FURTHER STUDIES OF THE INFECTIOUS UNIT OF VACCINIA

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In a previous communication (1) evidence was presented which was interpreted as indicating that, when vaccine virus was suitably introduced into the skin of a rabbit, the results of experiment were consistent with the hypothesis that a single particle (elementary body) of virus could give rise to infection. This conclusion rested in part upon knowledge of the physical characteristics of the virus and direct estimation of the number of elementary bodies comprising an infectious unit, but chiefly upon the results of applying certain statistical principles to the study. It has since been pointed out (2) that certain of the measurements we used were not fully satisfactory, and an alternative hypothesis has been presented (3), based on the assumption that host resistance plays the determining rôle in this as in other situations where a quantal response occurs. At the same time investigations of the problem in this laboratory have continued and a preliminary report of certain studies of a modified strain of virus has already been published (4). It is the purpose now to report fully on this modified vaccine virus, as well as to describe the results of a number of other experiments. Finally, these and other data will be considered in the light of the two hypotheses which have been put forward.

I. Composition of the Infectious Unit of a Cultured Strain of Vaccinia

Previous studies of the infectivity of vaccine virus have been carried out with strains which had a reasonably high virulence for the rabbit. The advantages of using a virulent virus in ease of preparing material and ease of determining end-points are evident. However, it is equally apparent that a complete statement of the mechanism of infection by a virus must include observations on strains of virus of low virulence. Accordingly, studies of the First Revived Strain (5) of vaccine virus, kindly made available to us by Dr. T. M. Rivers, were begun soon after the completion of the previous study.

EXPERIMENTAL

Experiments were planned to give evidence on the probable composition of the infectious unit of this strain of virus in terms of elementary bodies. Efforts

were made also to enhance the virulence for rabbits, and to learn if possible whether or not the suspensions of virus were homogenous with regard to rabbit virulence.

Methods and Materials

Virus.—The First Revived Strain was derived originally from the New York Board of Health strain. It was propagated by culture in a medium of chick embryonic tissue in Tyrode's solution, its rabbit virulence declining as passage was continued. After considerable reduction in rabbit virulence had occurred, it was passed serially in rabbits with rapid rise in pathogenicity for this animal. On further cultivation in chick embryonic tissue its virulence for rabbits again declined although more slowly than at first. In its present culture generation, which is well over 200 removed from the last rabbit passage, it has almost completely lost its virulence for this host. Undiluted culture of the virus gives rise to only a mild lesion on intradermal inoculation.

Chick Embryos.—Embryos for preparation of culture medium and for titration of virus were obtained by incubating fertile eggs of Leghorn hens at 37°C. for 10 to 14 days.

Rabbits.—Rabbits were of mixed breeds, selected only for the possession of clear skin and good health. Preference also was for immature animals about two-thirds grown.

Titration of Virus.—(*a*) In the Rabbit: Rabbit titrations of virus were made by inoculating 0.25 cc. (in the later experiments 0.1 cc.) of twofold dilutions of virus intradermally. The sites of inoculation were marked and the rabbits observed daily for a week or until fading of the lesions was well under way. An inoculation was considered "positive" if a lesion appeared which reached its maximum on the 4th to 6th day, and was recognizable for 2 successive days. Reddening of the skin was uniformly characteristic of a lesion; edema was usually evident, although it was never as extensive nor as sharply demarcated as with the more virulent strains.

(*b*) In the Chick Embryo: Titrations in the chorio-allantoic membrane of the 14 day old embryo were made according to the method of Burnet (6). An artificial air sac was made on the side of the egg, and 0.05 cc. of viral suspension dropped on the chorio-allantoic membrane. After 2 days the egg was opened and the inoculated membrane was removed, spread out on a shallow dish, and examined for the presence of lesions. Those considered due to the action of vaccine virus were discrete opaque areas, about 2 or 3 mm. in diameter. When high concentrations of virus were used, the membranes of the surviving embryos showed a diffuse thickening and opacity.

In the first experiment, virus was titrated simultaneously by rabbit intradermal and chick embryonic inoculation.

Experiment 1.—Virus in its forty-first culture generation in this laboratory was used. Cells, cell fragments, and viral clumps were removed from the suspension by centrifugation at 3000 R.P.M. for 10 minutes in the horizontal centrifuge. For titration in rabbits, twofold dilutions were made in Locke's solution, and inoculations (20 of each dilution) were made into several rabbits. The results are presented in Fig. 1.

For titrations in chick embryonic membranes, serial dilutions were made and used for inoculation. The results of this titration are presented in Table I.

Inspection of Fig. 1 reveals a reasonably close resemblance between the experimental curve (50 per cent point at dilution $10^{0.77}$), and the theoretical

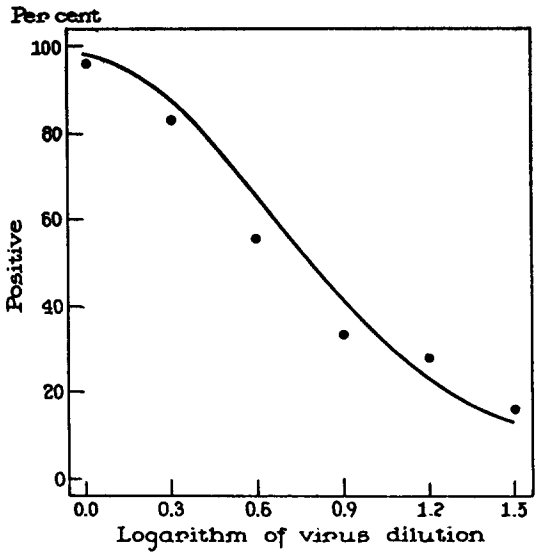

FIG. 1. Titration of C V I of vaccinia in rabbits. Per cent positive is percentage of the total inoculations giving rise to lesions.

TABLE I

Titration of C V I_{41} on Chick Embryonic Membrane

	Dilution of suspension		
	$10^{-2.5}$	10^{-3}	$10^{-3.5}$
Lesions on individual membranes	136	24	3
	40	32	40
	209	62	20
	271	15	19
	250	32	20
		40	2
		29	
Average.....	181.2	33.4	17.3

one which has been included, which is based on the assumption that a single particle is able to initiate infection. Calculation of χ^2 for goodness of fit according to the method of Haldane gives a P of 0.67, indicating that the hy-

pothesis is a tenable one. Applying this hypothesis, and assuming that at the 50 per cent dilution there are 0.69 particles[1] per unit inoculum of 0.25 cc., it would appear that the original suspension contained $10^{0.77} \times 4 \div 0.69$ particles per cc. However, the data of Table I do not agree with this. The relation between dilution and number of lesions produced is again consistent with the hypothesis that a single particle is giving rise to a lesion, but on this assumption it would appear that each cubic centimeter of original culture contained $10^{4.74} \times 20$ particles of virus (inoculum of 0.05 cc.). The discrepancy is considerably greater than any possible experimental error, and has been consistently demonstrated. Moreover, the ratio between chick and rabbit infectious units of virus is a reasonably constant one. It is perhaps easiest to explain the discrepancy by the assumption that particles of 2 sorts coexist in the suspension, one capable of infecting both chick and rabbit, and the other of infecting only the chick embryo.

This assumption is the one we were first inclined to accept (4), and was arrived at, apparently independently, by Gallagher and Woolpert (7) on the basis of their studies of a strain of vaccinia modified by passage through fetal rabbits. It has the merit of similarity to conditions known to exist among bacteria, in which virulent and avirulent members of a species may be carried together in culture for many generations. If this assumption be correct, regeneration of the rabbit virulent component should not be difficult, and further it should be possible to obtain the chick virulent strain in pure culture. Regeneration of the virus was first attempted.

Experiment 2.—For the purpose of regeneration of the rabbit virulent strain, a rabbit was injected in each testicle with 1.5 cc. of whole culture. After 5 days, although no evident disease had appeared, the organs were removed, ground with sand, and made into a 10 per cent emulsion with Locke's solution. 1 cc. of this emulsion was then inoculated into a second rabbit, and the process was repeated once, 3 rabbits being inoculated in series. No disease appeared, and inoculation of chick embryonic membranes with centrifuged emulsion resulted in the appearance of no specific lesions. The experiment was repeated twice with similar results.

Since simple serial passage in rabbits had resulted only in loss of the virus, a second method was tried.

Experiment 3.—In this experiment rabbit passage was alternated with culture *in vitro*. A rabbit was inoculated intratesticularly with whole culture and after 5 days the organs were removed. They were ground with sand and Locke's solution

[1] If it be assumed that 1 particle gives rise to a visible lesion, it can be shown by application of Poisson's binomial theorem that the suspension of particles giving rise to 50 per cent of positive results contains on the average 0.69 particles per unit volume, for $P = e^{-m}$ and for $P = 0.50$, $m = 0.69$.

to make a 10 per cent emulsion, and 0.2 cc. of this used to initiate a flask culture. After one subculture in flasks, the virus was returned to the testicles of a normal rabbit. This was repeated 8 times, and each time enough virus was recovered from the testicle to initiate growth in embryonic tissues, but not enough to survive a second rabbit passage.

This alternate rabbit and culture passage of virus failed to enhance the virulence of the virus sufficiently to allow successive rabbit passages. It was considered, however, that multiplication of the virus in the simultaneous presence of adult and embryonic cells might increase the rabbit virulence. This was tested as follows:—

Experiment 4.—Flask cultures were set up consisting of Tyrode's solution with 10 per cent of normal rabbit serum and an appropriate amount of minced tissue. This consisted of one-half chick embryonic tissue and one-half mouse kidney or rabbit kidney. It was first shown that medium containing mouse or rabbit kidney alone supported growth of the Board of Health strain of vaccine virus. The modified vaccine virus grew well under the conditions described and after 15 passages, 10 in chick embryo + mouse kidney and 5 in chick embryo + rabbit kidney, subcultures were made into medium containing rabbit tissue alone. No multiplication occurred.

These various attempts to enhance the growth of the rabbit virulent strain of virus in the presumed mixture of strains or to enhance its virulence having failed, efforts were made to secure a "pure culture" of the chick virulent strain by taking advantage of the wide disparity in the relative concentrations of the 2 sorts of virus.

Experiment 5.—A series of flasks of chick embryo-Tyrode solution culture medium was prepared and seeded with culture virus. Three flasks were inoculated with 0.1 cc. of each of a series of dilutions from 10^{-2} to 10^{-5} of the last previous culture. After 5 days incubation each flask was tested for the presence of virus: the flasks inoculated with virus diluted 10^{-4} contained virus, those inoculated with the 10^{-5} dilution did not. It was assumed that the flasks containing virus had been seeded with a minimal amount; the amount actually introduced was much less than that required to initiate recognizable infection in a rabbit. From the virus-containing flasks, a second series of dilutions of virus was prepared and medium seeded. Again virus appeared in large quantity in the flasks seeded with the 10^{-4} dilution, but none was present in those seeded with 10^{-5} dilution. The process was repeated once more. It was hoped that this serial culture of virus from a seed inoculum known to contain but a few chick infectious particles would rid the culture of rabbit infectious elements. The last culture when titrated in chick and rabbit gave these titers: estimated number of chick infectious units per cc., $10^{6.1}$; estimated number of rabbit infectious units per cc., $10^{2.1}$; difference, $10^{4.0}$.

It is evident from this experiment that a few chick infectious particles may give rise in culture to large quantities of virus, and that this virus when tested

has the same ratio between chick and rabbit titers as the initial material. It is evident then that the viral particles are essentially homogenous as far as this test is concerned.

RÉSUMÉ

Earlier studies of the First Revived Strain of vaccine virus seemed to indicate that infection in rabbits could follow the introduction of a single particle of virus. It was soon learned that many more viral particles were present than were infectious in the rabbit, and it was therefore assumed that particles of 2 sorts coexisted in the suspension. This conclusion was also arrived at by Gallagher and Woolpert (7), and the possibility is implied by Bryan and Beard (3) although with no experimental support. Attempts to favor the growth of the rabbit virulent component of the culture were unsuccessful, as were also attempts to free the chick virulent of rabbit virulent particles by using very dilute viral suspensions as seed inocula in cultures. These observations seemed to cast serious doubt on the first hypothesis and to suggest that the viral particles were essentially homogenous, a result requiring an entirely different concept of the mechanism of infection in the rabbit.

II. The Comparative Virulence of Vaccine Virus for Various Hosts

In the experiments outlined above a striking difference in virulence of vaccine virus for 2 different hosts was demonstrated. While it has long been known that not all animals are equally susceptible to all strains of a given virus, it seemed worth while to investigate the infectivity of a number of other strains of vaccinia for several hosts, applying quantitative methods as exact as conditions permitted.

EXPERIMENTAL

It was proposed to make simultaneous titrations of a number of strains of vaccine virus of widely differing virulence in a number of hosts; *viz.*, rabbit (intradermal inoculation), mouse (intracerebral inoculation), chick embryo (chorio-allantoic membrane), and guinea pig (intradermal inoculation).

Virus.—Virus of various sources was used, as follows:—

(*a*) Board of Health Strain (B H). Originally derived from the New York City Board of Health strain of virus, this strain has been propagated for several years in the testicles of rabbits. Subsequently it has been propagated by dermal inoculation.

(*b*) First Revived Strain of vaccine virus (C V I); derived from (*a*) and described in Part I above.

(*c*) Second Revived Strain of vaccine virus (C V II); derived from (*b*) by serial rabbit passages to enhance its virulence for this animal. Passages were made before the virulence had declined to its present level (5).

(*d*) Western Reserve Strain (W R); derived from (*a*) by 18 passages in mice by intracerebral inoculation (8).

(*e*) International Health Division (I H D). A strain of virus presumably derived originally from the B H strain, but propagated for many passages by intracerebral inoculation of mice, and kindly made available to us by Dr. J. E. Smadel.

(*f*) Connaught Laboratories (C L). Probably derived originally from the New York City Board of Health strain, it has been passed for many generations in rabbit skin, using elementary bodies as seed.

(*g*) Ohio State University—I (O S U). Originally a Lilly strain of vaccinia, propagated first by calf inoculation, and then on chick embryonic membranes, it was passed in fetal rabbits by Gallagher and Woolpert (7) with marked change in its virulence for adult rabbits. It was kindly made available to us by Dr. E. B. Adams and was received in its twenty-fifth fetal passage.

(*h*) Noguchi. Originally a dermal strain of vaccinia, this was adapted to rabbit testicular propagation by Noguchi (9). It has since been carried by rabbit testicular passage.

Animals.—Rabbits and chick embryos were selected and used as in the experiments of Part I. Large, white guinea pigs were used in order to facilitate the reading of skin lesions. Swiss mice weighing 15 to 25 gm. were secured from a single dealer.

Titration of Virus.—As end-point for all titrations the 50 per cent dilution was selected, with a theoretical inoculum of 1.0 cc. When intradermal titrations were made, a macroscopic lesion appearing after a latent period and present for 2 days was recorded as positive. Death of the animal was considered a positive result in the intracerebral titrations, since in the case of mice it was not considered possible to make sufficiently accurate clinical diagnoses of illness. Discrete lesions on the chick allantoic membrane were enumerated, and the results were adjusted on the assumption that 0.69 particles were present per unit inoculum when the 50 per cent end-point was computed. Accordingly the figures obtained with chick membrane were divided by 0.69 before inclusion in the tables.

A number of titrations of each virus were made in the available host animals.

As far as possible parallel inoculations were made at the same time from the same set of dilutions of virus. Thus a single series of dilutions of B H virus was divided into 3, and inoculations made intradermally in rabbits, intracerebrally in mice, and into embryonic membranes within 2 hours. At least 4 inoculations were made at each dilution, usually 5 or more. Membrane counts were based on at least 6 satisfactory membranes, and usually on 12, including membranes inoculated with 2 or 3 dilutions.

The results of these titrations are presented in Table II. To facilitate comparison the rabbit intradermal titer has been taken as standard, and titer in other hosts given in relation to this. A titer lower than that in the rabbit is expressed by a positive, a titer higher than that in the rabbit by a negative logarithm. Inspection of the table indicates that wide differences exist between the apparent titer in different hosts. Taking as a series the rabbit-mouse-chick embryo results, there appears to be a regular gradation in virulence

in the order I H D = W R > B H > C V II > C V I, expressed in the increasing amount of virus required to produce infection. Without final hypotheses as to the ultimate composition of the infectious unit, it is apparent that an inoculum producing disease in a given host contains a definite amount of virus. If then another host be inoculated, and require for the production of disease 100 times this viral concentration, it is reasonable to conclude that so far as the second host is concerned the virus is less virulent. In comparing the rabbit and mouse titers, it is seen that the W R strain gives only slightly higher titers in the rabbit skin than in the mouse brain. The original B H virus is definitely less virulent for the mouse, and with the C V II strain the difference

TABLE II

Titration of Vaccine Virus in Various Hosts

Strain of virus	Comparative titer (logarithm)*		
	Mouse (intracerebral)	Chick embryo	Guinea pig (intradermal)
WR	0.54		
I H D	0.54	0.56	2.97
B H	1.15	−0.53	2.45
C V II	3.42	0.32	2.48
C V I	—†	−4.83	—
O S U	—	—	3.10
Noguchi	−0.41	1.10	2.30
C L	5.30	0.33	1.55

* Figures represent logarithm of titer in rabbit skin minus log of titer in specified host. All reduced to a theoretical inoculum of 1.0 cc.

† No lesions with highest concentration of virus tested.

becomes impressive. The C V I strain by our criteria is avirulent for mice in concentrations we were able to secure.

The difference in virulence is expressed further in the character of the reaction, and in the type of the titration curve. In Figs. 2 and 3 are presented titration curves of W R and C V II strains, respectively, in mice. While the first departs definitely from the Poissonian curve, the second bears almost no resemblance to it. It appears that in addition to the requirement of greater quantity of virus to cause infection, other factors are operating, presumably to make the infection which does occur a milder one and allow host factors which are of minor importance in relation to a highly virulent virus to operate more effectively, thereby allowing difference between individual animals to become more apparent. This presumption is supported by data from the rabbit. The vaccinal lesions of B H strain resemble those of W R strain infection. Those of C V II strain are characterized by almost complete absence of massive necrosis, and no evident necrosis at all occurs with C V I strain

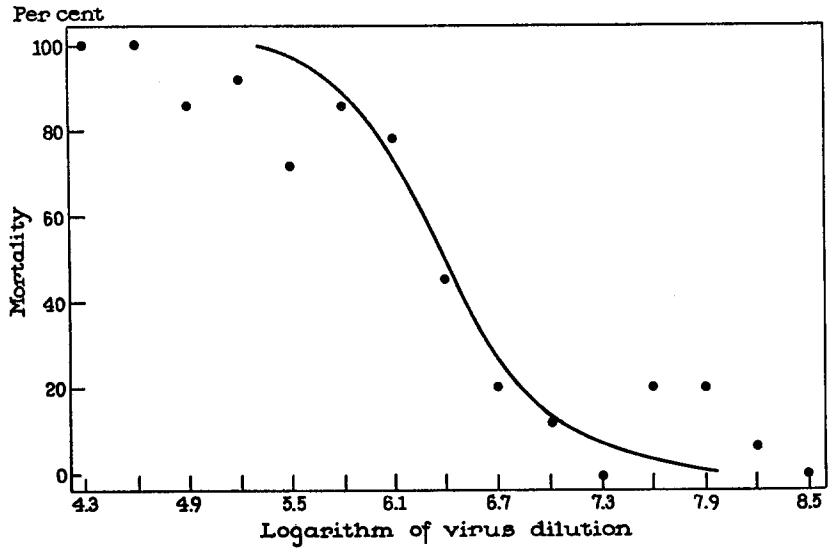

FIG. 2. Titration of W R strain of vaccinia in the mouse. Mortality per cent is percentage of mice succumbing within 14 days after intracerebral inoculation.

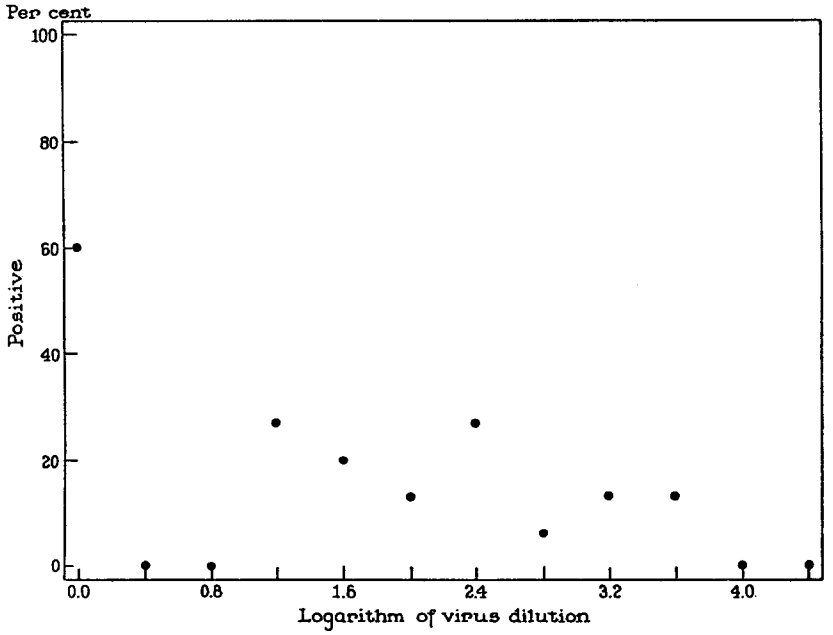

FIG. 3. Titration of C V II strain of vaccinia in the mouse. Per cent positive represents the number of mice dying within 14 days after intracerebral inoculation.

infection although examination with the microscope would probably reveal death of many individual cells. A similar tendency toward change in shape of the titration curve exists as was seen when virus was titrated in the mouse. With B H strain, or strains of similar virulence, no difficulty exists in demonstrating reasonably close correspondence between experimental and Poissonian curves when titrations in several rabbits are summed. With C V II strain the agreement is less good, and with C V I strain some difficulty exists in showing the relation. While the data on C V I strain presented in Part I do not repre-

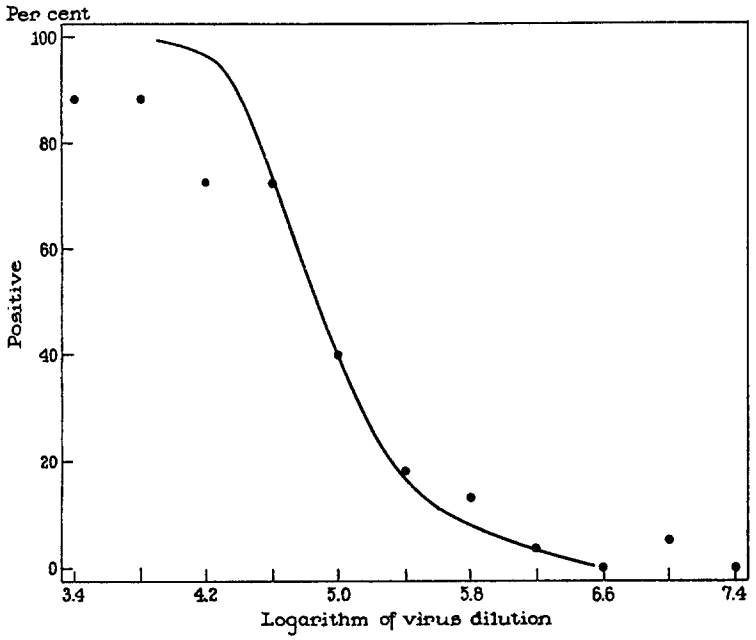

FIG. 4. Titration of C V II strain of vaccinia in rabbits by intradermal inoculation.

sent the results of unique experiments, most efforts to demonstrate the typical titration curve by summing the results obtained in several rabbits have failed. There are some data to support the possibility that results with this strain are more consistent when young animals are used for titration. In the case of C V II strain, it was pointed out (3) that the curves originally published do not fit the Poissonian distribution exactly and subsequent experiments have confirmed this. Results of such an experiment are presented in Fig. 4. As before, the change is in the direction of flattening of the curve, a characteristic which will be discussed in greater detail later.

The figures in column 4 (Table II) indicate that in general guinea pig titer is much below rabbit titer of the strains observed. They do not suggest any

striking gradation of virulence between the strains. That such a difference did exist was evident from observation of the character of the lesions. Those due to Noguchi virus characteristically proceeded to necrosis involving most of the original area of the lesion. Necrosis occurred following infection with B H strain but was much less extensive, while little if any followed inoculation with C V II or O S U strains. With all of the strains positive opinions as to the presence or absence of lesions due to the more dilute inocula were difficult to arrive at; the nature of the guinea pig skin probably contributed to this. Variation was observed between animals, but as with rabbits the range of dilution between that producing consistently positive and negative results was not much in excess of tenfold to fiftyfold. Exact observations were not attempted.

That the "attenuation" of these strains of vaccinia for the mouse first and then the rabbit does not represent a pure loss as far as the virus itself is concerned is shown by the results of chick embryonic inoculation. If ability to initiate infection in the passage host be taken as standard, no loss of virulence has occurred. More, an actual increase has been produced, for the lesions produced by C V I strain are larger and more diffuse than those of B H strain and whereas heavy inoculation with B H strain produces in 48 hours a membrane with confluent lesions, inoculation with C V I strain in similar amount produces almost always a dead embryo. Because of this, few membranes are seen with confluent lesions, and even with smaller inocula few embryos survive to the 3rd day.

RÉSUMÉ

Vaccine virus of several strains has been titrated in several hosts, and the results have been presented. Taking end-titers as indices, it is possible to arrange the strains in an order of virulence for mouse and rabbit with a mouse-brain-adapted strain (W R) first, then the original calf-rabbit strain (B H), a revived cultured strain (C V II), and finally a degraded culture strain (C V I). When titration curves are determined for each host a similar order is evident, although the data are not so exact. It is shown that while mouse and rabbit virulence are declining in the cultured strains, an enhancement of virulence for the passage host (chick embryo) is occurring and that the C V I strain, almost avirulent for the rabbit, is highly virulent for the chick embryo. In terms of end-titer all of the strains of vaccinia (which were primarily rabbit strains) were markedly less virulent for the guinea pig, although no such marked gradation in virulence was observed in this respect as has been noted above. Marked differences in the severity of the lesions produced were observed.

III. Statistical Considerations in Determination of the Infectious Unit

In Parts I and II of the present study, data were presented bearing on the infectivity of various strains of vaccinia for several hosts, and attempts to

separate single strains from a presumed mixture of strains of virus were described. It was shown that, both with regard to the end-titer of suspension and character of titration curve, gradation of virulence can be demonstrated when several strains are studied. It is proposed in the present section to consider these and other data in light of the hypothesis originally proposed that a single viral particle under the proper circumstances can give rise to infection.

It has been pointed out by Haldane that the commonly employed method of computation of χ^2 which we used to determine the goodness of fit of our curves was not fully adequate, and he suggested the use of a more accurate method. Application of this to the data for one strain of virus indicated that the hypothesis was a tenable one. Bryan and Beard (3) applied the method in a study of the virus of rabbit papilloma, as well as vaccinia, and pointed out that the proper calculation of goodness of fit for the data which we presented for other strains of vaccine virus indicated that for these strains the hypothesis which we accepted was not tenable, an observation which we have confirmed. They pointed out, moreover, that if the data for vaccinia were treated on the assumption that viral distribution was unimportant, but that the response of the rabbit skin was variable, and that the degree of resistance was normally distributed in terms of viral dose, a good agreement existed for all of the strains of virus between hypothesis and experiment. On this basis, they were led to conclude that the variation in response to small doses of virus was an expression simply of variable susceptibility of different rabbits, and of variation in susceptibility of different sites on the same rabbit. They showed that good fit between hypothesis and experiment could be obtained on the assumption that the degree of resistance to infection was normally distributed, applying here the technique of Gaddum (10).

EXPERIMENTAL

In this section it is proposed to reexamine certain already published data, and also to present the results of experiments designed to give information on the question of the possible rôle of host resistance in determining the infectious unit of vaccinia.

Materials and Methods

The experimental methods, strains of virus, and animals have been fully described in Parts I and II of this communication. Relevant details will be added in connection with individual experiments.

It was shown above that the infectious unit of virus for one host might represent a thousandfold multiple of the amount of virus which comprised an infectious unit for another host. Further, it appeared that as the virulence of a viral strain for a given host decreased, the titration curve in that host tended to depart more and more from the simple binomial expansion, but that even with a virus of low virulence for the rabbit, it was still possible to demonstrate that infection appeared to follow the introduction of a single viral particle. A definite departure from theory was found with the second revived strain of

culture virus, and an experiment was performed to gain more detailed information.

Experiment 6.—A suspension of virus of the C V II strain was freed of gross particles and clumped elementary bodies by strong centrifugation. Preliminary titration indicated a titer on rabbits of $10^{-4.5}$, and for accurate titration, therefore, suspensions were prepared in dilutions from $10^{-3.0}$ to $10^{-7.2}$, at intervals of $10^{-0.4}$. Rabbit intradermal inoculations were made in amounts of 0.1 cc., and 10 inoculations of each suspension made in each of 6 rabbits.

TABLE III

Results of Titration of C V II Strain of Vaccinia Simultaneously in Several Rabbits

	Dilution of suspension (logarithm)	Results of inoculation of individual rabbits													
		4-79		4-81		4-80		4-82		4-83		4-84		Total	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
	3.4	10	0			10	0	4	6	10	0	10	0	44	6
	3.8	10	0	10	0	10	0	4	6	9	1	10	0	53	7
	4.2	6	4	10	0	10	0	0	10	9	1	8	2	43	17
	4.6	10	0	8	2	10	0	0	10	7	3	8	2	43	17
	5.0	4	6	7	3	7	3	0	10	3	7	1	9	22	38
	5.4	1	9	1	9	3	7	0	10	2	8	3	7	10	50
	5.8	0	10	2	8	2	8	0	10	1	9	3	7	8	52
	6.2	0	10	0	10	0	10	0	10	1	9	0	10	1	59
	6.6	0	10	0	10	0	10	0	10	0	10	0	10	0	60
	7.0	1	9	0	10	1	9	0	10	0	10	0	10	2	58
	7.4	0	10	0	10	0	10	0	10	0	10	0	10	0	60
"50 per cent dilution"		4.84		5.13		5.29		3.49		4 84		4.86		5.00	
χ^2		1.18		3.26		0.15		3.9		5.3		7.2		102.00	
n		3.00		3.00		2.00		2.00		3.00		2.00			
P		0.79		0.36		0.92		0.15		0.26		0.03		<0.001	

The "average" titration curves, representing percentage of inoculations positive and negative for each viral dilution, are given in Fig. 4 of Part II. The lack of agreement between theory and experiment is evident, and is confirmed by the χ^2 test ($P < 0.001$). In Table III the data are given for the individual rabbits. It was postulated that each rabbit might constitute a separate universe in relation to the virus and the data have therefore been treated as 6 separate experiments. Inspection of the bottom row reveals in most cases a reasonably good agreement between theory and data. P for goodness of fit to the original Poissonian curve ranges from 0.03 to 0.92 and in no case is the value low enough to indicate that the hypothesis is certainly not tenable. For 5 of the 6 the P of 0.15 or more indicates reasonably good agreement.

The logical conclusion from this experiment would seem to be that, while a titration curve constructed for this strain by pooling results obtained with several animals is not a binomial one, the data for each rabbit which contribute to the total do follow the binomial expansion. It appears, moreover, that as the virulence of the virus increases, the summed curve tends to approach the binomial expansion as the limit. Additional evidence for this is obtained from physical and chemical studies.

Bryan and Beard pointed out that published data did not support the concept that one elementary body comprised an infectious unit. Reexamination of the papers quoted by them does not support their interpretation. Thus according to McFarlane's data (11) 1000 elementary bodies would be needed for an infectious unit (12*a*); however his preparations were apparently highly impure (12*b*). The figure arrived at by Sprunt, Marx, and Beard was 366 elementary bodies per infectious unit (13). This was based on physical characteristics determined by others, with different strains of virus, and the authors themselves gave very scanty data on the methods for purifying the suspension. They referred likewise to the figure of 42 elementary bodies per infectious unit based on work of Parker and Rivers (14). The preparations made in that study were not microscopically pure, as was pointed out at that time, although examination of the stained sediment did reveal a "minimum of extraneous material." Later intensive work on the physical and chemical constitution of vaccinia has continued to yield much information, and methods of purification have progressively been refined in that laboratory. The most recent relevant publication (12*b*) indicates a ratio of 4.2 elementary bodies per infectious unit, calculated from data on size and density obtained in the same laboratory. Further, the purity of the suspensions was checked and confirmed by ultracentrifugal analysis.

If the logic long honored in chemical research is applied to the data cited above, the conclusion seems inescapable that as purer suspensions of virus are obtained, the number of elementary bodies per infectious unit for virus of high virulence approaches 1, and the fact that with impure preparations it is higher substantiates not vitiates this conclusion.

As was noted in Part II the infectious unit of vaccine virus for the guinea pig contained many rabbit skin infectious units, and likewise some difference was observed in the reactivity of single pigs. While conditions of space as well as difficulties in exact classification of reactions precluded exact measurements, it was evident that the character of the titration curve for the individual pigs was not inconsistent with a binomial distribution of lesions. Similarly with C V I strain in rabbits, the infectious unit comprised many chick infectious particles. It was shown that with C V II strain, while the distribution calculated from the sum of several rabbit titrations did not follow a Poissonian distribution, the titration curve of each individual rabbit very definitely did. It appeared important, therefore, to reexamine the results obtained with C V I strain.

Experiment 7.—A large volume of culture of C V I strain was prepared, free of tissue, and concentrated by centrifugation. The virus was in its fifty-third culture passage in this laboratory. Titration in chick embryonic membranes indicated a concentration of chick infectious particles of $10^{6.1}$ per cc. From the concentrated suspension twofold dilutions were prepared, and inoculated into rabbits in 0.1 cc. amounts, 10 inoculations per rabbit per dilution. The rabbits were observed daily, and presence or absence of lesions at the site of inoculation was recorded. As before, difficulty was experienced in classifying the results with complete assurance; the data accepted represent the opinion of 2 observers. They are presented in Table IV.

TABLE IV

Titration of C V I Strain of Vaccine Virus in Rabbits

Dilution of suspension (logarithm)	Rabbit No.						
	5-76	5-77	5-78	5-79	5-80	5-81	Total
0.0	10*	10	10	10	10	8†	58
0.3	10	10	10	9	10	10	59
0.6	10	10	10	7	10	10	57
0.9	10	8	9	5	10	10	52
1.2	7	9	7	1	10	10	44
1.5	0	6	0	0	9	0	15
1.8	0	0	0	0	0	0	0
2.1	0	0	0	0	0	0	0
2.4	0	0	0	0	0	0	0
2.7	0	0	0	0	0	0	0
50 per cent dilution	1.29	1.47	1.26	0.72	1.63	1.35	1.32
P (1 particle)	<0.001	<0.001	<0.001	0.48	<0.001	<0.001	
P (4 particles)		0.07					
P (10 particles)	0.22	0.01	0.31	<0.001	0.14	0.06	

* Number of inoculations positive of a total of 10.

† Total of 8 inoculations of this dilution.

Inspection of Table IV reveals marked disparity between theory and experiment for the summed titration curve. Separating this into its component curves does not help much, as inspection of the row designated *P* (1 particle) indicates. In one set (R 5-79) good agreement is indicated, in the others the hypothesis that a single particle causes infection is obviously untenable. When the data are reconsidered on the assumption that 10 particles are required to cause infection, 4 of the sets indicate a possibility of agreement with the hypothesis and the remaining set can be harmonized with the assumption that the number of particles required is intermediate.

RÉSUMÉ

The data presented in this part appear to indicate that with increasing virulence of a viral strain, the titration curve derived from the results of inocula-

tion of several rabbits tends to approach as its limit the Poissonian binomial curve, and reconsideration of published data on the physical characteristics of an infectious unit indicates that with virus of maximum virulence an infectious unit is contained in a single elementary body. As virulence declines, the infectious unit comes to contain more and more elementary bodies, and the titration curve departs more and more from its original form. When the loss of virulence is moderate, as it is with the C V II strain, examination of the titration on individual rabbits indicates that for a single animal the binomial expansion, using the assumption that single particle initiates infection, describes the findings adequately. The lack of agreement with theory of the summed curve is largely due to variation between rabbits. When virulence is almost completely lost, as it is in the C V I strain, not only does the summed curve tend to depart from the original one, but the data of the individual animals also tend to become discrepant. Here the curve does not have a decreased but a sharply increased slope; the tendency is in the direction which would be expected on the assumption that more and more particles are required to initiate infection. As with the other strain of virus, variation in susceptibility of rabbits is observed.

DISCUSSION

It was postulated by early workers in the field that the viruses require an intracellular location for growth. Subsequent work has tended only to support that conception, and it seems well established now that while the virus of vaccinia will survive *in vitro* in the absence of living cells, for multiplication it requires living cells, and some evidence is already available that it requires cells of a particular sort. Thus it was shown by Rivers (15) that in the cornea it was principally the young cells, growing in response to the stimulus of trauma, which are readily available for vaccinal infection. Further, the data of Part II strongly suggest that difference exists between embryonic and fully differentiated cells with regard to susceptibility to infections. Much supporting evidence for this conception might be gathered from characteristics of other viruses; *e.g.*, the predilection of the poliomyelitic virus for anterior horn cells, of louping ill virus for the monkey's Purkinje cells, of viscerotropic yellow fever for hepatic, and of neurotropic yellow fever for nervous cells.

Obligate parasitism connotes an inability of the parasite to survive without deriving energy from a more complete organism, and the intracellular habitat of viruses strongly indicates that the source of energy for the virus is located near the heart of the cell's own economy. If this be true, adaptation on the part of a virus would consist in development of those energy-producing systems which would function most efficiently in the presence of the particular conditions existing within the available cells. Cells are known to differ widely in their composition and energy-producing mechanisms, a difference which would

be expected to reach its full development with the maximum differentiation of the cell. It is not implied that such differentiated cells bear no physiologic resemblance to other fully differentiated cells of another sort, but it seems apparent that profound differences may exist, and it does not seem unlikely that virus well adapted to survival under one set of conditions, might find another intracellular situation so unsuitable for multiplication as to render successful parasitism impossible. Accordingly, with regard to the initial phases of viral infection each host is to be regarded not as a single animal, but as a universe of cells differing widely in internal composition, some suitable, some unsuitable for viral multiplication. The possibility that such a situation might exist was suggested by Sprunt and McDearman (16), who pointed out that the "probability of insertion" of a particle was really the probability that the inoculated particle reached a susceptible cell. A virulent virus then would be one capable of deriving energy from a system common to many cells. Loss of virulence might be the expression of general loss of "growth potential," but it might also be the expression of the development on the part of a viral strain of capacity to parasitize cells having one sort of make-up at the expense of ability to parasitize a cell with different predominating energy systems. "Virulence" then has meaning only when both virus and host are exactly characterized. In developing this conception fully, it might be postulated that differences between individual animals with respect to viral infection are expressions of differences in the proportion of cells available and unavailable for parasitism. With a fully virulent virus, able to parasitize cells of a wide range, the probability of infection would be essentially the probability of insertion of a viral particle. With decline in virulence for a given host (perhaps in the course of adaptation to a different host) fewer and fewer cells would be available for parasitism, and 2 factors would then combine: the probability of inclusion of a viral particle in the inoculum, and the chance of a viral particle entering a susceptible cell. If but 1 host animal is available, no possibility exists of distinguishing between these possibilities on the basis of the results of inoculation. However, it is likely that with decreasing virulence and smaller number of cells available, individual animals will differ in the proportion of susceptible to resistant cells. Then, as far as the individual animal is concerned, the distribution of lesions in relation to viral concentration will remain the same, but combination of the data from several animals will no longer give a binomial distribution. To the binomial distribution obtained when the single animal is observed, has been added a normal distribution expressing the differences between animals. The data indicate that this is the case here.

It was shown that the difference in titer of C V I strain of virus as determined in 2 hosts was of the order of 10^5; the hypothesis elaborated above would imply that the ratio of C V I susceptible cells between chick embryo and rabbit is of the order of $1\text{–}10^5$. If this be true, it is difficult to visualize the way in which

infection, even if initiated, could be maintained, but in this circumstance other factors enter. It may be taken as axiomatic that only virus which enters susceptible cells can cause infection, but it must also be recognized that the viral particles do not necessarily have a predilection for entering such cells. In fact, examination of tissues after injection of identifiable particles shows that there is a marked tendency for the foreign material to be engulfed by cells of a particular type which have been variously designated, but may be described generally as phagocytes. Some of the material also enters lymphatic channels and is removed to a distance. It does not seem unreasonable to assume that the locally pathogenic action of viral particles carried away in lymphatics or engulfed by phagocytes may be reduced when not prevented completely. This leaves a proportion of particles, assumed to be constant for each animal, which are free to enter other cells of various sorts, and it is upon the chance that these cells are susceptible to viral growth that the chance of infection depends. Therefore, the infectivity ratio of $1–10^5$ does not necessarily mean that for each rabbit cell available to infection there are 10^5 unavailable, but simply that the chances of a particle entering a susceptible cell are 1 in 10^5. To this must be added the possibility that the infection which might arise from parasitization of a single cell might not cause a tissue reaction of sufficient magnitude to produce that erythema and edema which are essential to a diagnosis of local vaccinal infection. The combination of several such foci, each arising from a single cell, might, however, cause visible signs of inflammation. The data of Experiment 7 are consistent with this postulate. It is not to be anticipated that the number of foci involved in different rabbits will be uniform, for aside from the chance of combination of foci, there is added the variation in reactivity of rabbits to a uniformly noxious agent. Likewise too much stress is not to be laid on the fact that good agreement is obtained between an experimental curve and a theoretical one deduced from the assumption that 5 or 10 or any other number of foci must be present to cause visible signs of infection. Many factors are operating, not all of which are known, to produce the observed distribution. The essential element is that the slope obtained is much steeper than expected for a 1 particle distribution, which is consistent with the hypothesis proposed, and which would not be expected to follow the operation of the usual random errors.

CONCLUSIONS

A study has been made of the comparative virulence of several strains of vaccine virus for a number of hosts, and wide variation in animal susceptibility has been demonstrated. The results obtained in experiments with a chick-embryo-adapted strain are interpreted as indicating that the particles of virus are of essentially uniform virulence. Results of statistical analyses are presented which indicate that as the virulence of a strain of virus increases the

number of elementary bodies per infectious unit approaches 1, and at that limit the chance of infection is governed primarily by the presence or absence of virus in the inoculum. With lower virulence the chance of a lesion following inoculation of virus is still described by the binomial theorem, but the actual distribution is primarily of susceptible cells not of viral particles. It is postulated that with regard to the proportion of cells available for parasitism, differences exist between different animals of a species, and that this distribution is of a normal character.

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