STUDIES ON FILTERABLE VIRUSES *

III. FURTHER OBSERVATIONS ON VACCINE VIRUS

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In two previous papers,[1, 2] we have described certain experiments in which vaccine virus was successfully cultivated *in vitro*. It is our intention in this paper to report further studies on some of the cultural, biological and immunological aspects of this virus. Gordon [3] has recently published his exhaustive study of vaccine virus. In this, he has covered several points that we take up but since he was working with calf or rabbit lymph, while we used culture virus, we feel it worth while to report our results.

INCREASE OF VIRUS IN CULTURE

As described in one of the foregoing papers,[2] a strain of vaccine virus (VC 10) was cultivated for a period of fifty-six days, during which time it was transferred eleven times. Titrations of the virus content were made at various times and it was found that the eleventh generation contained 51,000 times as much virus as the original inoculum. We decided to repeat this experiment using the same methods for cultivation and titration.

The culture (VC 30) was started from bits of infected testis and normal plasma. The results are given in Table I. The actual virus content at the start was not determined, but after seven days incubation, it was equivalent to fifty skin doses per culture. The rate of increase on the whole was similar to that found in the previously described culture, VC 10. The twelfth generation of VC 30 contained a very large amount of virus. One-tenth of a cubic centimeter of a 1:50,000 dilution of two cultures gave a positive take. Since a culture weighed approximately ten milligrams or one-hundredth of a gram, a gram of this culture contained 25,000,000 skin doses. After the twelfth generation the potency rapidly diminished. The thirteenth contained only one-fifth as much virus, and the fifteenth less than one-hundredth as much virus as the twelfth gen-

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eration. Thereafter even the whole dilution failed to give a take. Such a record again demonstrates clearly that multiplication of the virus takes place under such conditions of cultivation.

The sudden drop in potency of VC 30 after about three months cultivation checks quite closely with another culture (VC 6A) with

TABLE I

Growth and Titration of Vaccine virus Culture, VC 30

Generation	Date	Split	Titration		Taking skin doses per culture	Virus content
			Amount used	Result		
	Aug.					
I	4		2 cultures	0.1 cc. 1:10+ 0.1 cc. 1:100−	50	1
II	11					
III	18					
IV	25		1 culture	0.1 cc. 1:500+ 0.1 cc. 1:1,000 not done	5,000	100
	Sept.					
V	1	yes				
VI	8		2 cultures	0.1 cc. 1:5,000+ 0.1 cc. 1:10,000 ±	25,000	1,000
VII	15	yes				
VIII	19					
IX	26		2 cultures	0.1 cc. 1:5,000+ 0.1 cc. 1:10,000−	25,000	2,000
	Oct.					
X	3					
XI	8	yes				
XII	15		2 cultures	0.1 cc. 1:50,000+ 0.1 cc. 1:100,000 not done	250,000	40,000
XIII	22	yes	1 culture	0.1 cc. 1:5,000+ 0.1 cc. 1:10,000−	50,000	16,000
XIV	29					
	Nov.					
XV	5		1 culture	0.1 cc. 1:50− 0.1 cc. 1:10 not done	500	320

which positive skin takes were not obtained after ninety-seven days incubation. The reasons for such decreases in potency are at present obscure. They might be any one or all of these three factors: (1) diminution in virulence of the virus even although growth continues; (2) cessation of growth; (3) development of viricidal properties on the part of the tissues in the cultures. Further studies are necessary to elucidate this problem.

Certain Factors Involved in Virus Growth

(*A*) *Necessity of Living Tissue:* Having convinced ourselves that growth does take place beyond question, we then attempted to ascertain some of the factors involved.

Steinhardt and Lambert [4] concluded that living tissue was necessary for the growth of the virus. In certain of our control experiments in which the tissue was not washed or renewed and therefore died, we found that the virus did not survive. This was in accordance with the above-mentioned workers' views. In order to demonstrate this point more conclusively, cultures were set up using testis killed by freezing with a control of living testis. The cultures were set up with pieces of normal testis (one set frozen, the other untreated) and plasma to which had been added a known amount of culture virus. The control showed a strongly positive take in the first generation after incubation, whereas the culture made with killed testis was negative in both the first and second generations. Such a result confirms the view expressed above: namely, that living tissue is essential not alone for the growth but even for the survival of the virus. It also would argue against the view that the increase of virus in cultures is not due to multiplication but due to disintegration of inclusion bodies with the consequent setting free of the virus.

(*B*) *Anaerobiosis:* Plotz [5] in 1922 described the successful cultivation of vaccine virus in Smith-Noguchi tubes in which conditions closely approaching complete anaerobiosis must have been obtained. We therefore attempted to determine the effect of such conditions applied to our methods. As our culture dishes were sealed with vaseline and contained growing tissue, it seemed possible that the oxygen in the cultures might be exhausted and that the virus consequently was growing anaerobically. Therefore, the air in such a plate was analyzed for its oxygen and carbon dioxide content. It was found that oxygen was present and was but slightly lower than that of air under normal conditions; the carbon dioxide was slightly increased. Hence our cultures were not anaerobic. Next, to study this point further, we incubated cultures in an atmosphere devoid of oxygen. In no instance was there evidence of growth or even survival of the virus, for we were never able to get a positive take even with the whole dilution of the first generation. This apparent disagreement with Plotz' results made it seem advisable to attempt to repeat his

work. Using exactly his technic, we repeated his experiments with absolutely negative results. With an original inoculum many times as potent as his, we were unable to demonstrate any increase or survival of the virus. Since it seemed probable that testis would be a more favorable tissue than kidney, several series of tubes were set up using this tissue. A questionable take was obtained in one tube in the third generation but the duplicate tube was negative as were later generations. In short, all our attempts to repeat Plotz' work were unsuccessful.

Effect of Temperature

Using culture vaccine virus, we carried out some experiments planned to show the effect of different temperatures on the survival of the virus. In every instance the virus content in skin doses was known. The results are given in Table II. Twenty minutes at 55° C apparently completely destroyed the virus while shorter

Table II

Survival of Virus at Various Temperatures

Temperature	Duration	Result
55° C	5 min.	+++
	10 min.	++
	20 min.	−
37.5° C	24 hours	−
6° C	14 days	++++

In this and the succeeding tables, the +'s indicate the degree of intensity of the positive takes; a negative take is shown by the sign −.

periods showed decrease in potency. Gordon [3] found that thirty minutes at 55° C completely destroyed most samples of calf lymph although a few gave weak takes after such treatment. It is interesting to speculate whether such diminution is due to diminished virulence of all the virus or to whether an increasing proportion is killed and the takes are due to the few surviving. After twenty-four hours at 37.5° C no takes could be obtained while at 6° C the virus survived at least fourteen days. The suspensions of the virus in each instance were made in Locke's solution.

Influence of Hydrogen Ion Concentration

Culture virus suspensions of known strength were made in 50 per cent glycerine containing 5 per cent disodium phosphate, adjusted to the desired hydrogen ion concentration. The final dilutions were such that 0.1 cc. originally contained 1,000 skin doses. The tubes were kept at 6° C. The results are given in Table III. At pH 5.0, the

Table III

Survival of Virus at Various $[H]^+$ at 6° C

$[H]^+$	2 days	7 days	14 days	28 days
8.0	++++	++++	±	–
7.0	++++	++++	+++	–
6.0	++++	++++	++	–
5.0	++++	+++	–	–

virus content was diminished after seven days and no virus could be demonstrated after fourteen days. At pH 6.0 and pH 8.0, after seven days the virus was unchanged but at fourteen days was markedly weaker, especially in the pH 8.0 tube. At pH 7.0, the potency was undiminished after seven days and but slightly decreased after fourteen days. All tubes were negative after twenty-eight days. It would appear that the optimum pH for the survival of the virus in glycerine at icebox temperature is in the neighborhood of pH 7.0.

Effect of Centrifugalization

If vaccine virus is particulate, it is reasonable to expect to concentrate it by centrifuging. MacCallum and Oppenheimer [6] have described a differential method for such concentration. In our experiments a culture virus containing less than five skin doses per 0.1 cc. was centrifuged at about 2,800 revolutions per minute for one hour. The fluid at the top gave a moderately positive take when undiluted but diluted 1:10 was negative. The fluid at the bottom gave a strongly positive take and, diluted 1:10, a weak but definite take. Gordon [3] obtained similar results. In one of his experiments, the fluid before centrifuging was positive up to a 1:10,000 dilution, whereas after centrifuging, the top layer was positive only up to 1:1,000 while the botton layer gave a take at 1:50,000. Such evi-

dence would favor either the particulate nature of the virus or the association of the virus with particulate matter.

Filtration

Casagrandi[7] in 1909 was the first to filter vaccine virus successfully through a porcelain filter. Since then, both successful and unsuccessful results have been reported. Since we were working with a culture virus free from cells, we felt that conditions were favorable for filtration if such a thing was possible. Two experiments were carried out using a small Mandler filter. The tightness of the filter was controlled by adding to the virus suspension a loopful of bacteria from a twenty-hour throat culture on blood serum. Subcultures of the filtrate to plain broth were negative. The first virus suspension was such that 0.1 cc. contained 2,500 skin doses. The filtrate did not give a take. To rule out the possibility that the virus had passed through the filter but not in sufficient amounts to give a take, some of the filtrate was used to dilute normal plasma and cultures were set up with this and pieces of normal testis. The cultures were carried through three transfers and then tested with negative results. The second virus suspension was so diluted that 0.1 cc. contained five skin doses. All tests with the filtrate were negative. Gordon,[4] likewise, was unable to pass the virus through Berkefeld filters.

Viricidal Action of Hyperimmune Calf Serum

These experiments were made possible through the kindness of Dr. Benjamin White of the Massachusetts State Antitoxin and Vaccine Laboratory. A calf was hyperimmunized against vaccine virus by primary scarification followed by subcutaneous and then intravenous injections over a period of three months. Two weeks after the the last injection the calf was bled. Some of this serum was used by us in the following experiments. The normal serum employed was obtained from the same calf before immunization.

In the first set of experiments, cultures were set up in the usual manner except that instead of diluting the plasma with Locke's solution, it was diluted with two parts of either 50 per cent normal or 50 per cent immune calf serum. After seven days incubation each culture was ground with sand in the usual way and various dilutions were made and tested. The results are given in Table IV. The cul-

ture containing normal serum showed definite takes as high as 1:100 and questionable takes at 1:1,000 and 1:5,000 whereas that to which immune serum was added was negative at 1:10.

In order to titrate the viricidal action of the immune serum *in vitro*, varying dilutions of culture virus were incubated for one hour

TABLE IV

Viricidal Action of Hyperimmune Calf Serum in Cultures

Culture with Normal Serum		Culture with Immune Serum	
Dilutions	Results	Dilutions	Results
1:10	++++	1:10	−
1:100	++	1:100	−
1:1,000	±	1:1,000	−
1:5,000	±	1:5,000	−

TABLE V

Viricidal Action of Hyperimmune Calf Serum in Vitro
(Varying dilutions of virus + an equal amount of undiluted calf serum)

Virus Dilutions	Results with Normal Serum	Results with Immune Serum
1:1,000	−	−
1:100	+++	−
1:10	++	−

at 37.5° C with equal amounts of undiluted immune and normal serum. The various tubes were then tested as usual by intradermal injections. The results are shown in Table V. The virus was of such potency that 0.1 cc. of a 1:10 dilution contained 5,000 skin doses. The normal serum neutralized the 1:1,000 dilution but did not affect the 1:100 and 1:10 dilutions. The immune serum neutralized through the 1:10 dilution. Since 0.1 cc. of 1:1,000 virus contained $\frac{5000}{100}$ or 50 skin doses and since 0.1 cc. of normal serum neutralized this amount, then 1 cc. of normal serum could have neutralized 500 skin doses. Applying the same method of calculation, 1 cc. of the immune could have neutralized at least 50,000 skin doses.

Since the limit of viricidal or neutralizing power of the immune serum was not determined in the last experiment, another was set up using fixed amounts of virus, 0.1 cc. containing 500 skin doses, and

equal amounts of increasing dilutions of normal and immune serum. The tubes were incubated and tested as before (see Table VI). The normal serum failed to neutralize even when undiluted whereas the immune serum neutralized definitely at a 1:1,000 dilution. On the

TABLE VI

Viricidal Action of Hyperimmune Calf Serum in Vitro
(Varying dilutions of serum + an equal amount of virus, diluted 1: 10)

Serum Dilution	Results with Normal Serum	Results with Immune Serum
Undiluted	++	–
1: 10	++++	–
1: 100	+++	–
1: 1,000	++++	–
1: 10,000	++++	±

basis of this experiment, 1 cc. of normal serum could have neutralized less than 5,000 skin doses while the immune serum was capable of neutralizing as many as 5,000,000 skin doses. In the one experiment tried by Gordon, 1 cc. of immune serum neutralized 25,000 skin doses.

SUMMARY

1. Further experiments are described demonstrating the multiplication of vaccine virus in tissue cultures.

2. Vaccine virus could not be cultivated using killed tissues or under anaerobic conditions.

3. The virus when suspended in Locke's solution, was destroyed if heated to 55° C for twenty minutes or to 37.5° C for twenty-four hours.

4. The optimum hydrogen ion concentration for survival of the virus in glycerine was found to be pH 7.0.

5. After centrifugalization, the bottom layer contained more virus than the top layer.

6. The virus could not be filtered through a Mandler filter.

7. Experiments on the viricidal action of hyperimmune calf serum are described.

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