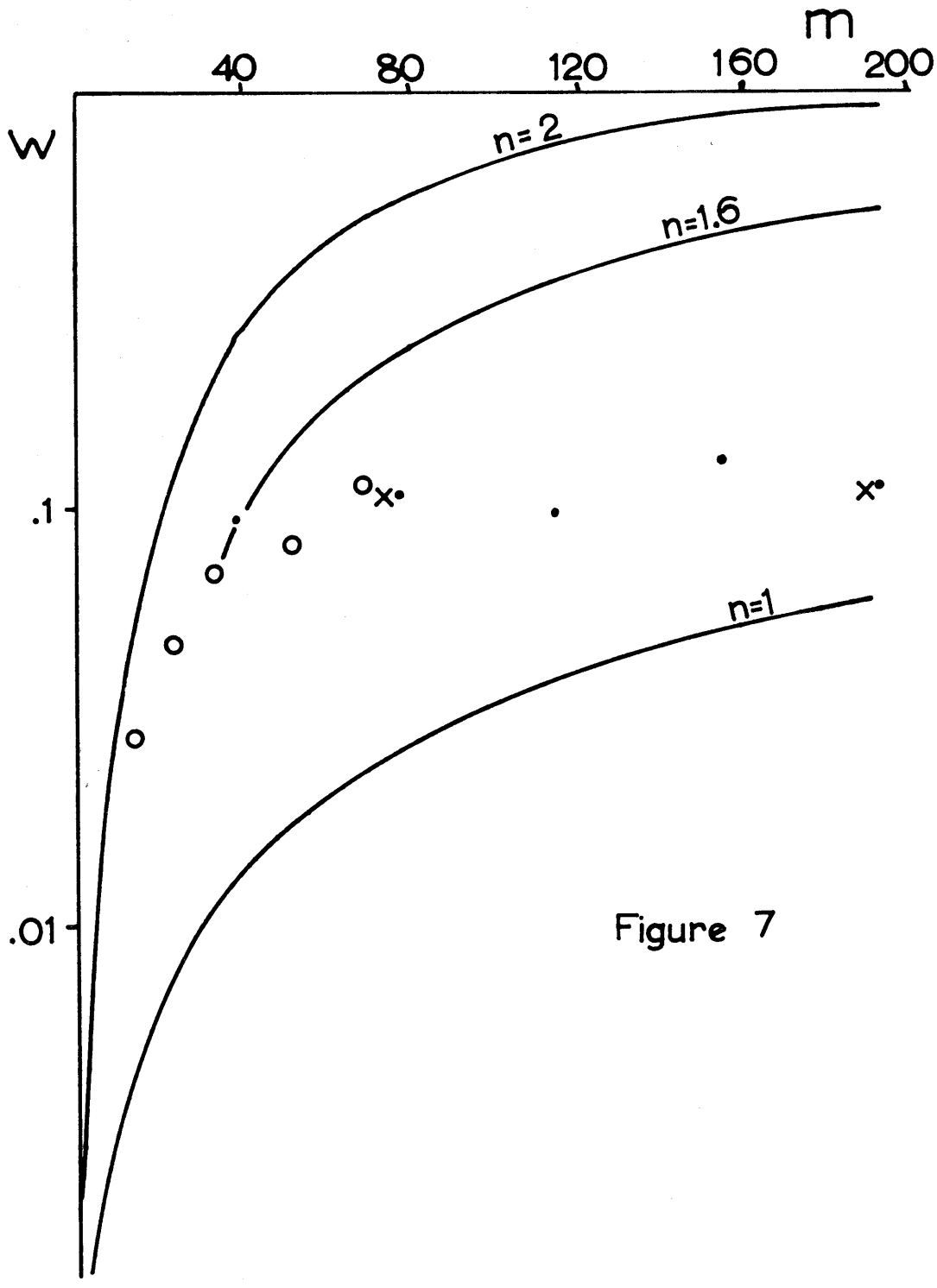


Figure 7

Figure 7. Fraction of yielders w (= I) plotted against multiplicity m. Crosses, 7.4 hits; circles, 7.5 hits; dots, 7.8 hits. Curves are equation 1 for r = 8 and values of n as indicated.

Table XV

Min UV	S[a]	r[b]	I[c]	w[d]
0	1	0	1	1
4	4.7×10^{-2}	3.1	2.6×10^{-1}	3.7×10^{-1}
6	1.4×10^{-2}	4.3	7.1×10^{-2}	1.0×10^{-1}
8	3.5×10^{-3}	5.7	2.8×10^{-2}	3.9×10^{-2}
10	7.3×10^{-4}	7.2	7.4×10^{-3}	1.0×10^{-2}
14	3.7×10^{-5}	10.2	1.3×10^{-4}	1.8×10^{-4}

[a] UV survivors (V_p^*/V_p). These UV stocks passed through one more freeze-thaw cycle than the live virus; a plot of V_p against min. UV had an intercept of 5.32×10^7, which is taken as V_p.

[b] UV lethal hits; $r = -\frac{\log S}{.4343}$.

[c] I = normalized fraction of infected cells (58% infected at live virus multiplicity of about 8).

[d] $V_c = 8.6 \times 10^7$ (corrected for extra freeze-thaw cycle); using 0.4 ml virus, 3 million cells, virus stock diluted by 3, and adsorption value of 65.6%,

$$m = \frac{8.6 \times 10^7 \times 0.4 \times 0.656}{3 \times 3 \times 10^6} = 2.5$$

$w = \frac{I}{1 - (m+1)e^{-m}}$, the denominator is 0.713 for a multiplicity of 2.5.

Table XVI

Exp. No.	Symbol[a]	m	Slope	$\frac{\text{Slope}^{b}}{-.434}$	Intercept[c]	n[d]
A	cross	2.5	.46	1.1	1.1×10^{1}	2.6
B	circle	11	.40	0.9	1.1×10^{1}	1.0
C	square	20.5	.45	1.0	1.5×10^{2}	1.7
D	dot	29	.43	1.0	7.4×10^{1}	1.3
E	triangle	38	.46	1.1	1.7×10^{2}	1.4
Ave				1.01		

[a] As in Figure 8.

[b] Observed slope compared to slope of virus titered at very low multiplicities.

[c] Back-extrapolate to zero-dose of points in Figure 8.

[d] Calculated from equation 1: as r becomes large, w approximately approaches $m^{n}\ e^{-r}$ and at the zero-dose intercept, n = log intercept/log m.

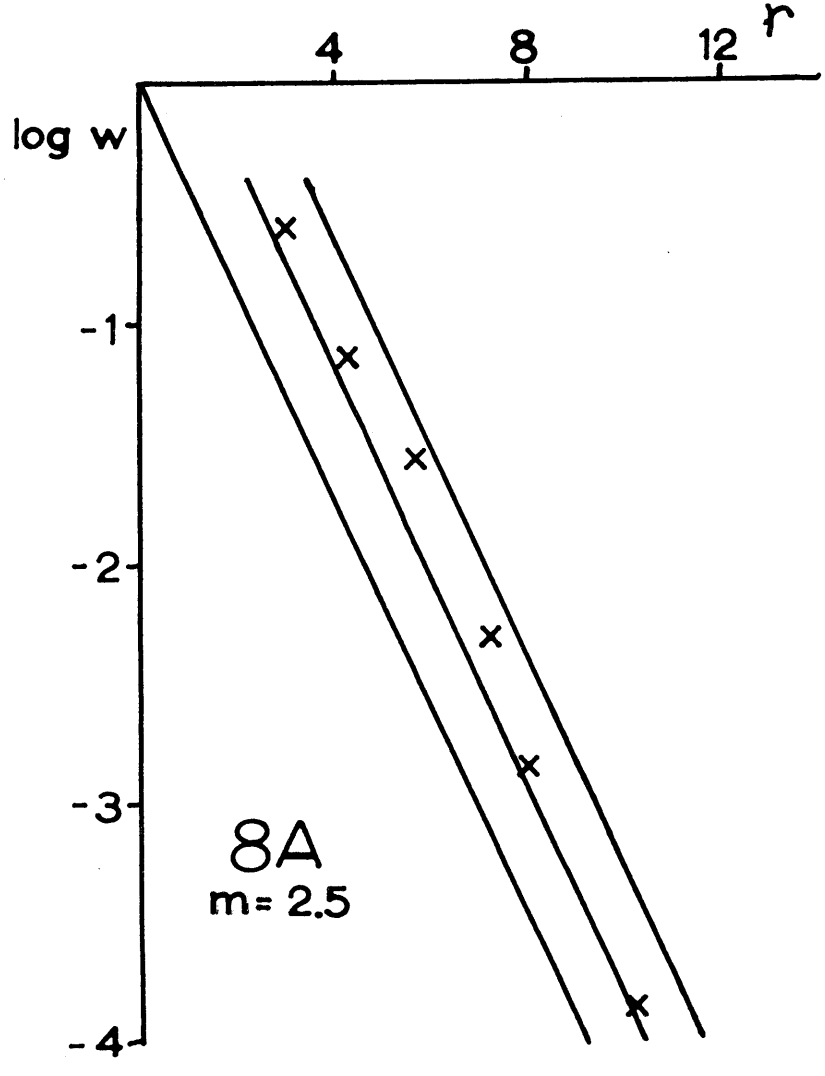
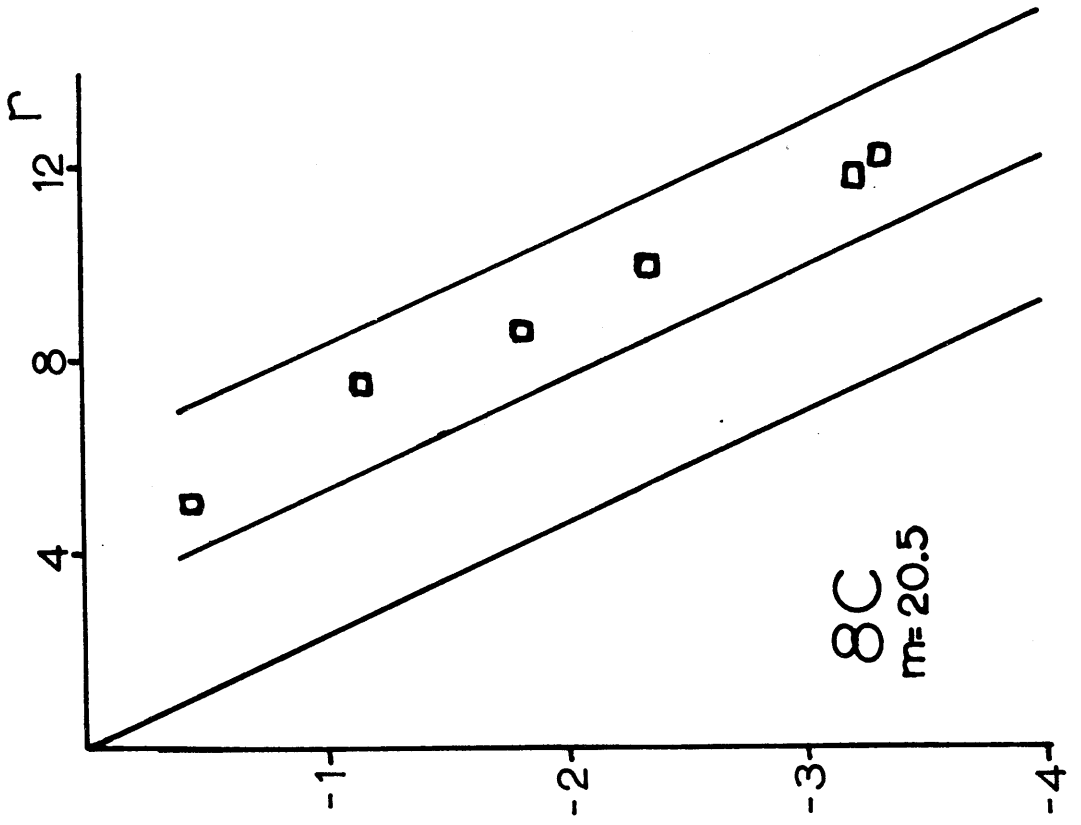
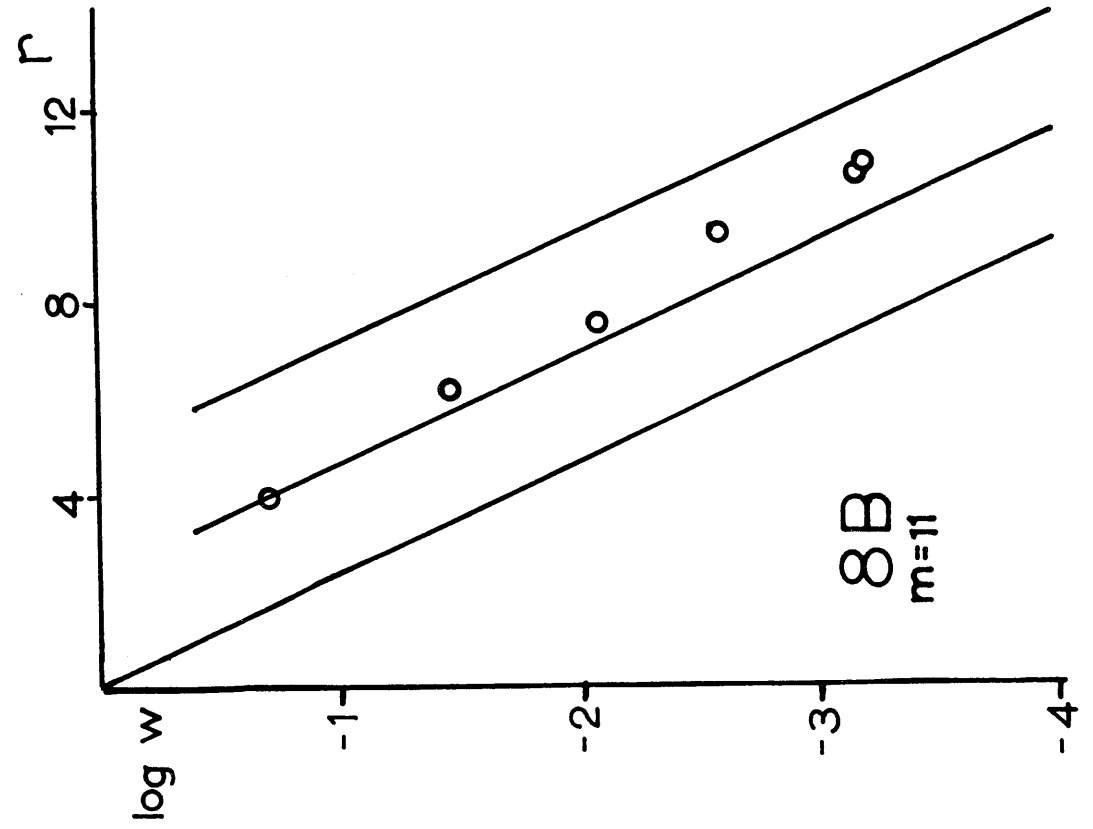
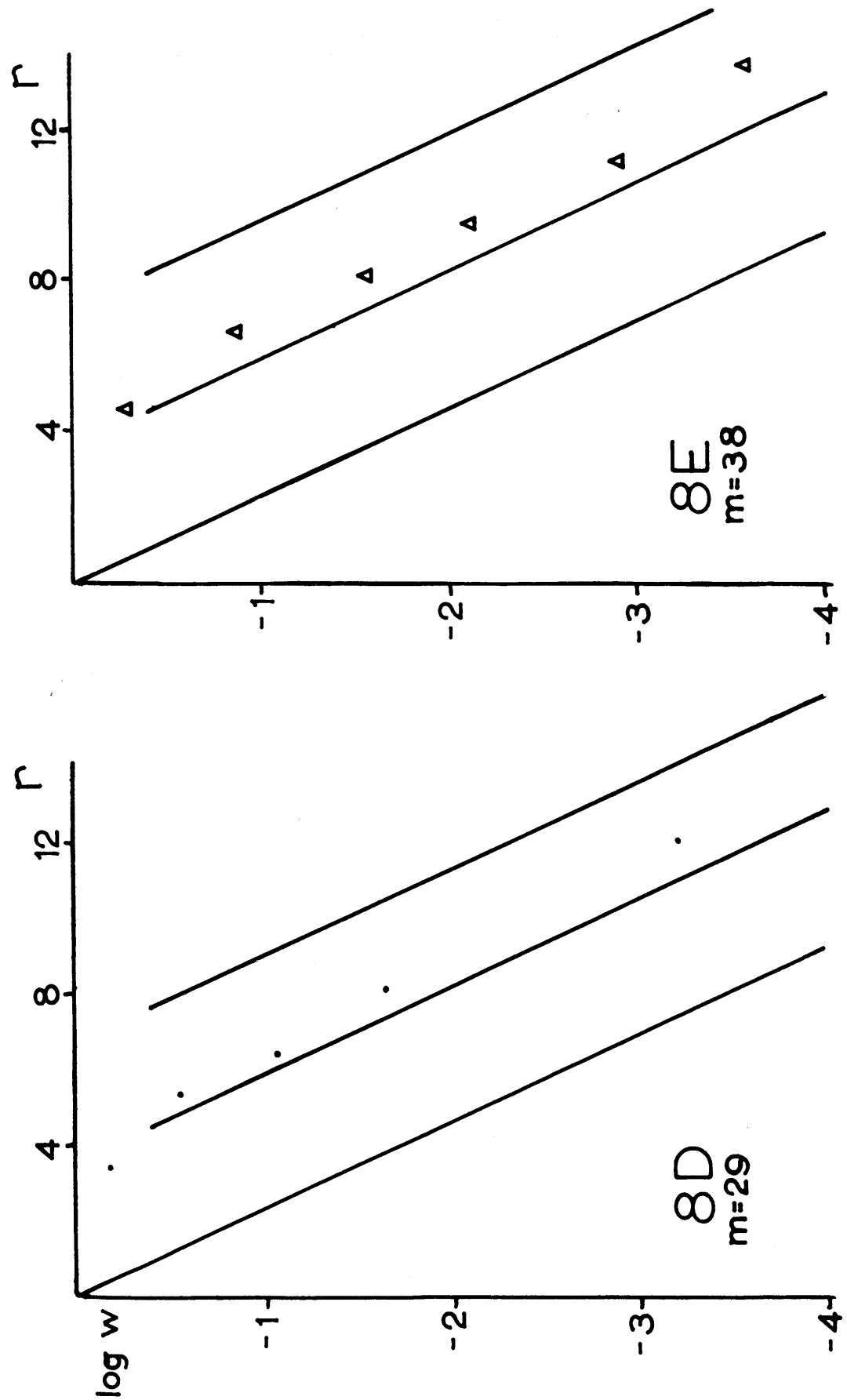


Figure 8 A-E. Fraction of yielders w (=I for all except 8A) plotted against dose r in hits. The multiplicity is constant within each experiment, and is indicated on the graph. In each graph the bottom line is the survival of free virus (e^{-r}); the middle line is equation 1 in asymptotic form for large r with n = 1 and the given multiplicity; and the top line is the asymptotic form of equation 1 with n = 2 and the given multiplicity.


r
12
8
4
-1
-2
-3
-4
8C
m=20.5


r
12
8
4
log w
-1
-2
-3
-4
8B
m=11


r
12
8
4
-1
-2
-3
-4
8E
m=38
r
12
8
4
log w
-1
-2
-3
-4
8D
m=29

DISCUSSION

The main object of this work was to study various interactions among polioviruses infecting the same cell. The first kind of interaction studied was the ability of virus particles to interfere with the multiplication of superinfecting viruses. Although poliovirus inactivated by ultraviolet irradiation possessed no interfering abilities, live virus was capable of interfering with heterotypic polioviruses. Each of the three types of polioviruses exhibited about the same interference, and in addition there was suggestive evidence for co-interference: the total fraction of cells yielding either virus after double infection was depressed some 50% below the fraction of yielders after single infection.

The second type of interaction investigated was of a positive nature. It was found that a proportion of the progeny virus from cells infected by two heterotypic polioviruses, under conditions in which interference was not a powerful factor, was neutralized by antisera active against either of the parental virus types. The existence of such particles suggests that polioviruses infecting a single cell can interact in the formation of coat material composed of protein specified by both of the input virus types, in a manner similar to phenotypic mixing. The existence of mixed coat material would imply the presence of "pools" of genetic and/or protein viral components within a multiple infected cell. However, since the possible participation in the

origin of these mixed particles of some artefact must still be studied, this is not conclusive evidence.

The third kind of interaction studied was multiplicity reactivation (MR) among homotypic poliovirus inactivated by ultraviolet light. This work was undertaken because it is likely that MR involves an interaction among the genetic material of the viruses (see Introduction). If, therefore, MR occurs in polioviruses, it could be considered as strong presumptive evidence for the occurrence of genetic interactions. Such a result would be desirable since information is lacking about genetic recombination in polioviruses.

One difficulty in the poliovirus MR system is that the fraction of cells releasing virus as a consequence of MR in these experiments is only a few times greater than that which one would expect because of input UV survivors. Thus a great deal of attention was given to the study of phenomena which may conceivably produce an apparent MR. It was clearly shown that reactivation is not produced by heat-killed or originally uninfectious virus, and that UV-irradiated but not heat-killed virus gives MR. Extraneous agents, such as visible light and non-viral or antiserum-inactivated heterotypic lysates, do not facilitate the MR system. As a consequence of these controls, the occurrence of type I poliovirus MR as a genuine phenomenon appears to be well established.

In the MR experiments the live virus titer V_c was determined under the same conditions as MR was measured. The controls showing that V_c is not changed by the presence of a

UV'd lysate, and that killed virus is as efficiently adsorbed as are plaque-forming particles, furthermore imply that it is allowable to calculate the multiplicity in the manner chosen. Therefore, it is very improbable that an as yet unmeasured fraction of live virus is responsible for the MR, or that the multiplicities we have measured are underestimated.

The manner in which the input virus is distributed among the cells is of great importance. It was found that the proportion of infective centers is a function of the virus concentration which corresponds approximately to a random distribution of virus particles among the cells; the fraction of uninfected cells in fact disappears according to the first term of a Poisson distribution with a mean multiplicity close to that measured by the disappearance of free virus. In these experiments infection was extended over more than 60% of the cells capable of forming plaques. Over this range the slope of the curve was nearly constant; thus a considerable majority of the S3 cells are infected by polioviruses with an approximately constant probability.

This result is in agreement with the finding of Vogt and Dulbecco (27) that 95% of the cells of the S3 line are killed in a one-hit manner by adsorbed virus with an efficiency (killers/PFU) approaching unity. Only about 5% of the cells are killed with a reduced efficiency.* The cells of the

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*In the experiments which measured the fraction of uninfected cells (1 - I) as a function of multiplicity, the observed fraction of plaque-forming cells was divided by the

resistant fraction adsorbed virus with the same probability per cell as did the majority fraction of cells. These results, therefore, demonstrate that the adsorbed virus is distributed nearly randomly among the cells, including the cells not appearing as plaque-formers, and that the multiplicity of infection can be approximately calculated from the proportion of infected cells by using the first term of the Poisson distribution. More specifically, the coincidence of the multiplicity as determined from the proportion of virus adsorbed with the multiplicity of infection as determined from the proportion of cells yielding virus, and with the multiplicity of killing as determined from the proportion of cells surviving as clone formers, shows that the adsorbed virus is nearly equally distributed among that fraction of infected cells able to form plaques and that fraction unable to do so.

The results of the MR experiments were analyzed by using the Luria-Dulbecco model based on an assortment of units. The slope of the survival curve for multicomplexes (dose dependence of the yielding fraction of cells) was

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fraction infected at a large multiplicity (ca. 20). Since this multiplicity was sufficient to infect the 5% of cells having a higher multiplicity requirement, whereas the multiplicities used to determine the uninfected fraction were not greater than 1.4, which is not sufficient to infect this 5% of the cells, the data should be further corrected by dividing not by the cells forming plaques at high multiplicities, but by this fraction minus 0.05. When this correction is added to the correction for the cell size distribution, the observed value of 1 - I agrees with the expectation based on the value of adsorbed virus, m_{ads}.

equal to that of the free virus, but a non-integer average value of n was found. The use of this formal model does not imply that the results obtained necessarily depend upon the assortment of genetic units. This will be discussed later. Considering the rather weak MR present in this system and the lack of experiments revealing an increase of genetic interaction in irradiated over unirradiated polioviruses, a non-genetic, physiological mechanism cannot as yet be ruled out.

The analysis of the polio data according to the Luria-Dulbecco model yields a number of units, n, equalling about 1.6. The significance of the parameter n and of the slope of the MR dose dependence can be more fully appreciated in the light of the following considerations.

The parameter n was originally intended to represent a number of segments of equal size into which the viral genome disintegrated within the host cell. There is to date no physical evidence of equally sized pieces in phage DNA corresponding to the number of units estimated by Luria and Dulbecco, but phage DNA in vitro is clearly multipartite (88, 89). Furthermore, breaks in the phage genetic material may not appear in the same locations in each phage; for example, the model linking recombination and heterozygosis in phage (90) assumes randomly located overlap regions which might be particularly susceptible to breakage.

Barricelli (68) has suggested that the phage genome is divided into two groups of segments, one consisting of pieces

of very small UV cross section and the other of a few pieces of large cross section. In this model the damage to the small pieces would be mostly eliminated by MR and the survival curves of multicomplexes at intermediate UV doses and multiplicities would be determined primarily by the behavior of the large pieces. Except at very large UV doses these curves would have a flatter slope than the free virus curve.

In view of the small value of n found for poliovirus, one may ask whether n may be affected by conditions independent of the mechanism of reactivation. One such condition would arise if only a fraction of the adsorbed viruses were actually involved in a reactivation attempt. In this case the value of the multiplicity used to calculate n would be overestimated, and n would consequently be underestimated.

Another possible manner in which adsorbed viruses could have a reduced interacting potential would be the formation of independent pools. Due to the large size of the host cell, particles which could otherwise interact may be physically separated intracellularily. That this is the case may be suggested by the incomplete phenotypic mixing obtained from polioviruses coinfecting a cell, compared to the nearly completely random mixing of serological markers in phenotypic mixing among phages.

One may also ask whether a small value of n for poliovirus may be due to the small size of this virus. In fact, we may expect that the number of segments within a virus particle may be a function of the absolute size of the viral

genome and of the probability per unit length for an interaction such as a crossover to occur. All other factors being equal, two viruses which differ either in the size of their genomes (perhaps equal to the total weights of their nucleic acids) or in the chance of an interaction per unit length of the genome, would also differ in their MR. In practice, however, the amount of viral nucleic acid is not closely correlated with the value of n among the T-phages.

In conclusion, the question arises what experimental approaches are now suggested for the further study of intracellular interactions among polioviruses. One of the first steps towards simplifying poliovirus-cell systems should be an attempt to devise conditions such that all cells adsorbing viruses will appear as plaque-formers. A further desirable simplification would be to devise conditions of cell growth which will give populations of cells uniform in their susceptibility to infection by polioviruses, selecting by some indirect means against cells not efficiently infected by adsorbed virus, and perhaps also narrowing the cell size distribution.

Several problems have been raised by the results of the experiments reported here. Are titer discrepancies indeed a reflection of phenotypic mixing, or perhaps even of genetic recombination? Conditions for maximizing titer discrepancies should be investigated, and any possibility of virus aggregation should be eliminated. The genetic composition of a virus suspension exhibiting a large titer

discrepancy should be further examined by picking plaques formed from such a suspension and testing their phenotypes. If possible, other than antigenic characters should be examined in this manner; a suitable marker might be heat resistance in type I poliovirus. A possible role for phenotypic mixing in interference (see Section E of Experiments and Results) should also be investigated.

It would be very useful if conditions could be established to infect cells simultaneously with two or more polioviruses. In this way interference might be circumvented, and genetic recombinants might be more easily obtained than with unsynchronized infection. If it develops that recombination is still difficult to demonstrate between live viruses, which well may be the case if interference is powerful and is very rapidly established, then recombinants might be more readily obtained by means of cross-reactivation or rescue experiments, in which a UV'd and a live virus infect the same cell and give rise to progeny viruses containing genetic markers from the irradiated parent.

REFERENCES

1. Dulbecco, R. (1952) Proc. Nat. Acad. Sci. 38: 747-752.

2. Dulbecco, R. and Vogt, M. (1954) J. Exp. Med. 99: 167-182, 183-199.

3. Schwerdt, C. E. and Schaffer, F. L. (1955) Ann. N. Y. Acad. Sci. 61: 740-753.

4. Schwerdt, C. E. Williams, R. C., Stanley, W. M., Schaffer, F. L. and McClain, M. E. (1954) Proc. Soc. Exp. Biol. Med. 86: 310-312.

5. Briefs, A., Breese, S. S., Jr., Warren, J. and Huebner, R. J. (1952) J. Bact. 64: 237-246.

6. Bang, F. B. (1955) Ann. Rev. Microbiol. 9: 21-44.

7. Luria, S. E. (1953) General Virology, New York, John Wiley & Sons, p. 57.

8. Burnet, R. M. (1955) Principles of Animal Virology, New York, Academic Press, pp. 89-102.

9. Schaffer, F. L. and Schwerdt, C. E. (1955) Proc. Nat. Acad. Sci. 41: 1020-1023.

10. Schwerdt, C. E. and Schaffer, F. L. (1956) Virology 2: 665-678.

11. Fogh, J. and Schwerdt, C. E. (1956) Federation Proc. 15: 253-254.

12. Lwoff, A., Dulbecco, R., Vogt, M. and Lwoff, M. (1955) Virology 1: 128-139.

13. Rubin, H. (1957) In press.

14. Hoyle, L. (1952) J. Hyg. 50: 229-245.

15. Hoyle, L., Jolles, B. and Mitchell, R. G. (1954) J. Hyg. 52: 119-127.

16. Frisch-Niggemeyer, W. and Hoyle, L. (1956) J. Hyg. 54: 201-212.

17. Uhler, M. and Gard, S. (1954) Nature 173: 1041.

18. Miller, H. K. (1956) Virology 2: 312-320.

19. Ada, G. L. and Perry, B. T. (1954) Aust. J. Exp. Biol. 32: 453-468.

20. Hershey, A. D. (1955) Virology 1: 108-127.

21. Meselson, M. (personal communication).

22. Schaffer, F. L. (1957) (in press).

23. Burnet, F. M. and Lind, P. E. (1951) J. Gen. Microbiol. 5: 59-66.

24. Ada, G. L. and Perry, B. T. (1956) J. Gen. Microbiol. 14: 623-633.

25. Roca-Garcia, M. and Jervis, G. A. (1955) Ann. N. Y. Acad. Sci. 61: 911-923.

26. Kibrick, S. (1955) Ann. N. Y. Acad. Sci. 61: 822-829.

27. Dulbecco, R. and Vogt, M. (personal communication).

28. Pojahnpelto, P. (personal communication).

29. Dulbecco, R. and Vogt, M. (1955) Ann. N. Y. Acad. Sci. 61: 790-800.

30. Dulbecco, R. and Vogt, M. (1953) Symposium, "Interaction of Viruses and Cells," Rome, pp. 73-80.

31. Tenenbaum, E. (personal communication).

32. Tenenbaum, E. and Drake, J. W. (unpublished).

33. Puck, T. T. and Marcus, P. I. (1956) J. Exp. Med. 103: 653-666.

34. Dunnebacke, T. H. (1956) Virology 2: 811-819.

35. Baluda, M. (1957) Thesis, California Institute of Technology.

36. Delbrück, M. (1945) J. Bact. 50: 151-170.

37. Visconti, N. (1953) J. Bact. 66: 247-253.

38. Benzer, S. (1952) J. Bact. 63: 59-72.

39. Drake, J. W. (unpublished).

40. Puck, T. T. and Lee, H. H. (1954) J. Exp. Med. 99: 181-194.

41. Dulbecco, R. (1952) J. Bact. 63: 209-217.

42. Weigle, J. J. and Delbrück, M. (1951) J. Bact. 62: 301-318.

43. Lesley, S. M., French, A. C., Graham, A. F. and van Rooyen, C. E. (1951) Canadian J. Med. Sci. 29: 144-148.

44. Wecker, E. (personal communication).

45. Ledinko, N. and Melnick, J. L. (1954) J. Exp. Med. 100: 247-268.

46. Chanock, R. M. (1955) Proc. Soc. Exp. Med. Biol. 89: 379-381.

47. Novick, A. and Szilard, L. (1951) Science 113: 34-35.

48. Hershey, A. D., Roesel, C., Chase, J. and Forman, S. (1951) Carnegie Inst. Wash. Yearbook 50: 195-200.

49. Appleyard, R. K., McGregor, J. F. and Baird, K. M. (1956) Virology 2: 565-574.

50. Streisinger, G. (1956) Virology 2: 388-398.

51. Quersin-Thiry, L. (1955) Monograph, Institut Pasteur de Bruxelles.

52. Burnet, F. M. and Lind, P. E. (1951) Aust. J. Exp. Biol. Med. Sci. 32: 145-152.

53. Burnet, F. M. and Lind, P. E. (1953) Cold Spring Harbor Symp. Quant. Biol. 18: 21-24.

54. Fraser, K. B. (1953) Brit. J. Exp. Path. 34: 319-328.

55. Hirst, G. K. and Gotlieb, T. (1953) J. Exp. Med. 98: 41-52.

56. Hirst, G. K. and Gotlieb, T. (1953) J. Exp. Med. 98: 53-70.

57. Granoff, A. and Hirst, G. K. (1954) Proc. Soc. Exp. Biol. Med. 86: 84-88.

58. Gotlieb, T. and Hirst, G. K. (1954) J. Exp. Med. 99: 307-320.

59. Sprunt, K., Mountain, I. M., Redman, W. M. and Alexander, H. (1955) Virology 1: 236-249.

60. Melnick, J. L. (1955) Proc. Soc. Exp. Med. Biol. 89: 131-133.

61. Wenner, H. A., Kamitsuka, P. and Lenahan, M. (1956) J. Immunol. 77: 220-231.

62. Luria, S. E. (1947) Proc. Nat. Acad. Sci. 33: 253-264.

63. Luria, S. E. and Dulbecco, R. (1949) Genetics 34: 93-125.

64. Dulbecco, R. (1952) J. Bact. 63: 199-208.

65. Cairns, H. J. F. and Watson, G. S. (1956) Nature 177: 131-132.

66. Harm, W. (1956) Virology 2: 559-564.

67. Bernstein, A. (1957) Virology 3: 286-298.

68. Barricelli, N. A. (1956) Acta Biotheoretica 11: 107-120.

69. Benzer, S. (1955) Proc. Nat. Acad. Sci. 41: 344-354.

70. Streisinger, G. (1956) Virology 2: 1-12.

71. Doermann, A. H., Chase, M. and Stahl, F. W. (1955) J. Cell. Comp. Physiol. 45 Suppl. 2: 51-74.

72. Harm, W. (1958) (in press).

73. Park, B. H. (1956) Virology 2: 711-718.

74. Weigle, J. (1953) Proc. Nat. Acad. Sci. 39: 628-636.

75. Stent, G. S. (1958) Adv. Virus Res. (in press).

76. Watson, J. D. (1950) J. Bact. 60: 697-718.

77. Weigle, J. J. and Bertani, G. (1956) Virology 2: 344-355.

78. Stent, G. S. and Fuerst, C. R. (1955) J. Gen. Physiol. 38: 441-458.

79. Mutsaars, W. (1957) Ann. Inst. Past. 92: 1-14.

80. Hershey, A. D. and Chase, M. (1952) J. Gen. Physiol. 36: 39-56.

81. Cohen, S. S. and Arbogast, R. (1950) J. Exp. Med. 91: 637-650.

82. Henle, W. and Liu, O. C. (1951) J. Exp. Med. 94: 305-322.

83. Cairns, H. J. F. (1955) J. Immunol. 75: 326-329.

84. Kilbourne, E. D. (1955) J. Exp. Med. 101: 437-450.

85. Puck, T. T. and Fisher, H. W. (1956) J. Exp. Med. 104: 427-434.

86. Dulbecco, R. (1950) J. Bact. 59: 329-348.

87. Cairns, H. J. F. (1957) Virology 3: 1-14.

88. Meselson, M., Stahl, F. W. and Vinograd, J. (1957) Proc. Nat. Acad. Sci. 43: 581-588.

89. Levinthal, C. (1956) Proc. Nat. Acad. Sci. 42: 394-404.

90. Levinthal, C. (1954) Genetics 39: 169-184.