PLAQUE FORMATION AND ISOLATION OF PURE LINES WITH POLIOMYELITIS VIRUSES*

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(Received for publication, June 1, 1953)

The production of plaques on a monolayer of chicken embryonic cells with Western equine encephalomyelitis virus (1) has opened the possibility of studying animal virus-host cell systems along lines similar to those followed in bacteriophage work. In trying to extend the previous results to other animal viruses, attention was directed to the viruses of the poliomyelitis group. These viruses, besides their practical importance, have many advantageous properties: great stability, good growth in tissue culture, and marked cytopathogenicity (2). In addition they display a certain degree of genetic variability, as inferred from their occurrence in various types and laboratory-adapted lines, so that they may lend themselves to studies of genetic variation.

The present work concerns the production of plaques on monolayer cultures of monkey tissue by poliomyelitis viruses. It will be shown that the plaques can be efficiently used for titration purposes, and that pure lines of the viruses can be isolated from single plaques. In addition, virus stocks of high titer have been obtained during the development of this work.

Material and Methods

Solutions and Media

Phosphate-Buffered Saline (PBS).—(*a*) NaCl 8.0 gm., KCl 0.2 gm., Na_2HPO_4 1.15 gm. KH_2PO_4 0.2 gr., water 800 ml.; (*b*) $CaCl_2$ 0.1 gm., water 100 ml.; (*c*) $MgCl_2 \cdot 6H_2O$ 0.1 gm., water 100 ml. Autoclave (*a*), (*b*), and (*c*) separately; mix when cooled. Trypsin (Nutritional Biochemical Co.) 0.25 per cent in PBS, sterilized by filtration.

Tissue Culture Fluid.—Earle's saline (ES) (3), 8 parts, horse serum, 6 parts, chicken embryo extract (1:1 in ES), 3 parts; Earle's saline and horse serum were sterilized by pressure filtration through a Selas filter of porosity 03; the embryo extract was prepared under sterile conditions. An amount of 100 μg. of streptomycin and 100 units of penicillin per ml. were added to the saline solutions.

Agar Overlay.—One pound of Difco agar was washed in twenty changes of tap water, and two changes of distilled water (10 to 15 liters per change); after decanting the last water, the agar was freed of excess water by squeezing it in a towel. 3 liters of acetone was then added to the agar. After 1 hour, the acetone was replaced with the same volume of fresh acetone, and a

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

similar change of acetone was repeated after a further hour. Finally, the acetone was eliminated by suction in a Buchner funnel. The agar was then collected from the funnel and spread on a sheet of aluminum foil. The agar was allowed to dry by the heat of three normal lamps and was collected when completely dry.

(*A*) 2.7 per cent solution of washed agar in hot distilled water was distributed into a series of test tubes which were then autoclaved and tightly closed with sterile rubber stoppers. Care was taken not to autoclave them again, since on reautoclaving toxic products arise from the agar.

(*B*) A 1/10,000 *solution of neutral red* in distilled water, sterilized by autoclaving.

(*C*) *Fourfold Earle's saline.* The pH of this solution was brought to 7.4 by bubbling CO_2 into it immediately after solution of all the ingredients; it was sterilized by pressure filtration through a Selas filter, porosity 03, and distributed into test tubes which were kept well stoppered in the refrigerator.

(*D*) *Chicken embryo extract* (1:1 in ES).

The agar overlay, used to overlay the cultures after infection, consisted of 12 parts of 2.7 per cent agar (*A*), 12 parts of neutral red solution (*B*), 8 parts of fourfold Earle's saline (*C*), and 5 parts of embryo extract (*D*). The agar tubes were first heated in boiling water until the agar melted, and subsequently transferred to a 43°C. water bath; the ingredients (*B*), (*C*), and (*D*) were prewarmed at 43°C., and added to the melted agar. The agar mixture was kept at 43°C. until used.

Preparation of Tissue Cultures

In most of the experiments, the source of tissue was *cynomolgus* monkey kidney, and in few experiments the testis. Two kidneys (or testes) at a time were minced into small pieces (1 to 4 $mm.^3$ in size), washed several times in PBS, and transferred into a small flask containing 60 ml. of a prewarmed (37°C.) 0.25 per cent trypsin solution. After 10 minutes' incubation at 37°C., the trypsin was decanted and substituted by approximately 20 ml. of fresh prewarmed trypsin. The tissue was then pipetted several (10 to 15) times back and forth with an automatic pipette having a bore of about 3 mm. diameter. The pipetting was done fairly vigorously to break up the tissue fragments into clusters of cells and single cells. After having allowed the large fragments to settle down, the turbid supernatant containing single cells, cell clusters, and cell debris, was poured into a centrifuge tube. About 20 ml. of fresh prewarmed trypsin was added to the fragments left in the flask; the tissue was again pipetted back and forth, and the supernatant collected. The same procedure was repeated 15 to 20 times until the parenchymatous kidney tissue had been entirely converted into cell clusters or single cells. This moment was recognizable by the fact that the original fragments had lost their brownish color and were now mere whitish connective tissue masses that tended to stick to each other. All supernatants were centrifuged at 600 R.P.M. for 2 minutes, and the pellets resuspended in PBS in one-half of the original volume. Two additional, similar centrifugations in which the pellets were again resuspended in half of the previous volume of PBS were carried out. The final pellets were resuspended in 20 to 40 times their volume of Earle's saline. 2 ml. of this suspension containing cell clusters and single cells, 1.5 ml. of horse serum, and 0.75 ml. of embryo extract were used to start a culture in a 60 mm. pyrex dish. From 15 to 30 cultures could be obtained from one kidney. The cultures were incubated at 38°C. in a well humified incubator. To control the pH of the culture fluid in the unsealed Petri dishes, the incubator received a continuous flow of air containing 3 per cent of CO_2. The cultures were incubated until the bottom of the Petri dishes was covered by a continuous cell layer. The culture fluid was changed every 3 days.

Formation of a Monolayer

The first signs of the formation of a monolayer consisted of patches of polygonal epithelial-like cells and were usually observed 48 hours after the preparation of the cultures. These patches originated mainly from the cell clusters present in the original suspension. The time required for the cell patches to fuse and to form a continuous cell layer, containing approximately 2×10^6 cells, varied from 3 to 7 days. The monolayers of the kidney cultures were always formed by polygonal cells, independently of the the time required for their formation. In the case of testes however, only the fast developing cultures formed cellular sheets similar to those of the kidneys; slow developing testis cultures contained mostly elongated cells of fibroblastic appearance.

Plating of the Virus

Cultures showing a continuous cell layer were washed twice with 2 ml. of PBS. After removing the last washing fluid, a volume of 0.3 ml. of virus suspension in PBS, at the appropriate dilution, was pipetted onto the cell layer. After 30 minutes' incubation at 37°C. to permit adsorption of the virus onto the cells, the infected cultures were covered with 3 ml. of melted (at 43°C.) agar mixture (agar overlay); the cultures were then kept at room temperature for about 10 minutes to allow the agar to solidify, and were finally placed in an incubator supplied with the CO_2-air mixture.

Virus

Type 1, Brunhilde strain (SC-1949 pool), of poliomyelitis virus, kindly supplied by Dr. C. F. Pait, was used in the majority of the experiments. The virus was supplied as a 20 per cent suspension of spinal cord of *rhesus* monkey in distilled water (4). In a number of experiments, Type 1 virus obtained from passage through the monolayer kidney cultures was used. Type 2, Yale-SK strain, and Type 3, Leon strain, were kindly supplied by Dr. J. L. Melnick in form of tissue culture supernatants.

Preparation of Virus Stocks

Cultures showing a continuous cell layer were washed and infected as described in the section Plating of the Virus. Instead of the 3 ml. of agar overlay, 3 ml. of Earle's saline containing 20 per cent embryo extract was added to each Petri dish, which was then incubated in an incubator continuously supplied with the CO_2-air mixture. The supernatants were collected when the cells began to lose their attachment to the glass, usually 24 hours after infection.

Antisera

Normal monkey serum and type-specific poliomyelitis antisera were kindly supplied by Dr. C. F. Pait. The specific antisera used were: Type 1—Pool A-453, 1/23/50, and Type 2—Pool A-300, 6/20/49. (4)

RESULTS

Characteristics of Plaque Formation

In an infected culture, both the aspect and the number of plaques varied with the time of incubation. The formation of plaques with poliomyelitis virus Type 1 and Type 2 could be seen macroscopically 24 hours after infection. In

oblique light, against a dark background, the plaques stand out as small, brilliant areas. After 48 hours, they were best seen against a clear, green background; they appeared as round, uncolored areas contrasting with the red color of the surrounding living cells, stained intravitally with neutral red (Fig. 1). At this time, the diameter of the plaques varies from 1 to 3 mm. During subsequent incubation, the plaques increased progressively in size until they became confluent. On a given plate, the number of plaques approximately doubled between the 1st and 4th day, and remained constant thereafter.

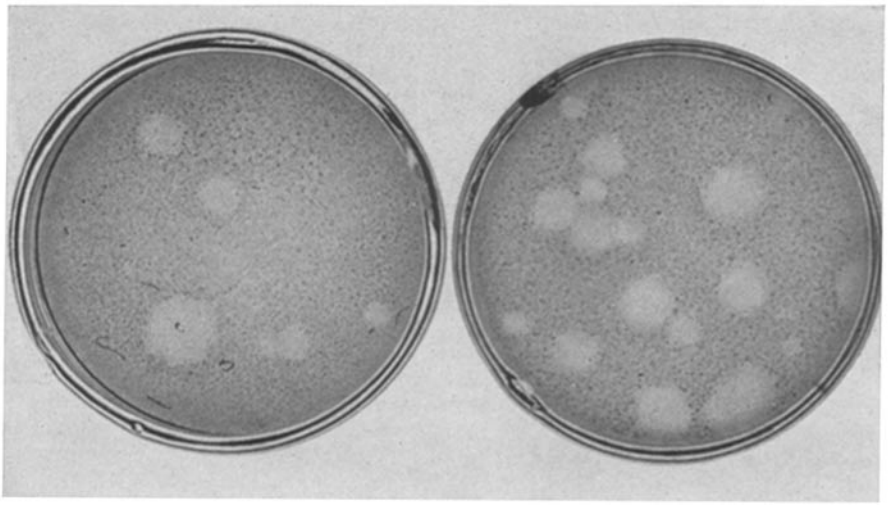

FIG. 1. Plaques of poliomyelitis virus, Type 1, on monolayer monkey kidney cultures, 72 hours after infection. The plate to the right was infected with double the amount of virus. Approximately natural size.

Plaques of Type 3 virus appeared only after 48 hours, and increased in number up to the 5th day. Owing to their later appearance, plaques of Type 3 virus were on corresponding days always smaller than those produced by the other two types.

When comparing plaque formation on kidney and testis cultures, it was observed that kidney cultures were all equally and highly susceptible to the virus. In the case of testis cultures, however, the morphological differences between fast and slow growing cultures (see section Formation of a Monolayer) proved to be correlated with a difference in susceptibility of the two types of cultures: fast growing testis cultures were almost as susceptible as kidney cultures while slow growing testis cultures were much less susceptible to the virus. For this reason, all quantitative experiments have been done with kidney cultures, or fast growing testis cultures.

The number of plaques produced by a given virus sample was reproducible (with the restriction just mentioned), and inversely proportional to the dilution of the virus, provided the number of plaques per plate was sufficiently small (around 40 or less) to avoid an overlapping of plaques (Table I). The amount of virus capable of producing one plaque will be called a plaque-forming dose.

TABLE I

Proportionality between the Number of Plaques and the Concentration of Virus

Each culture was infected with 0.1 ml. of the appropriate virus dilution.

No. of experiment	Virus stock	Relative concentration of virus	No. of plaques on each plate	Average No. per plate	Calculated titer per ml.
1	Monkey spinal cord suspension	1	69; 104; 132	102	8.1×10^5
		½	46; 62; 62; 68	60	9.5×10^5
2	Monkey spinal cord suspension	1	47	47	7.5×10^5
		½	21; 25	23	7.4×10^5
3	Plaque stock 2	1	29; 53	41	2.5×10^5
		⅓	8; 12; 12; 17; 17; 18	14	2.5×10^5
4	Plaque stock 2	1	10; 36	23	1.2×10^5
		⅓	5; 5; 7; 11; 11; 22	10	1.5×10^5
		1/9	0; 1; 1; 2; 2; 5; 5; 5; 6; 9	4	1.6×10^5
5	Liquid stock 2-1	1	18; 20; 31; 33	26	2.6×10^8
		½	8; 9; 9; 12; 13; 15; 15; 18	12	2.5×10^8
		¼	1; 2; 3; 4; 4; 5; 5; 6; 6; 7; 7; 7; 7; 7; 9; 12	6	2.3×10^8

Plaque formation with Type 1 virus could be prevented by pretreatment of the virus with specific Type 1 antiserum, whereas pretreatment with normal monkey serum or specific Type 2 antiserum would not prevent plaque formation (Table II). This result shows that plaques are produced by a factor that has the same antigenic properties as the virus.

The titer obtained by counting the plaque-forming doses was slightly higher than the infectivity titer obtained by monkey titration. Thus, the 20 per cent spinal cord suspension of Type 1 virus was found to have a plaque-forming titer of 7.4×10^5 plaque-forming doses per ml. Its PD_{50} titer per one-half milliliter had been found to be $10^{5.4}$ (4). Since a PD_{50} corresponded to an average of 0.7 infectious doses (as shown in the discussion), a titer of $10^{5.4}$ PD_{50} corresponded to a titer of 1.75×10^5 infectious doses per ½ ml. (or 3.5×10^5 infectious doses per ml.).

The plaque titer was, therefore, slightly higher than the monkey infectivity titer. It has also been found that the plaque titer was somewhat higher than that obtained in roller tubes of monkey kidney (5).

TABLE II

Suppression of Plaque Formation by Type-Specific Antiserum

The virus was incubated for 1 hour at 37°C. in serum diluted 1:20 in PBS; aliquots of 0.1 ml. of the mixtures were subsequently plated at the dilutions indicated.

No. of experiment	Virus stock	Type of serum	Dilution of virus-serum mixture	No. of plaques on each plate
6	Monkey spinal cord suspension	Type 1 antiserum	1	0
			10	0
			100	0
		Type 2 antiserum	10	40
			100	7
		Normal monkey serum	10	>100
			100	22
7	Plaque stock 1	Type 1 antiserum	1	0*
			10	6; 4‡
			50	0; 0
		Normal monkey serum	50	> 40; confluent
			200	18; 7
8	Plaque stock 2	Type 1 antiserum	1	0
			10	0; 0
			50	0; 0; 0
		Normal monkey serum	50	Confluent; confluent
			200	68; confluent; confluent

* The absence of plaques in this undiluted sample, in contrast to the presence of plaques in the 10 times more diluted sample, is due to the high concentration of antiserum carried over to the plate.

‡ In Experiments 7 and 8, aliquots of the higher dilutions were plated in duplicate or triplicate.

Isolation of Virus from the Plaques and Determination of Its Characteristics

The virus was recovered from a plaque by picking up with a Pasteur pipette an agar cylinder, 2 mm. in diameter, from the center of the plaque (6); the agar cylinder was suspended in 1 ml. of Earle's saline containing 20 per cent embryo extract and frozen; this preparation will be called "plaque suspension." Twenty suspensions from different plaques were assayed and found to contain from 10^4 to 10^5 plaque-forming doses. From each plaque suspension a "plaque

stock" was prepared as described under Methods. Since each plaque stock originated from a single virus particle (as proved in the Discussion), these stocks constitute the purest lines of virus presently available. The question of purity of these lines will be expanded further in the discussion.

The virus originating within a plaque diffuses out, and may, therefore, contaminate an adjacent plaque. An experiment was devised to determine the distance covered by the virus in diffusing away from a plaque. Several plates were infected with diluted Type 1 virus, so as to obtain very few plaques. One plate developed a single plaque. After 4 days of incubation, the plaque had a diameter of 5 mm. Agar cylinders were picked up from different regions on the plate: (1), from the center of the plaque; (2), at 6 mm.; (3), at 13 mm.; and (4), at 20 mm. from the center. All agar cylinders were suspended in 1 ml. volume. The various suspensions were then assayed for virus, suspension (1) at a proper dilution, the others undiluted. The virus content of the suspensions was found to be: (1), 1.8×10^4; (2), 10; (3), no plaques in 0.6 ml. (the remaining 0.4 ml. was lost by contamination of a plate); (4), no plaques.

It appears, therefore, that virus from a plaque is still present at a distance of 3 to 4 mm. from the rim of the plaque; its concentration decreases, however, very rapidly with the distance, as expected from the laws of diffusion. At a distance of 10 mm. or more from the rim, diffused virus is entirely absent.

It is interesting that after 3 days the plaque reached the position of cylinder (3); two new agar cylinders were picked up at this time, contiguous to cylinders (2) and (3), respectively. Both of them now contained virus (about 10^4 plaque-forming doses).

Several characteristics of the virus contained in a number of different plaque stocks were determined; *i.e.*, the serological type of the virus, its pathogenicity for monkeys, and the type of plaques produced on replating.

As can be seen from Experiments 7 and 8 in Table II, the plaque stocks studied were of the same serological type as the source material.

The pathogenicity of the plaque stocks was determined by inoculating intracerebrally into *cynomolgus* monkeys aliquots of 0.2 to 0.4 ml. of six plaque stocks—two of which had been obtained after a second plaque passage. Two monkeys were used for each stock. Each monkey showed characteristic signs of poliomyelitis, with a flaccid paralysis between the 4th and 7th day. Since the original Brunhilde stock, containing 3.5×10^5 infectious doses per ml., had been diluted before inoculation by a factor of 1.45×10^9 in the case of the first passage stocks and by a factor of 4.3×10^{16} [1] in the case of the second

[1] These dilution factors arose in the following way: (*a*) first-passage stock: dilution of Brunhilde stock into the agar overlay of the first plate: 1.6×10^4; $1/600$ of the agar overlay picked and diluted 1:30 to make a stock; of this 0.2 ml. inoculated into the animal. (*b*) second passage stock: similar to the first passage stock, but with an additional dilution $1/500$ in replating the first plaque suspension, and $1/600$ in the second picking; in addition the second plaque suspension was diluted $1/300$ instead of $1/30$ to make a stock.

passage stocks, the experiment proves that pathogenic virus was produced inside the plaques.

To test whether the differences in plaque size that had been observed were hereditary with the virus responsible for them, stocks from large and small plaques were plated separately and scored for plaque size. The plaques produced by stocks of both types were indistinguishable and showed again the same variability in plaque size. It was, therefore, concluded that differences in plaque size are non-hereditary.

Part of the variability in plaque size can be attributed to differences in the time of adsorption of individual particles onto the cells of the same culture, as proved by the following experiment:—

Four cultures were washed and infected; after 30 minutes' adsorption, two cultures were overlaid with agar while the remaining two cultures were washed by two changes of 2 ml. of PBS, so as to eliminate the majority of the free virus, and then overlaid with agar. All the plates were incubated. It was found that the washed cultures showed only one-third of the number of plaques present in the non-washed plates; this shows that two-thirds of the plaque-forming doses were adsorbed during the incubation of the plates, that is after the 30 minutes' adsorption period.

However, this is not the only cause that produces a variability in the size of the plaques, since the plaques developing on the washed plates still showed differences in size. The other causes are, so far, unknown.

Independent Infection of the Individual Cells in a Culture

To show that all cells of a culture can be infected independently, the following experiment was performed.

Cultures were infected with virus inocula of increasing concentration, overlayed after 30 minutes' adsorption with agar (from which the neutral red had been omitted), and incubated for 10 hours. At the end of this period, the cultures were stained with neutral red for 1 hour, and then observed microscopically.

In cultures infected with high virus concentrations, most of the cells were unstained, whereas in cultures with low virus concentrations only a few cells had not taken up the stain. A count of the two classes of cells in several microscopic fields of the different cultures showed that the fraction of unstained cells increased proportionally to the concentration of the virus; they were therefore, the cells infected by the inoculum. Stained and unstained cells were randomly mixed. In cultures in which only few unstained cells were present, these were always single; patches of unstained cells, suggestive of units of infection containing many cells, were never found.

Thus, the findings that at low virus concentrations infected cells were distributed randomly and that at high concentrations (usually) all the cells

showed signs of infection are evidence that in a monolayer culture individual cells can all be infected independently.

Production of High Titer Stocks

Monolayer kidney cultures lend themselves to the production of high titer stocks. The highest titers, ranging between 2 and 7 $\times$ 10^8 plaque-forming doses per ml. supernatant, were obtained when the inocula were sufficiently large to infect almost every cell of a culture; *i.e.*, contained approximately 5 $\times$ 10^6 plaque-forming doses. This was deduced from the following observation. By using inocula of increasing concentration, the virus titer in the supernatant increased correspondingly, until a maximum was reached. The minimum number of plaque-forming doses required in the inoculum to obtain this maximum virus titer was equivalent to two to three times the number of cells present in the culture (as determined by direct cell count). When such an inoculum was used, all the cells of a culture showed a complete degeneration approximately at the same time (around 18 hours), whereas with lower inocula, many cells were at this time still apparently intact. Larger inocula did not increase the titer further.

The highest concentration of the virus was obtained 24 hours after infection when all cells had degenerated and virus production had ceased. This last fact was shown by the following experiment:—

The supernatant culture fluid was removed from a culture 24 hours after inoculation with an inoculum insuring infection of most of the cells; all the cells were degenerated but the majority was still sticking onto the glass. 3 ml. of fresh fluid was added to the culture, which was incubated for an additional period of 24 hours. At this time, a second supernatant was collected. Upon assay it was found that the second supernatant had only $\frac{1}{10}$ the titer of the first, which could be entirely accounted for by the residual first supernatant.

Since a fully grown culture contained approximately two millions of cells, each cell in cultures giving high titer supernatants produced (on the average) an amount of virus ranging between 300 and 1000 plaque-forming doses.

DISCUSSION

The present work shows that plaque formation by poliomyelitis viruses of the three types has been obtained on monolayer cultures of monkey kidney and, in the case of Type 1, of monkey testis. Kidney cultures, however, proved preferable due to their uniform susceptibility. Other tissues might lend themselves equally well to plaque production. Among these the HeLa cells derived from a human cancer, might be a material of choice for future work, since these cells grow on glass, are perpetuated easily, and show necrosis under the influence of the poliomyelitis viruses (7).

The production of plaques with poliomyelitis viruses offers two points of interest: a theoretical and a practical one.

From a theoretical standpoint, the formation of plaques affords the strongest evidence that a single virus particle is sufficient to initiate the infection of a cell. We will define later the characteristics of this particle.

The proof that a single virus particle is sufficient for infection is based on the results of the dilution experiments reported in Table II and Fig. 2, which show an accurately *linear* relationship between the number of plaques and the virus concentration within each experiment:—

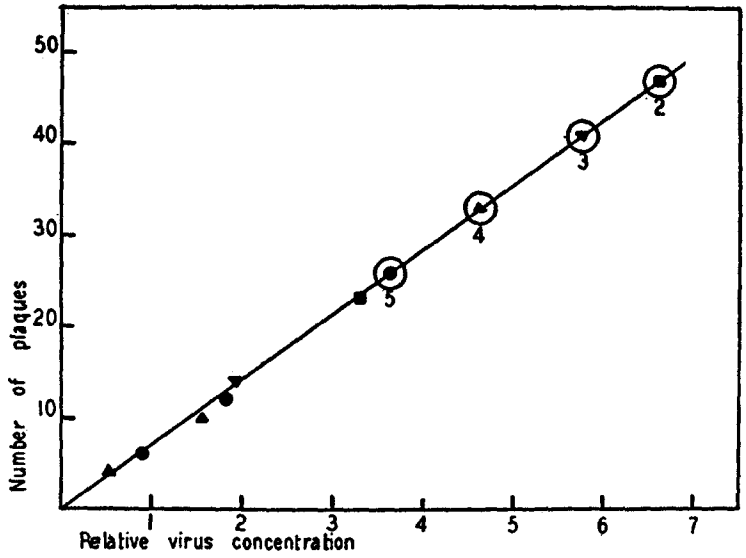

FIG. 2. Linear relationship between the number of plaques and the virus concentration. The points represent the data of Experiments 2, 3, 4 and 5 of Table I. Similar symbols belong to the same experiment. The points surrounded by circles represent the number of plaques at the highest virus concentration within each of the four experiments; for convenience, the values for the corresponding relative virus concentrations were chosen arbitrarily, so as to bring the four points onto the same line. The relative virus concentrations of the other points within each experiment correspond to the respective dilution factors as given in Table I.

Proof That a Linear Relationship Indicates a One-Particle Phenomenon.—A monolayer culture of cells can be ideally subdivided into a large number, N, of cellular units, which could be single cells or groups of cells. We now adsorb v virus particles *at random* onto the cell-layer; the *average number* of virus particles per cellular unit is, therefore, $\frac{v}{N}$. If the adsorption of *one particle* on a cellular unit is sufficient to produce a plaque (one-particle hypothesis), the number of plaques (Pl) is given, according to the Poisson distribution by:

$$Pl = N\ (1 - e^{-av/N}) \qquad (1)$$

in which a is a constant defining the efficiency of the system. The number N, of cellular units, is equal to the number of the cells in a culture; this is inferred from the fact that all the cells of a culture can be infected at appropriate virus concentration and infection of individual cells occurs independently, as shown under *Results*. N is, therefore, of the order of 2×10^6.

Since we are interested in the case in which only a relatively small number (less than 50) plaques is produced; *i.e.*, in which $\frac{av}{N}$ is very small, we can approximate equation 1 to

$$Pl = av \tag{1 a}$$

A linear relation between number of plaques and virus concentration is, therefore, expected.

It has now to be shown that such linear relation cannot be obtained under other conditions. Let us consider therefore as second hypothesis, the case that *more than one* particle per cellular unit is required to form a plaque, for instance, at least *two* particles. In this case, according to the Poisson distribution, the number of plaques is given by

$$Pl = N\left[1 - e^{av/N}\left(\frac{av}{N} + 1\right)\right] \tag{2}$$

which, for small $\frac{av}{N}$, approximates to

$$Pl = \tfrac{1}{2}\frac{(av)^2}{N} \tag{2 a}$$

The number of plaques would now be proportional to the square of the virus concentration. As shown in Fig. 3, the curve drawn according to equation (2 *a*) does not fit the experimental points.

If a higher number of virus particles per cellular unit were required, the number of plaques would be proportional to a correspondingly higher power of the virus concentration; the respective curve would fit the experimental points even less well.

Let us turn to a third hypothesis. It has been concluded that for some virus-cell systems the quantitatively different effects of different doses of viruses can be attributed to a variation in the regional susceptibility of the hosts, and that the relation between virus concentration and effect could be best explained by assuming that the logarithm of the minimal effective concentration per cellular unit is normally distributed. Under this assumption, the dependence of the number of plaques on virus concentration would be described by an integrated normal distribution (8, 9). This assumption is rather vague as long as the three parameters of the normal distribution are chosen at will; *i.e.*, the number of cellular units, the concentration of virus at which 50 per cent of the cellular units develop lesions, and the variance of the distribution. By arbitrarily assuming appropriate values of the three parameters, the relation between virus concentration and plaque number predicted by this theory be-

comes nearly linear over certain ranges; these segments of the curve could not be experimentally differentiated from a true straight line. To test the hypothesis properly, the three parameters must be determined on the basis of the experimental data. In our case, this can be done since we know the number of the cellular units. We have already shown that this number is of the order of 2×10^6. The other two parameters are determined from any two experimental points of the curve.

The points used were the following: (1) the point at which the fraction of infected cells per culture was 0.83 (measured by microscopic count in the experiment showing

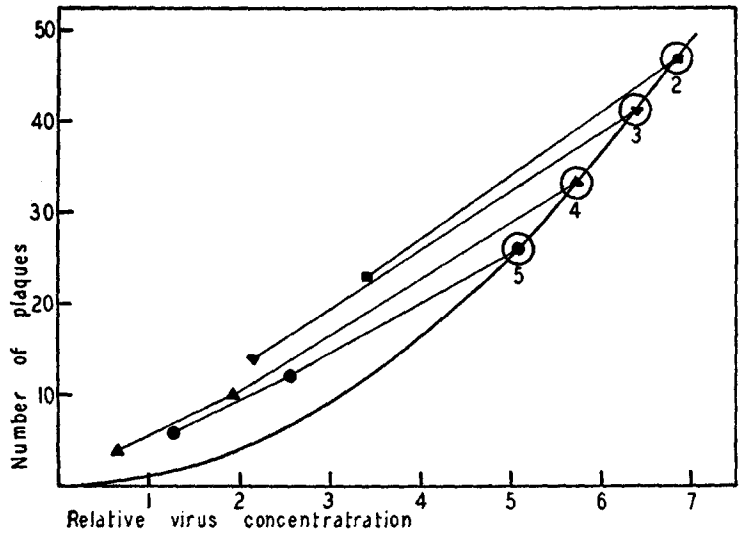

FIG. 3. Comparison of experimental data with hypothesis 2. The curve has been drawn according to equation (2 *a*). The points surrounded by circles have been placed arbitrarily on the curve. The relative virus concentrations of the other points correspond, as in Fig. 2, to the respective dilution factors. Points of the same experiment are connected by lines.

the independent infection of the cells); (2) the point at which the fraction of infected cells per culture was 5×10^{-7}, corresponding to one infected cell in a culture (determined by the development of one plaque per plate as average). On the normal probability curve, these points lie at +0.95 and −4.9 standard units, respectively, from the center of the curve. Since the virus concentrations used to obtain the two points consisted of 4×10^6 plaque-forming doses and of one plaque-forming dose, respectively, the ratio between the corresponding virus concentrations was $10^{6.6}$. Hence, 5.85 standard units corresponded to 6.6 $\log_{10}$ units of virus concentration, and the standard deviation of our curve was equal to 1.12 $\log_{10}$ units of virus concentration. The center of the curve corresponded to a virus concentration of $10^{5.54}$.

All the parameters of the normal curve being thus determined, we can now predict the dependence on the concentration of the virus of the number of plaques, for the experimental case that the number of plaques is less than 50 (corresponding to a distance of −3.4 and more standard units from the center of the curve). Fig. 4 shows

that there is a strong disagreement between the theoretical curve and the experimental points; the third hypothesis can, therefore, be discarded.

Our data, thus, conclusively prove that a single virus particle is sufficient to produce a plaque, and that the unit of cellular infection is a single cell.

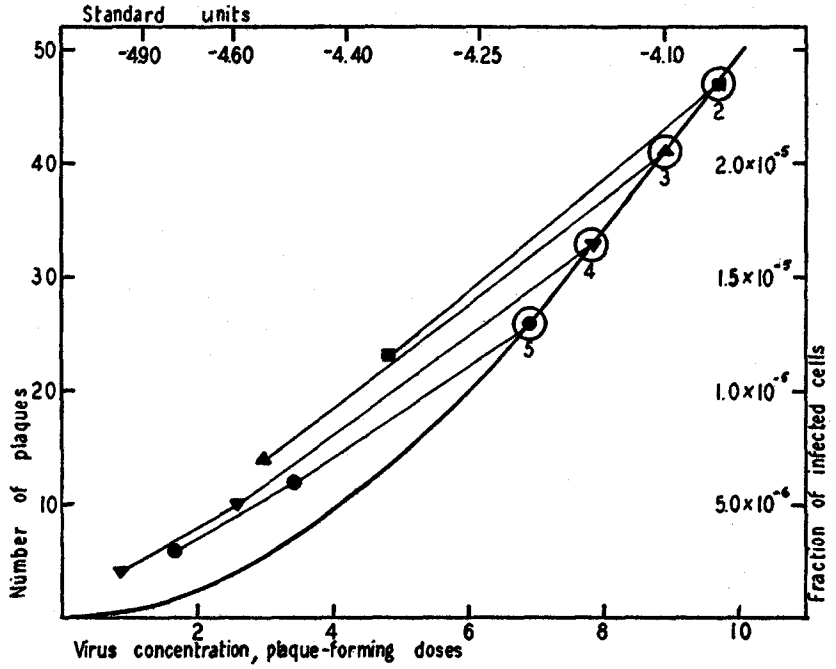

FIG. 4. Comparison of experimental data with hypothesis 3, that the number of plaques is proportional to an integrated error function of the logarithm of the virus concentration. The center of this curve corresponds to $10^{5.54}$ plaque-forming doses of virus, the standard deviation to 1.12 $\log_{10}$ ratio of virus concentrations. In the figure the concentration is represented in a linear scale. The distances from the center of the curve in standard units are given in the upper scale. The number of plaque-forming doses, given in the lower scale, is equal to $10^{5.54 + \text{standard units} \times 1.12}$. For additional information, see text. The number of plaques (left scale) is given by the product of the probability (right scale) times the number of cells in the culture (2×10^6). The experimental points have been placed as in Fig. 3.

Having arrived at this point, it is now possible to define properly the characteristics of the virus particle detected by a plaque. Owing to its all-or-none effect, it has the character of a particle. It corresponds to a unit of the virus which is not further subdivisible at high dilution. From the property by which it is recognized, we call it a plaque-forming particle. We do not know its morphological or genetic properties. It might be a single elementary body, or a clump of them, provided that the clump persists indefinitely at high dilution.

A plaque-forming dose contains at least one plaque-forming particle. It may contain more than one if the coefficient a of equation (1) is less than unity.

The finding that the plaque-forming titer of a given virus stock is very similar to its monkey-infectious titer (see under Results) is a strong indication that also for the infection of the monkey one single virus particle is sufficient. A PD_{50}, which corresponds to a probability of infection of 0.5, can be, therefore, expressed in "monkey-infectious doses," one PD_{50} corresponding to 0.7 infectious doses according to the Poisson distribution ($e^{-0.7} = 0.5$).

We want now to discuss briefly two conditions which apparently contradict the general conclusion that a single virus particle is sufficient to produce infection.

On one hand, a virus sample containing a single plaque-forming dose may actually contain more than one physical virus particle. This may occur for a variety of reasons, which do not invalidate the general conclusions: some of the physical particles may be non-infectious, or they may give rise to non-infectious progeny, or they may be infectious but have a small probability of coming into contact with a cell under the conditions of the experiment. The last condition may explain the slight differences between plaque-forming dose and monkey-infectious dose.

On the other hand, a cell may well be infected by more than one plaque-forming particle. It will be shown in a subsequent article that this has been accomplished with the virus of Western equine encephalomyelitis on a suspension of susceptible cells (10). Under the conditions of plaque formation however in which less than 100 virus particles come into contact with more than a million cells, the probability that a cell will be infected with more than one particle is entirely negligible. Under the circumstances outlined a plaque is, therefore, *always* initiated by a single plaque-forming particle.

The practical interest of the production of plaques is a consequence of their properties, as defined in the preceding theoretical discussion, and consists in the fact that they offer a new, sensitive, and reproducible method of titration, and a reliable method for isolating pure lines of the poliomyelitis viruses.

Justification of using the plaque technique as a method of titration is based on the following experimental results: (1) the plaques are due to the specific action of the virus; (2) the number of plaques is closely reproducible and proportional to the concentration of the virus, provided the plates are not too crowded; (3) the method appears to be slightly more sensitive than the titration in monkeys or in roller tube tissue cultures. From a practical point of view, there should not be more than 40 to 50 plaques per plate, so that the loss by overlapping of plaques be less than 10 per cent.

The overlapping of plaques can be avoided by using an inoculum producing a small number of plaques, as seen from the following calculation:—

A distance of 3 mm. between the centers of the two neighboring plaques is approximately the minimum distance required to recognize the separate origin of two plaques. The probability that two or more plaques originate within a circle of 3 mm. diameter can be calculated from equation (2), in which, now v is the number of virus particles on a plate and n the number of non-overlapping 3 mm. circles on the bottom of a 60 mm. Petri dish; *i.e.* = 270. The total number of plaques is given by equation (1). Using these equations, it can be seen that 100 plaque-forming particles on a plate produce 84 plaques, 15 of which are of multiple origin; 50 particles on a plate produce 46 plaques, 4 of which are of multiple origin; and 10 particles on a plate produce 10 plaques, with practically none multiple (only one multiple plaque in 10 such plates).

Using as maximum 40 to 50 plaques per plate, the plaque technique compares favorably with the roller tube technique as assay method:—fifteen to thirty cultures prepared from one monkey kidney allow an accurate count of approximately 600 to 1200 plaques, which are statistically equivalent to about 1200 to 2400 roller tubes.

The production of pure virus strains has been obtained by Burnet *et al.* (11) and by Hirst and Gotlieb (12) in influenza virus by egg passages at limiting dilutions, and by Downie and Haddock (13) in vaccinia by resuspending pocks developing on the chorio-allantoic membrane of the chicken embryo.

The recovery of virus from single plaques provides another method for virus purification. Purity of the plaque virus is assured if the following three requirements are fulfilled: (1) absence of plaques of multiple origin due to secondary overlapping; (2) no contamination of the plaque picked by virus diffusing from an adjacent plaque, or (3) by residual non-adsorbed seed virus.

The conditions fulfilling requirements (1) and (2) have already been discussed; they consist in the use of plates having less than 10 plaques, and in the picking of plaques at a distance of at least 10 mm. from the margin of an adjacent plaque. Requirement (3) can be fulfilled by washing the cell layer free of unadsorbed virus at the end of the adsorption period. It should be noted, in addition, that possibly contaminating virus particles could be eliminated by using two or more serial plaque passages. After fulfilling these three requirements, the purity of the plaque virus is limited only by the possible existence of heterogeneous components within the same plaque-forming virus particle.

SUMMARY

Plaques have been produced with the three types of poliomyelitis viruses on monolayer tissue cultures of monkey kidney and monkey testis. The number of plaques was proportional to the concentration of the virus. Each plaque originates, therefore, from a single virus particle, defined as the virus unit that is unseparable by dilution. The plaques are due to the specific action of the virus since they are suppressed by type-specific antiserum.

Pure virus lines were established by isolating the virus population produced in single plaques. These derived virus lines had the same morphological, serological, and pathogenic properties as the parent strain.

High titer virus stocks, with titers up to 7×10^8 plaque-forming particles per ml., were obtained.

Grateful acknowledgment is made to Dr. C. F. Pait for numerous discussions and for performing the intracerebral inoculation of the monkeys; to Mrs. Kathy Hayden and to Mrs. Patricia Knuth for their valuable technical assistance.

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