THE FIXATION AND PROTECTION OF VIRUSES BY THE CELLS OF SUSCEPTIBLE ANIMALS

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PLATE 31

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The nature of the relation existing between viruses and the cells they affect is fundamental amongst the problems presented by these causes of disease. Some authorities believe that virus phenomena should be interpreted in terms of what is known of bacteria, while at the other extreme are those who hold that certain viruses at least are the inanimate products of disordered cells. The association with the latter is evidently very close. But is it obligatory? Are the viruses intracellular as a class? If so how do they first come to influence cells, and what is the nature of the later relationship? Experiments *in vitro* with tissue fragments have failed to answer these questions because of the complexities inevitable with organized material. Were it possible, however, to liberate from it the cells as individuals and keep them alive during tests with viruses, something might be learnt. This has been done in the work to be presented here. One of the two viruses studied, vaccinia, causes necrotizing lesions; and many facts indicate that it is a particulate, living entity. The other, the Shope fibroma virus (1), which has only recently become available for study, engenders growths with the appearance of cellular, rapidly proliferating, connective tissue tumors. Instead of killing cells it stimulates them to multiply; and in this respect it resembles the agents responsible for the chicken sarcomata, as Andrewes has pointed out (2). But the Shope virus gives rise on occasion to inclusion bodies in the overlying epithelium, and eosinophilic inclusions are not infrequently visible in the proliferating connective tissue cells; while

furthermore the growths induced by the virus regularly retrogress and disappear, and they have sometimes a large inflammatory element.

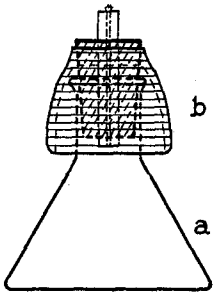
The Cell Material

When a culture containing tissue that has grown out into a plasma clot is submitted to a trypsin solution (3) many of the proliferating cells "let go hands" as the clot digests and round up separately like leukocytes. They can now be washed and plated in plasma, there to grow anew. For present purposes they have been exposed instead to virus-containing fluids, treated in various ways, and inoculated into animals susceptible to the viruses.

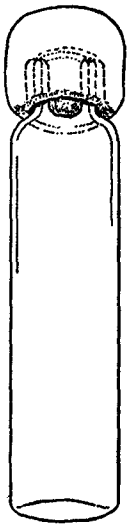
Rabbit embryos 18 to 24 days old were procured aseptically, decapitated, and hashed in a grinder. The fragments were washed three times by allowing them to settle out of suspension in Tyrode's solution containing ⅛ per cent gelatin, and were then implanted in a 1:3 mixture of rabbit plasma and Tyrode solution. To every 25 cc. of plasma, 1.8 cc. of 1 in 1000 heparin solution was added at the time of collection. Pyrex flasks of special shape served as culture chambers. They were Ehrlenmeyer in type, of about 35 cc. capacity, with an unusually broad and flat bottom (Text-fig. 1 *a*); and they were closed with flanged stoppers (*b*),—made by cutting off both ends of an ovoid 20 cc. rubber bulb and slipping it over a rubber stopper,—thus protecting the lip of the flask from bacterial contaminants which might "creep" into the medium. A thick-walled, cotton-plugged, capillary tube, extending through the stopper, provided for readjustments of air pressure. 1½ cc. of the suspension of the tissue fragments was put into each flask, followed by ½ cc. of rabbit plasma, with brief agitation; and after a few minutes, when clotting was complete, 2 cc. of a 1:3 mixture of rabbit serum and Tyrode was superimposed. From eight to ten flasks were used for each sowing,—which was done in a room ventilated with filtered air and sprayed beforehand to carry down dust. The cultures were washed every 2–3 days with plain Tyrode, and the supernatant fluid renewed. Profuse growth by the end of the period was the rule. Contamination was infrequent. Just prior to the digestion to free the cells, after 2 to 7 days of incubation, the cultures were searched with a microscope, and any showing bacterial colonies were discarded.

The trypsin solution was prepared by dialyzing Fairchild's trypsin against several changes of distilled water at 4°C. until the enzyme precipitated out. The supernatant was discarded, the sediment made to 0.9 per cent NaCl by adding concentrated salt solution, further diluted to 5 per cent in terms of the original material, and passed through a Berkefeld filter. With phenol red the reaction of the fluid proved to be about pH 6.5. It was stored at −5° to −8°C. in pyrex tubes capped with rubber corks; and just prior to use a tube was thawed and diluted with Tyrode to ½ per cent or 1 per cent, depending on its activity. No impor-

tant deterioration of the enzyme took place in several weeks. Standardization tests of its rate of digestion at 37°C. were carried out with specimen cultures. A solution strong enough to digest the clot in 45 to 60 minutes proved best, as yielding numerous living cells in not too long a time, stronger solutions tending to kill them. Digestion was started with 4 cc. of trypsin solution to each culture, poured on after removal of the supernatant fluid; and 30 to 60 minutes later this was replaced with 2 cc. more, and sometimes replaced yet again in case the skim of clot immediately about the liberated tissue fragments had not wholly digested away. A magnifying glass was required to make certain of this. All the digests as poured off were added to the supernatant culture fluid, the serum component of the latter acting to check further digestion; and a final dilution was done to 160



TEXT-FIG. 1. × ½.



TEXT-FIG. 2. × ½.

cc. with gelatin Tyrode. Except when the liberated cells were to be submitted to ultraviolet light the Tyrode employed with them regularly contained ⅛ per cent gelatin, since this acts to prevent mechanical injury to cells repeatedly pipetted while in suspension (4). The abbreviations p. Ty. and gel. Ty. will be used in the protocols for plain and gelatin Tyrode respectively.

The diluted digest was cloudy with individual cells. Forcible pipetting of the tissue fragments to set more of them free liberated fibrils as well and was avoided. The undigested debris was removed as follows:—

The cell suspension was passed through two layers of very fine gauze tied like a bag into a thistle tube, and then through two of washed, close-textured lens paper. Slow centrifugation was done for 5 minutes in four 50 cc. pyrex tubes closed with corks having a central core to keep them in place and a flange to protect the lip

(Text-fig. 2). The bottoms of the tubes had been flattened, so that the cells might come down in a thin layer; for they proved difficult to resuspend without damage when gathered into a mass. Flat discs of rubber and wood at the bottom of the centrifuge shells supported the tubes. The cloudy supernatant was drawn off through a capillary with a recurved end to prevent sucking up of the sediment, which was resuspended in 45 cc. of gel. Ty. for each tube. A drop of the material now showed great numbers of rounded, individual cells and occasional small, loose, grape-like clusters. Repeated filtrations and pipettings, with the end of the 50 cc. pipette held against the tube bottom, sufficed to remove or break up the latter. Centrifugation was now done as before, for 10 minutes, the supernatant discarded, the cells made into a suspension of slightly greater bulk than was needed for the experiment; and they were passed again through doubled lens paper, examined microscopically, and repipetted and refiltered if necessary. The ultimate, somewhat cloudy suspension showed the cells as separate spheres (Fig. 1) with, rarely, two or three joined by flat surfaces. Preparations stained with Loeffler's methylene blue were searched for bacteria. They were not always absent, since the liberation by trypsin of a single, overlooked colony in one of the many flasks of each digestion sufficed to contaminate the material. But they were found in only three of twelve instances and then in small number. All three instances happened to be those of experiments selected for detailed report here because of their comprehensiveness. In each case the contaminating organism proved to be non-pathogenic, as will appear when the protocols are given.

For some test mixtures the cells were killed by heating a part of the final suspension in a water bath at 53°C. for 15 minutes, or by exposing it to ultraviolet light. When light was used the preliminary washings and suspensions were carried out with p. Ty., the material was put in a flat-bottomed dish, covered with a sheet of quartz glass, and exposed to a mercury vapor, quartz lamp placed vertically above and 48–50 cm. away. A thick sheet of aluminum under the dish effectually conducted heat away during the exposure, the thermometer showing a rise of only a fraction of a degree C. at most. The cells killed by heat withstood the subsequent washings well, whereas those rayed for 10 minutes became so fragile that some break-up and loss occurred during even the most gentle pipetting. This loss did not alter the experimental results significantly as the protocols attest. Cells rayed for only 5 minutes stained like dead cells with neutral red, and had but a slightly increased fragility.

Several drops of the ultimate inocula, cells which had been exposed to a virus, were examined microscopically just prior to injection, on a slide coated with neutral red and ringed with vaseline. No secondary clumping of the cells had occurred. A large proportion of those not heated or rayed segregated the dye into vacuoles, showing that they were alive; and the appearance of others indicated that they were living but of different sort. Dead cells did not segregate the dye or show protoplasmic movement, and their nuclei were sharply outlined. Rayed ones were often fragmented or fatty.

The Viruses and the Immune Sera

Dr. Rivers kindly provided us with vaccine virus of the New York City Board of Health strain, in the form of a 28th subculture with chick embryo tissue and Tyrode *in vitro*. The pathogenicity of the strain had been revived twice by intratesticular inoculation into susceptible rabbits (5). For our own purposes it was propagated further in the testicles of rabbits.

The lesions caused by intradermal injection of the culture strain of virus have been studied by Rivers and Ward (6). When the liberated cells of our experiments were exposed to it briefly and washed and injected intradermally in rabbits the characteristic lesions of vaccinia resulted. In evaluating the results the rate at which the lesions appeared and spread, as well as their character, proved of importance. When but little virus was carried by the cells, or it was attenuated, the lesions were small and nodular, each nodule consisting of a necrotic focus with profuse cellular proliferation round about, as the microscope showed. Such lesions appeared relatively late, slowly increasing in size at a time when those due to more active material were already retrogressing in the same animal. Active virus caused discoid, cutaneous thickenings that were often several centimeters across, with more or less extensive vesiculation and superficial necrosis, in some instances hemorrhagic. Generalized pocking was rarely encountered, even when a number of large local lesions had been induced.

We are indebted to Dr. Shope for three strains of the virus causing the fibroma of rabbits. It was received as glycerinated tissue. The first strain tested gave rise to discoid, raised, red thickenings when a Tyrode extract of the ground tissue, cleared with the centrifuge, was inoculated into the skin; but the thickenings consisted in the main of a brawny edema with secondary pressure necrosis, pronounced reactive inflammation round about, and, exceptionally, small foci of highly abnormal, large cells of fibroblastic type, showing occasional mitotic figures. This strain (Strain A of Shope) did not fulfil our need for a virus inducing frank cell proliferation. However the others (B and C) yielded fibromatous growths like those pictured by Shope. They were used in the work. The rapidly growing, ruddy, sharply defined, spherical or discoid skin nodules frequently became capped with a broad vesicle, and later underwent a central pressure necrosis and ulceration (Fig. 2). When attenuated the virus yielded merely discoid thickenings or red indurations. Andrewes has already noted the differences in the virus strains (7).

Variations in individual susceptibility of the inoculated rabbits were pronounced, and they proved to be now a help now a hindrance in the work, bringing out or obscuring differences in the pathogenicity of the inocula. The Shope lesions were much more markedly conditioned by the body state than those of vaccinia. Thin or sick individuals tended to yield small growths or none at all; and a change for the better soon after inoculation was not infrequently attended by the appearance of the fibroma at a time when the large growths in vigorous, susceptible animals receiving the same material were already retrogressing.

The virus suspensions were mostly procured by grinding the infected tissue in a mortar with gel. Ty. and a little sterile sand. Glycerinated or fresh vaccinial testicle was used, and subcutaneous fibroma procured on the 7th to 10th day of growth. A special technique was developed to remove cells and particulate matter from the extracts. The fluid was first spun slowly, and the supernatant was transferred to 15 cc. tubes and centrifuged at high speed for 20–25 minutes with renewed transfer and rapid spinning once again. After each centrifugation a red-hot monel metal disc fixed on a wire was held above the meniscus until the surface layer began to bubble. Cells that had risen to the surface with air or fatty material were thus killed. Then the clear fluid from the middle of the tube was aspirated through a needle 16 cm. long into a sterile 10 cc. syringe, with due care that the needle point did not approach the side of the tube or extend into its slanting lower portion. A different syringe and needle was used with each tube, and the needle was disconnected while still immersed. The total fluid thus procured after the final centrifugation was not more than one-eighth to one-sixteenth of the original quantity; and hence centrifugation was begun with 100 to 200 cc. The ultimate material, searched in thick layer with the microscope, appeared free from cells, but showed particles close to the limit of visibility and very occasional fatty fragments of protoplasm or refractile granules having the same specific gravity as the fluid. The virus percentages or dilutions given in the protocols are calculated in terms of weight of infected tissue and cc. of extracting fluid.

In a few experiments the pellucid, supernatant fluid from cultures of embryo tissue infected with vaccinia was utilized as the virus suspension. When it had been "de-celled" in the way just described, it proved highly infective yet appeared almost free from particles. Sometimes a vaccinial supernatant was mixed with an extract of testicular tissue prior to decelling. None of the virus suspensions showed bacteria in stained smears.[1]

The neutralizing sera were procured from rabbits recently recovered from vaccinia, and from others that had been repeatedly inoculated with the fibroma virus after disappearance of an initial growth. While the Shope fibroma is still growing the blood becomes capable of neutralizing the virus (8), but a first reinoculation not infrequently gives rise to a small nodule. Hence the need for further reinoculations with material of known activity. Bleeding for serum was done only when these had yielded negative results. The sera were ordinarily procured 2 or 3 days prior to use,—to ensure the presence of alexin; and they were centrifugalized several times at high speed to remove any cells. Many of our experiments were so arranged as to demonstrate the neutralizing capacity of the antisera used; but when this was not the case separate tests were done to make sure of it.

[1] Virus suspension as such mixed in equal amount with gel. Ty. sometimes gave rise to larger lesions, and in other experiments to smaller ones, than did cell materials exposed to more considerable amounts of the same suspension and repeatedly washed thereafter. In one uncharted test with vaccinia the virus as such caused no lesions, whereas the cells carrying it yielded large ones.

The Initial Association of Cells and Viruses

Do viruses become fixed upon the cells of susceptible animals, and if so, under what conditions? Are viruses protected by cells? To obtain answers to these questions living and dead cells were exposed to a virus suspension, and, after they had been washed, submitted to neutralizing sera, and repeatedly washed yet again, they were inoculated into rabbits.

General Technique.—Portions of cell suspension (½ or 1 cc.) were mixed at room temperature with a decelled virus suspension (1 to 3 cc.); after various intervals gel. Ty. was added to a total of 45 cc.; the cells were recovered by brief, slow spinning in flat-bottomed tubes; washed once more with 45 cc. of gel. Ty.; made to ½ or 1 cc. with gel. Ty.; and placed in a large excess of antiserum neutralizing for the virus to which they had been exposed. To keep them suspended the container was slowly rotated on its long axis, during incubation at 37°C. Said container was a tube 1.5 cm. in internal diameter and 6.5 cm. long when stoppered. A bubble remained between stopper and serum when 10 cc. of fluid had been introduced, and the change in position of this bubble as the tube turned over kept the fluid stirred. The several tubes of each experiment were held with rubber bands against a square board fixed vertically at right angles to the shaft of a small motor making 3½ revolutions per minute, in an incubator at 37°C. The control mixtures in normal serum or gel. Ty. were also turned or stood at room temperature. No clumping of the cells took place during the 45–120 minutes of exposure to serum. Afterwards all of the specimens were made to 45 cc. with gel. Ty., and the cells were collected by slow centrifugation, washed again in 45 cc. gel. Ty., and resuspended in 0.8 cc. or 1.0 cc., according as inoculation material for three or four rabbits was required. 0.2 cc. of each inoculum was injected into the shaved skin of the side, always by the same operator, at the same approximate level, and, as a rule, after all of the suspensions had been assembled. The injections were so arranged that those materials which supposedly would give the largest lesions remained *in vitro* the longest time; and when there were many inocula, those of which negative results were expected were alternated in situation with others of positive promise. The lesions near the groin tended to be largest. Hence the site of each material was varied from animal to animal, and, when possible, each control inoculation was made at the same situation relative to the groin as the corresponding experimental one on the opposite side. The lesions never became confluent. They were measured and charted from day to day.

At each of the repeated washings the supernatant was drawn off as completely as possible, and at each resuspension the cells were distributed thoroughly. The centrifugings were done at a definite, slow rate. To prevent disturbance of the sediment in the flat-bottomed 50 cc. tubes it proved necessary to throw off the centrifuge brushes before the centrifuge stopped, and do the last braking with

the finger. The final inocula shimmered slightly with cells. In one instance in which they were unusually abundant, producing a definite cloudiness, an average of 610 per c.mm. was found on count.

Each experiment, from digestion to inoculation, was done in 1 day, and in each some of the test materials gave rise to lesions. Their size and character were recorded daily until retrogression began. In the charts a broken line indicates a more or less well defined, ruddy thickening of the skin, that is to say either a beginning lesion or an abortive one. Characteristic Shope tumors and vaccinia lesions are recorded in solid black, with hatching where there is vesiculation or necrosis. The charts are carried up to the beginning of retrogression only in the case of vaccinia, and through the first days of growth in that of the Shope fibroma.

Comment on the Individual Experiments.—The findings were essentially the same with the Shope virus as with vaccinia. Charts 1–4 record them for two experiments with each virus. Certain points relating to each will be commented upon before they are submitted to analysis as a group. A number of less comprehensive tests were made which yielded corroboratory findings.

Experiment 1 (Chart 1) was planned to disclose whether Shope virus becomes associated with living and killed cells respectively, and whether in such association it is protected against the action of immune serum.

Living and killed cells in suspension were exposed to the virus, and washed and turned at 37° with immune serum, normal serum, and gel. Ty. respectively. The effects of the sera and of gel. Ty. respectively on the virus material as such were tested by injecting into the skin of the upper back 0.2 cc. of fresh mixtures in equal parts. The resulting lesions are not charted because of their exceptional situation. The virus-gel. Ty. mixture gave rise to lesions in all three rabbits, the mixture with normal serum in two, but that with immune serum in none.

Experiment 2 (Chart 2), also with the Shope virus, besides covering much of the ground of Experiment 1, was designed to test whether antibodies become fixed on killed cells subjected to immune serum and are carried over with them into the host, there to neutralize virus introduced at the same time.

Living cells submitted to virus and, after washing, to immune serum in the usual way (to neutralize any free virus remaining), were mixed after further washings with equal portions of killed cells washed after exposure to immune serum or Tyrode only (Inocula *E, I, F, J*). The mixing was done just prior to injection, and the resulting suspensions were made to the same bulk as the ordinary inocula,

Procedures	Summary	Rab.	Days: 3 5 6 7 8 9 11 13
1 cc. *Living* Cell Suspension	*Living* Cells Submitted to Shope Virus		5 cm.
(*B*) Mixed with 2 cc. 8% virus, left 15 min., made to 43 cc., washed 4 times, and suspended in immune serum for injection (Injection 6:15–6:17 p.m.)	Injected with immune serum		Negative
(*A*) Treated like *B* except that finally suspended in gel.Ty. (5:54–5:57 p.m.)	Injected with gel.Ty.	3 2 4 1	
(*E*) Submitted to virus, washed twice, then rotated 45 min. with 9 cc. gel.Ty. and again washed twice (5:50–5:52 p.m.)	Rotated with gel.Ty.	3 2 4 1	
(*D*) Treated like *E* except that rotated with 9 cc. normal serum instead of gel.Ty. (5:46–5:49 p.m.)	Rotated with normal serum	3 2 4 1	
(*C*) Treated like *E* except that rotated with 9 cc. immune serum instead of gel. Ty. (5:42–5:45 p.m.)	Rotated with immune serum	3 2 4 1	
(*F*) Mixed with 42 cc. of 0.4% virus, at once washed twice, rotated with immune serum, and again washed twice (5:39–5:41 p.m.)	Submitted briefly to same amount of virus in dilute suspension; then rotated with immune serum	3 2 4 1	
1 Cc. *Heated* Cell Suspension	*Heated* Cells Submitted to Virus		
(*J*) Treated like *E* (5:28–5:30 p.m.)	Rotated with gel.Ty.	3 2 4 1	
(*I*) Treated like *E* except that rotated with 1 cc. normal serum plus 8 cc. gel.Ty. (5:25–5:27 p.m.)	Rotated with 1:9 mixture of normal serum and gel. Ty.	3 2 4 1	
(*H*) Treated like *D* (5:22–5:24 p.m.)	Rotated with normal serum	3 2 4 1	
(*G*) Treated like *C* (5:18–5:21 p.m.)	Rotated with immune serum		Negative
1 Cc. *Rayed* Cell Suspension	*Rayed* Cells Submitted to Virus		
(*L*) Treated like *E* except that rotated with 1 cc. normal serum plus 8 cc. gel.Ty. (5:14–5:16 p.m.)	Rotated with 1:9 mixture of normal serum and gel. Ty.	3 2 4 1	
(*K*) Treated like *C* (5:08–5:10 p.m.)	Rotated with immune serum		Negative

CHART 1

Procedures	Summary	Rab.	Days 4	5	6	7	8	9	10
1 cc. *Living* Cell Suspension	*Living* Cells						5 cm.		
(*K*) Double portion, washed 4 times (Injection 5:41–5:43 p.m.)	As such (double portion)		Negative						
(*A*) Mixed with 3 cc. of 10% virus, left 15 min., made to 45 cc., and washed 4 times (5:47–5:49 p.m.)	Submitted to Shope virus	2	●	●	●	●	●	○	○
		3	●	●	●	●	●	●	●
		1	○	○	●	●	●	●	
		4	○	–	–	–	◐	◐	◐
(*B*) Submitted to virus like *A*, washed twice, rotated 50 min. with 8 cc. immune serum, and washed twice (6:49–6:52 p.m.)	Submitted to virus and then to immune serum	2	●	●	●	●	●	–	
		3	+?	○	●	●	●	●	●
		1	–	–	●	●	●	●	
		4	–	–	○	●	●	●	–
(*F*) Treated like *B* + 1 cc. *heated* cell suspension washed 4 times (6:12–6:14 p.m.)	Treated like *B* + *heated* cells	2	○	○?	+	–	–	–	–
		3	–	–	●	●	●	●	◎
		1	–	–	●	●	●	○	
		4	–	–	–	+?	○	?	–
(*E*) Treated like *B* + 1 cc. *heated* cell suspension rotated 50 min. with 8 cc. immune serum and washed twice (6:35–6:39 p.m.)	Treated like *B* + *heated* cells submitted to immune serum	2	–	–	●	●	●	●	◎
		3	–	–	–	●	●	●	○
		1	–	–	○	●	○	○	
		4	–	–	–	–	+	○	○
(*J*) Treated like *B* + 1 cc. *rayed* cell suspension washed 4 times (6:30–6:33 p.m.)	Treated like *B* + *rayed* cells	2	–	–	●	◎	◎	◎	◎
		3	–	–	●	●	●	●	◎
		1	–	–	●	●	●	○	
		4	–	–	–	–	●	●	◐
(*I*) Treated like *B* + 1 cc. *rayed* cell suspension rotated 50 min. with 8 cc. immune serum and washed twice (6:44–6:47 p.m.)	Treated like *B* + *rayed* cells submitted to immune serum	2	?	–	●	◎	◎	●	●
		3	○	○	●	●	●	●	●
		1	–	–	●	●	●	○	
		4	–	–	–	–	○	○	?
1 Cc. *Heated* Cell Suspension	*Heated* Cells								
(*D*) Treated like *A* (6:00–6:03 p.m.)	Submitted to virus	2	○	○	●	●	●	○	○
		3	–	–	–	○	●	●	●
		1	–	–	○	○	●	●	
		4	–	–	–	–	–	–	–
(*C*) Treated like *B* (6:56–6:59 p.m.)	Submitted to virus and then to immune serum	2	?	?	+	+	–	–	–
		3	–	–	–	–	–	–	–
		1	–	–	○	○	○	○	
		4	–	–	–	–	–	–	–
1 Cc. *Rayed* Cell Suspension	*Rayed* cells								
(*H*) Treated like *A* (5:33–5:36 p.m.)	Submitted to virus	2	–	–	–	–	○	○	○
		3	–	–	–	–	◎	◎	◎
		1	–	–	○	?	?	?	
		4	–	–	–	–	–	–	–
(*G*) Treated like *B* (7:03–7:06 p.m.)	Submitted to virus and then to immune serum		Negative						

CHART 2

with result that they contained twice as many cells per cc. Many of the cells killed by raying (10 minutes) fragmented during the subsequent washings and were lost. The experiment was done in the summer, and the control specimen *A* stood with gel. Ty. at a room temperature of 92°F. until the time of inoculation.

The initial cell suspension showed occasional large diplococci deriving from the tryptic digest. Inoculation of double portions of the cell material, after four washings like those of the test portions, gave rise to no lesions. The virus suspension alone, in 0.2 cc. amounts, gave rise to large lesions save in Rabbit 4, a naturally resistant animal. These lesions are not charted.

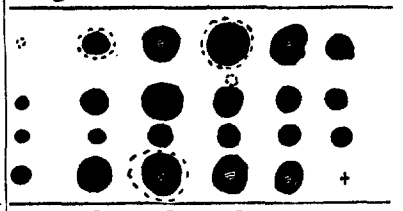
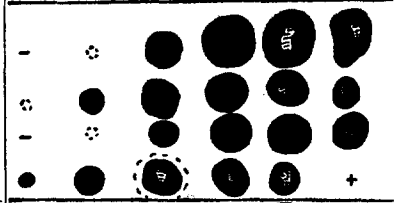
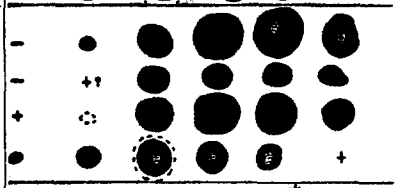
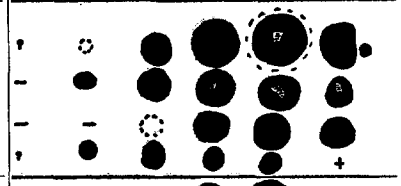
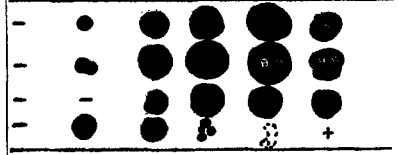
Experiment 3 (Chart 3) records the result of a nearly similar experiment with vaccinia.

To test whether serum antibodies were carried over into the host on killed cells, portions of these, washed after exposure to immune serum but not to virus, were mixed just prior to injection with other killed cells submitted to virus only and then washed (Inocula *D* and *H*), with appropriate controls. Two of the test rabbits showed generalized pocking on the 6th day after inoculation; and at this time of reduced resistance (9) lesions suddenly appeared where the heated cells turned with immune serum after exposure to virus had been injected. The other inoculations with killed materials submitted first to virus and then to immune serum yielded negative results, as had those of Charts 1 and 2. An occasional coccus was present in the initial cell suspension; but evidently it was non-pathogenic, since the inocula of living cells as such, and double portions of heated and rayed cells unexposed to virus, gave rise to no lesions, while cells exposed to virus gave typical ones.

In Experiment 4 (Chart 4) tests were made of the influence of time and of virus dilution on the fixation of vaccinia upon cells,—points already considered in Experiments 1 (Inocula *B* and *F*) and 3 (*B* and *C*). To see whether a preliminary exposure of living cells to immune serum would result in destruction of such virus as might become fixed on them later, a portion of them was turned with immune serum, washed, exposed to virus, and, after renewed washing, was turned with more immune serum (Inoculum *I*).

The cells killed by raying had been exposed for but 5 minutes and were well preserved.

Interpretation of the Findings.—The charts show that the inocula containing living cells repeatedly washed after exposure to virus gave rise regularly to lesions. The inoculation of living cells exposed to virus, rotated with immune serum, and washed also resulted in lesions, and so too did that of killed cells merely washed after exposure to virus. On the other hand killed cells that were exposed to virus, rotated with immune serum, and washed gave rise to no lesions or

Procedures	Summary	Rab.	Days 2 3 4 5 6 7 — 5 cm.
Living Cell Suspension	*Living* Cells		
(*L*) Washed 4 times (Injection 5:03–5:05 p.m.)	As such		Negative
(*A*) ½ cc. mixed with 0.4 cc. 2½% virus 15 min., thus diluting virus to 1.11% (or 1:90), made to 45 cc. with gel. Ty. and washed 4 times (5:14–5:16 p.m.)	Submitted to virus	1 2 4 3	
(*B*) ½ cc. mixed with virus 15 min., diluted to 45 cc. and washed twice, mixed with 9 cc. immune serum, rotated 1 hr., and washed twice (5:11–5:13 p.m.)	Submitted to virus, then to immune serum	1 2 4 3	
(*C*) Same as *B* except 0.4 cc. virus was made to 45 cc. with gel. Ty. before addition of cells = cells submitted to 0.02% virus (1:5000) (5:08–5:10 p.m.)	Submitted to same amount of virus in dilute suspension, then to immune serum	1 2 4 3	
Heated Cell Suspension	*Heated* Cells		
(*G*) Double portion (1 cc.) washed 4 times (5:19–5:21 p.m.)	Double portion as such		Negative
(*E*) ½ cc. treated like *A* (submitted to virus and washed 4 times) + ½ cc. washed twice (5:28–5:30 p.m.)	½ cc. submitted to virus + ½ cc. as such	1 2 4 3	
(*D*) ½ cc. treated like *A* + ½ cc. rotated 1 hr. with immune serum and washed twice (5:32–5:34 p.m.)	½ cc. submitted to virus + ½ cc. submitted to immune serum	1 2 4 3	
(*F*) ½ cc. mixed with virus like *A* but washed only twice, then rotated 1 hr. with immune serum and washed twice + ½ cc. washed twice (5:25–5:27 p.m.)	½ cc. submitted to virus, then immune serum + ½ cc. as such	1 2 4 3	
Rayed Cell Suspension	*Rayed* Cells		
(*K*) Double portion washed 4 times†	Double portion as such		Negative
(*I*) ½ cc. treated like *A* + ½ cc. washed twice (5:50–5:52 p.m.)	½ cc. submitted to virus + ½ cc. as such	1 2 4 3	
(*H*) ½ cc. treated like *A* + ½ cc. rotated 1 hr. with immune serum and washed twice (5:46–5:48 p.m.)	½ cc. submitted to virus + ½ cc. submitted to immune serum	1 2 4 3	
(*J*) ½ cc. mixed with virus like *A* but washed only twice, then rotated 1 hr. with immune serum and washed twice (5:44–5:46 p.m.)	½ cc. submitted to virus, then immune serum + ½ cc. as such		Negative

* Generalized pocking now. † Injection 5:40–5:42 p.m.

CHART 3

Procedures	Summary	Rab.	Days 2	3	4	5	7
½ Cc. *Living* Cell Suspension	*Living* Cells		5 cm.				
(*J*) Washed 4 times	As such		Negative				
(*A*) Mixed with 1.0 cc. virus fluid,* left 1 hr., made to 45 cc., and washed 4 times (Injection 5:24–5:26 p.m.)	Submitted to virus	5					
		6					
		7					
		8					
(*C*) Mixed with virus fluid like *A*, then washed twice, rotated 1 hr. with 5 cc. immune serum, and washed twice (5:11–5:13 p.m.)	Submitted to virus, then to immune serum	5					
		6					
		7					
		8					
(*B*) Mixed with virus fluid only 2 min., then diluted to 45 cc. and treated like *C* (5:07–5:09 p.m.)	Submitted *briefly* to virus, then to immune serum	5					
		6					
		7					
		8					
(*D*) Mixed with a 1:40 dilution of virus fluid for 2 min., then treated like *C* and *B* (5:04–5:06 p.m.)	Submitted *briefly* to same amount of virus in dilute suspension, then to immune serum	5					
		6					
		7					
		8					
(*I*) Rotated 1 hr. with 9½ cc. immune serum, washed twice, and treated like *C*—6 washings in all (5:42–5:44 p.m.)	Submitted to immune serum, to virus, and to immune serum again	5					
		6					
		7					
		8					
½ Cc. *Heated* Cell Suspension	*Heated* Cells						
(*H*) Treated like *A* (5:32–5:34 p.m.)	Submitted to virus	5					
		6					
		7					
		8					
(*F*) Treated like *C* (5:28–5:30 p.m.)	Submitted to virus, then to immune serum	5					
		6					
		7					
		8					
½ Cc. *Rayed* Cell Suspension†	*Rayed* Cells						
(*G*) Treated like *A* (5:22–5:24 p.m.)	Submitted to virus	5					
		6					
		7					
		8					
(*E*) Treated like *C* (5:20–5:22 p.m.)	Submitted to virus, then to immune serum	5					
		6					
		7					
		8					

* Decelled mixture of gel. Ty. extract from infected testicle and supernatant fluid from a culture of vaccinial tissue. † Raying was for only 5 minutes.

CHART 4

to but trifling ones. The experiments seemed to show that the viruses became fixed on both living and dead cells, and were carried through the washings with them. When now the living cells were exposed to immune serum such virus as they carried was not in the least affected, whereas that associated with killed cells underwent neutralization.

Before these implications of the findings can be accepted some alternative possibilities must be considered.

Will a persistence of virus in the free state throughout the manipulations explain the results?

The four successive washings of the cells with gel. Ty. after exposure to virus should on calculation have diluted any free virus more than one million times. The steps taken to remove particles which might contain virus from suspensions of the latter have already been described. If the persistence into the final inoculum of such particles, or of suspended free virus, had been responsible for the lesions, the killed cell material exposed to virus and then to immune serum should have produced lesions, since living materials similarly treated did so.

Some tissue extracts are known to enhance the activity of vaccinia injected with them (Reynals factor). May not a failure of killed cells to enhance the lesions explain, in part at least, the differing results with them? This can scarcely be since the controls with killed cells yielded large lesions (Charts 3 and 4).

Will the presence of neutralizing serum principles in the inocula explain the differences found?

The washings of the cells exposed to serum diluted the latter to between 1–1500 and 1–3000 in the inocula. On comparing the lesions from Inocula *E* and *I* of Chart 2 with *F* and *J*, as also *D* and *H* with *E* and *I* of Chart 3, it will be seen that when material containing serum thus diluted was mixed, just prior to inoculation, with other material carrying virus in minimal amount, as good lesions were obtained as with similar cell mixtures from which serum was absent. Evidently the serum as such cannot have reached the inocula in effective amount. Nor, as the tests show, can neutralizing serum principles have been carried on the dead cells into the animal, there to neutralize virus introduced at the same time.

From all this it is plain that the lesions produced by inoculation of the cells must have resulted from virus fixed upon them. This fixation was so firm as to withstand agitation of the cells with serum during a considerable period, and repeated pipettings with large quantities of wash fluid, together with as many as six changes of the latter. So small was the cell content of the inocula that they appeared almost pellucid. Yet they gave rise to lesions save when nearly all the cells

had been lost, as in some instances when treatments with ultraviolet rays had rendered them friable (Chart 2, *H*) or when they had been killed before they were submitted to neutralizing serum. The differences in the results with living and killed cells exposed to such serum cannot be laid to a carrying over into the final inocula of the serum as such.

The fixation of virus on the cells took place rapidly.

In Experiment 1 (Chart 1), 1 cc. of living cell suspension was mixed with 42 cc. of a 1 in 21 dilution of 8 per cent Shope virus (0.4 per cent virus) in a tube already balanced for centrifugation; and this was forthwith spun at low speed for 10 minutes with another tube in which the same quantity of cell suspension had been standing with 1 cc. of 8 per cent virus for 15 minutes prior to dilution to 0.4 per cent just before the spinning. After the usual two washings both cell portions were exposed to immune serum and washed twice again. They gave identical results on inoculation (Chart 1, *C* and *F*). In Experiment 4 cells that had stood with virus for 2 minutes prior to washing and exposure to immune serum gave lesions as large as those that had stood for an hour (Chart 4, *B* and *C*).

The virus suspensions were free from particles that could be readily thrown down with the centrifuge. Nevertheless several tests were made to see whether fixation occurred while the cells were in suspension, or whether it was due to virus caught between the tube bottom and the sedimented cells with result that it became attached to them. In the latter case cells that had stood with a dilute virus suspension should have yielded as large lesions as when they had stood with virus not diluted until just before the tubes were spun. In one instance just discussed the two specimens did give identical results. In two other tests, however, (Chart 3, *B* and *C*; Chart 4, *B* and *D*) the lesions from cells that had stood with virus already diluted were the smaller. The conditions did not permit of great virus dilution, and no more considerable differences in result could have been expected than were encountered.

While the test mixtures proper were rotating with serum in the incubator, the control of living cells merely exposed to virus and washed was in most instances let stand at room temperature, the object being to minimize the deterioration of virus that takes place in salt solutions. In Experiment 1 (Chart 1, *A* and *E*) the effects of these procedures on the Shope virus were compared and no difference was found. (See also Chart 7, *A* and *B*.) In the case of vaccinia, however, the control lesions were decidedly the larger. No great difference was observed in experiments yet to be described, with cells long exposed to vaccinia in tissue cultures (Chart 5, *A* and *D*; Chart 9, *B* and *C*). It seems probable that in the present instances of brief exposure to the virus, some of it was so loosely fixed as to have been still accessible to the immune serum, or else it was dislodged during the turning. Exposure of the virus to 37° during the period of turning cannot have been

an important factor, since 53° for 15 minutes had almost no effect upon it (Charts 5 and 6, *A* and *C*).

The fixation of Shope virus upon killed cells exposed briefly thereto would appear from the lesions of Charts 1 and 2 to have been considerably less, or less firm, than upon living ones; but in the vaccinia experiment (of Chart 4) as well as in some with cells from cultures of both sorts (Charts 5, 6, 8, 9) the magnitude of the lesions indicated that almost or quite as much virus was carried by the dead material.

The adsorption of viruses upon inanimate matter is known to be much affected by the pH and other qualities of the fluid medium. The association of the Shope virus with the living cells upon which it had become fixed would appear, however, to have been wholly unaffected by incidental differences in the fluids used in our experiments. Lesions of the same magnitude were produced by virus-laden cells that had been rotated with immune serum, normal serum, and gel. Ty. respectively, or had been merely let stand with the latter (Chart 1). It seemed possible, however, that virus might come away from dead cells, as not from the living, during rotation with serum and be lost in the subsequent washings. The results with Inocula *J*, *I*, *H*, and *G* of Chart 1 bear upon this point. The virus-laden dead cells were rotated with gel. Ty., with normal serum as such or diluted, and with immune serum respectively. Only in the case of the last did the inocula fail to give lesions. In Experiment 10 (Chart 9) water-killed cells rotated in a dilute mixture of immune serum and gel. Ty. gave rise to lesions almost as large as those from material thus killed and merely washed at room temperature and injected forthwith. The cells turned with undiluted immune serum on the other hand yielded lesions which were relatively insignificant.

It seems clear from the results as a whole that vaccinia and the Shope virus become quickly, firmly, and abundantly fixed upon individual cells derived from cultures of rabbit embryo. Fixation takes place on both living and killed cells. Exposure *in vitro* to neutralizing serum does not affect the virus when the cells with which it is associated are alive, as is proven by the size of the lesions resulting from inoculation of the material. If they are dead, though, when exposed to the serum, the material gives rise to no lesions or to negligible ones.

It was repeatedly noted that no lesions develop when living cells

carrying the viruses are injected together with immune serum. This fact will be considered further in the light of experiments now to be described.

The Association of Viruses with the Cells of Tissue Cultures

Vaccinia can be demonstrated in abundance in the fluid of tissue cultures in which it is under propagation. Does there exist, in addition, a fixation of the virus in or upon the growing tissue cells? If so, are the latter capable of protecting the virus from neutralization with serum? These points were investigated by liberating cells from tissue cultures of vaccinia, and subjecting them to experiments of the sort just described. Similar tests were made with cells from cultures of the Shope tumor.

The Vaccinial Cultures.—Cultures of rabbit embryo tissue were made by our usual technique except that just prior to implantation one part of vaccinial material was added to six parts of tissue suspension. Ordinarily a 10 per cent centrifugalized extract in Tyrode of glycerinated or fresh testicular material on the 3rd to 5th day of infection was added; but in occasional instances the supernatant fluid from cultures infected with such material was utilized to start new ones which were employed for the experiment proper. As a rule 8–10 flask cultures were prepared at one time, together with others containing embryo tissue only. They were washed with gel. Ty. every 2–3 days and utilized after 5 to 7 days in all.

Injections of the decelled supernatant fluid from the cultures resulted in large vaccinial lesions, and inoculation of the cells liberated with trypsin regularly showed the presence of virus in quantity. The inoculated cultures grew far less well than did the uninoculated controls; the tryptic digest was more clouded with debris; the living cells that had been freed were mostly fatty; and great numbers of refractile bodies were present having some resemblance to those liberated by trypsin from fowl pox tissue (10).

The bodies mentioned were 10 to 50 μ in diameter,—not larger than some of the associated cells. They had the form of slightly irregular, pale buff spheres, with an ill-defined, coarse facetting. The small ones were frequently intracellular, but the large appeared free. They were present in quantity in the supernatant fluid drawn off after the first slow spinning of 5 to 7 day old vaccinial cultures, though some were entangled and carried down with the tissue debris. Rapid centrifugation did not bring them either to the surface or the bottom,—so closely did their specific gravity approximate that of the fluid. Unlike the inclusions of fowl pox studied by Goodpasture and Woodruff (11) they did not disintegrate when subjected to strong trypsin in the presence of sodium bicarbonate, nor did they swell on exposure to distilled water. Like such inclusions, however,

they ruptured at the moment when water evaporated from about them. Pressure on the cover-glass also caused them to rupture, forcing out a single, round, highly refractile globule from a thin, transparent, wrinkly sac, probably the remains of an enveloping cell. The globules thus obtained looked precisely like the fatty ones that could be pressed out of degenerating culture cells, and they readily dissolved in alcohol and ether. When dried bodies were subjected to these reagents there was left only the sac. Morosow's stain colored them a homogeneous yellow like the cell fat, not black like the particles described by Paschen. Altogether the evidence indicates that we were dealing with the metabolic products of cells injured by the virus, a view supported by the very occasional recovery of elements of identical appearance and behavior from cultures uninfected with vaccinia.

Character of the Experiments.—The methods were those already described. The digests of eight to ten cultures were pooled, and the freed cells were separated from the tissue fragments by filtration, twice washed, and suspended for test. Debris was practically absent from the ultimate suspensions, and neutral red showed nearly all of its cells to be alive. Heating at 53° for 15 minutes was ordinarily used to kill them. The heat affected the virus little if at all (Charts 5 and 6).

Tests were made to see if the washed, living and killed cells carried virus, and whether they protected it from the influence of neutralizing serum of proven potency.

Comment on the Findings.—The experiments yielded consistent results. Charts 5 and 6 of Experiments 5 and 6 record their outcome in two typical instances.

Living culture cells that were twice washed with 45 cc. of gel. Ty.,—in addition to the several washings incident to preparation of the suspension,—regularly gave rise to characteristic vaccinial lesions. When they were also exposed to a neutralizing serum the lesions were almost as large; and so too when they were killed with heat and then washed. Cells subjected to immune serum, washed, and killed with heat, produced lesions that were slightly less marked, though characteristic,—as if the slightly detrimental effects of heating and of immune serum had been added together. In pronounced contrast were the almost completely negative results with cells killed with heat prior to exposure to serum. Either they caused no lesions or small nodular ones appearing late.

The persistence of free virus and of neutralizing antibodies, as influencing the outcome of the tests, can be excluded on the same grounds as in the experiments already described (*q.v.*), and so too can the possibility that serum antibodies became fixed on the dead cells and were carried over into the host, there to neutralize the virus introduced with them.

The experiments show that vaccine virus was carried by the culture cells, and was so closely associated with them as not to be removed by washings and pipettings, even after the cells had been killed by heat.

Procedures	Summary	Rab.	Days 2	3	4	5	7
1 Cc. Cell Suspension from Vaccinial Cultures	*Vaccinial Cells*					5 cm.	
(*B*) Washed twice and suspended in immune serum for injection* (Injected 3.52–3.55 p.m.)	Injected with immune serum*		Negative throughout				
(*A*) Washed twice and suspended in gel.Ty. for injection (3:58–4.01 p.m.)	Injected with gel.Ty.	2 1 4 3					
(*C*) *Heated* at 53° for 15 min., washed twice, and injected (3:42–3:45 p.m.)	Killed with heat and washed	2 1 4 3					
(*D*) Rotated 1 hr. with 9 cc. of immune serum and washed twice (3:35–3:38 p.m.)	Cells rotated with immune serum	2 1 4 3					+
(*E*) Treated like *D* and *heated* at 53° for 15 min. just prior to injection (3:29–3:31 p.m.)	Rotated with immune serum and killed with heat	2 1 4 3					
(*F*) *Heated* as was *C*, rotated in 9 cc. immune serum and washed twice (3:18–3:23 p.m.)	Cells killed with heat and rotated with immune serum	2 1 4 3		?			+

* All other final suspensions were made in gel. Ty.

CHART 5

Procedures	Summary	Rab.	Days 2	3	4	6
1 Cc. Cell Suspension from Vaccinial Cultures	*Vaccinial Cells*		5 cm.			
(*B*) Washed twice and suspended in immune serum for injection* (Injected 4:34–4:37 p.m.)	Injected with immune serum*	2 4 3 1				
(*A*) Washed twice and suspended in gel.Ty. for injection (4:27–4:30 p.m.)	Injected with gel.Ty.	2 4 3 1				
(*C*) *Heated* at 53° for 15 min. and washed twice (4:23–4:26 p.m.)	Killed with heat and washed	2 4 3 1				
(*D*) Rotated 1 hr. with 9 cc. immune serum, and washed twice (4:18–4:21 p.m.)	Rotated with immune serum	2 4 3 1				
(*E*) Treated like *D* and *heated* at 53° for 15 min. just prior to injection (4:12–4:15 p.m.)	Rotated with immune serum and killed with heat	2 4 3 1				
(*F*) *Heated* as was *C*, rotated with 9 cc. immune serum, and washed twice (4:04–4:10 p.m.)	Killed with heat and rotated with immune serum	2 4 3 1				

* All other final suspensions were made with gel. Ty.

CHART 6

The immune serum failed to affect the virus associated with living cells, whereas that associated with killed cells was neutralized by it.

The Cultures Containing Shope Virus.—To obtain the Shope virus in cultures the hashed tissue of young, growing fibromas was cut up and implanted precisely as was rabbit embryo in the work with vaccinia. Large, individual cells moved out early from the tissue bits and occasionally entered the supernatant fluid in considerable number. They segregated neutral red promptly in granules of highly various size and had the general appearance of clasmatocytes. After the first 2 or 3 days elements of fibroblastic type began to extend out in strands, which were fairly numerous by the 6th or 7th day. Only when this had happened were the cultures digested.

Character of the Experiments.—Some early tests were directed to determining whether the virus responsible for the fibroma was present in the supernatant fluid of the cultures and in the fluid portion of the tryptic digest. As a rule these materials were decelled and 0.2 cc. was injected.

The suspensions of culture cells were procured as in the case of vaccinia. Heating to 53° for 15 minutes, or raying for 5 minutes rendered the virus inactive, and hence could not be utilized to kill the cells. Water was employed instead. The cells from standard portions of well washed suspension were thrown down, resuspended in 2 or more cc. of twice distilled water, and allowed to stand for 56–60 minutes at room temperature, the controls meantime standing in the same amount of gel. Ty. As result the cells became greatly swollen, and the staining of the nuclei with neutral red showed that they were dead. They did not completely return to normal size when isotonicity was restored by washing them in an excess of ordinary gel. Ty. or by adding in appropriate quantity "triple" Tyrode,—a solution containing the salts of Tyrode in triple strength; and they tended to fragment when washed, necessitating longer centrifugations than usual to prevent loss. The gel. Ty. controls were centrifuged at the same time. Each of the ultimate materials was made to a bulk of 0.7–0.75 cc., and three rabbits were injected with 0.2 cc. of this.

Comment on the Findings.—Chart 7 portrays the results of an orienting experiment, Experiment 7, typical of several of the sort. The supernatant culture fluid contained living cells of makrophage type in fair number. It was inoculated as such, whereas the fluid portion of the tryptic digest was decelled prior to inoculation. The suspension of washed culture cells was divided into three portions of 1.2 cc., of which one was let stand in gel. Ty. at room temperature, while the other two were rotated with gel. Ty. and with immune serum respectively for 2 hours.

The three cell materials all gave rise after approximately the same time to characteristic tumors which grew at the same general rate. The tryptic digest fluid on the other hand caused a lesion in but one animal, and this consisted of several small, shotty growths appearing late and coalescing. The supernatant from the cultures gave rise late to more numerous growths of the same sort.

They were scattered over an area of several cm. on the rabbit's side, but have been charted close together to save space, a procedure employed in some of the other chartings also.

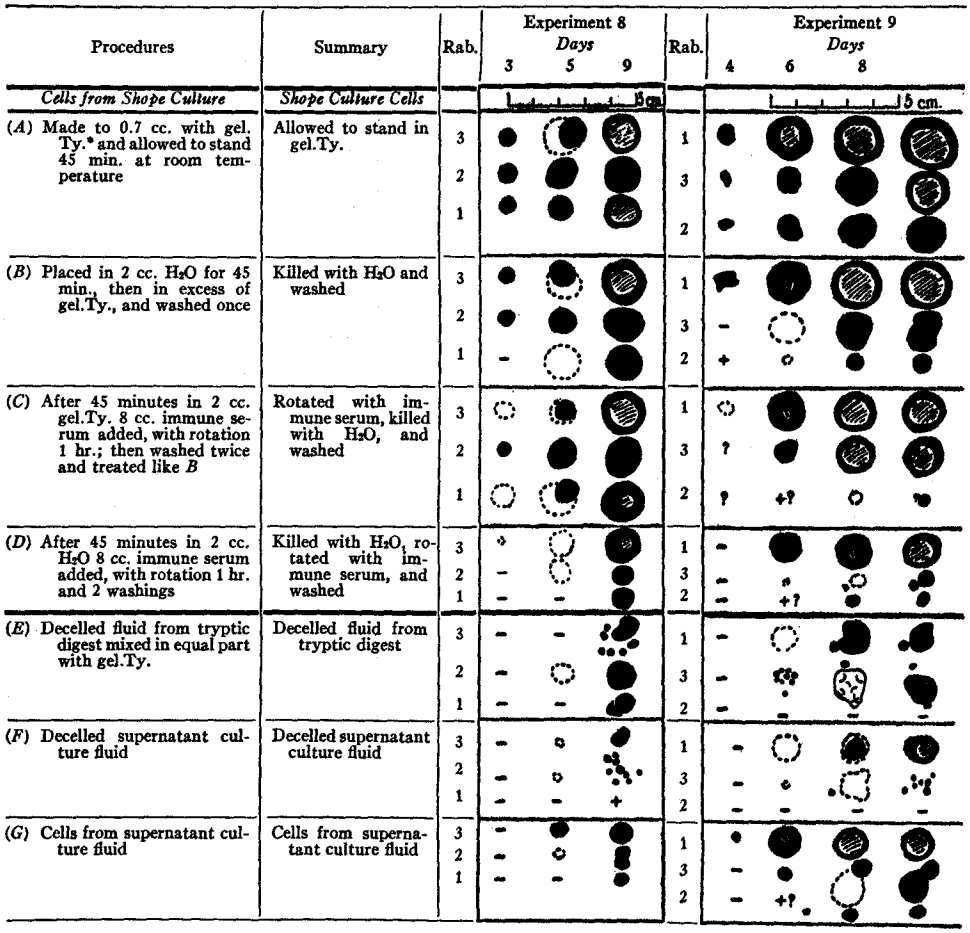
Procedures	Summary	Rab.	Days 7	9	14	23
Cells from Shope Culture	*Shope Culture Cells*					1.5 cm.
(*A*) Suspended in 1.2 cc. gel. Locke's and let stand 2 hrs. at room temperature	Cells let stand at room temperature	3	◌			
		2				+
		1				+
(*B*) Rotated 2 hrs. in 10 cc. gel. Locke's, twice washed, and made to 1.2 cc.	Cells rotated with gel. Locke's	3	–	–		
		2				+
		1		**	–	–
(*C*) Rotated 2 hrs. in 10 cc. immune serum, twice washed, and made to 1.2 cc.	Cells rotated with immune serum	3	◌			
		2				+
		1		**	–	–
(*D*) Decelled fluid from tryptic digest	Decelled fluid from tryptic digest	3	–	–		
		2	–	–	–	–
		1	–	–	–	–
(*E*) Supernatant fluid from cultures*	Supernatant* from cultures	3	–	–		
		2	–	–	–	–
		1	–	◌		–

* Contained some inwandered cells.
** Measured on 8th day and excised.

CHART 7

Experiments 8 and 9 (Chart 8) were more extensive. The supernatant from the cultures was decelled in both cases. In each it had been only 2 days on a

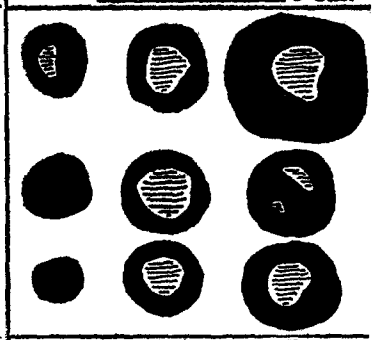
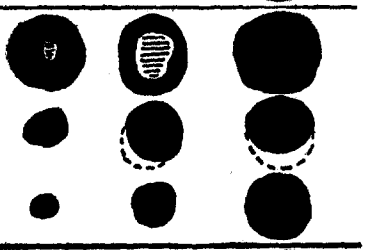
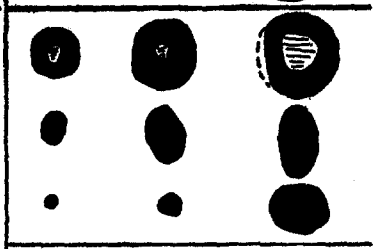
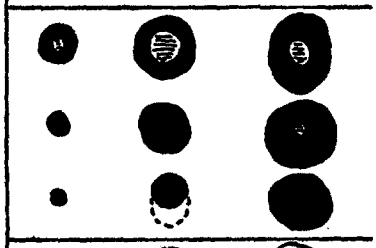
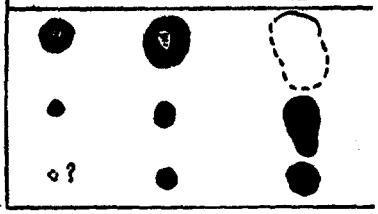
culture 4 days old. It gave rise late to nodules that tended to be small, scattered, and shotty, as did also the decelled tryptic supernatant. In Experiment 9 this contained some bacteria, evidently from a colony overlooked in the examination



* Ultimate amount of all inocula in Experiment 8, 0.7 cc., in Experiment 9, 0.75 cc.

CHART 8

of the cultures; but these were non-pathogenic as the outcome of the inoculations attested. The cells collected from the supernatant of Experiment 9 during the decelling were relatively few and of makrophage type; yet they gave rise, after two washings, to discrete, small growths like the larger ones resulting from the culture cells proper. In each experiment one portion of the latter was allowed to stand

Procedures	Summary	Rab.	Experiment 10 *Days* 3 4 5
1 Cc. Cell Suspension from Vaccinial Cultures	*Vaccinial Culture Cells*		5 cm.
(*A*) Washed twice after standing 45 min. in gel.Ty. (Injected 4:25–4:27 p.m.)	Washed after standing	2 1 3	
(*B*) *Killed* with H_2O,* made isotonic, and washed twice (4:28–4:30 p.m.)	Killed with H_2O and washed	2 1 3	
(*C*) Rotated 1 hr. with 8½ cc. immune serum washed twice, *killed* with H_2O, made isotonic, and again washed twice (7:03–7:05 p.m.)	Rotated with immune serum and killed with H_2O	2 1 3	
(*D*) *Killed* with H_2O, made isotonic, rotated 1 hr. with 2 cc. immune serum and 6½ cc. gel.Ty. (5:52–5:54 p.m.)	Killed with H_2O and rotated with dilute immune serum	2 1 3	
(*E*) *Killed* with H_2O, made isotonic, rotated 1 hr. with 8½ cc. immune serum, and washed twice (5:47–5:48 p.m.)	Killed with water and rotated with immune serum	2 1 3	

* Cells thrown down, suspended in excess of twice distilled water (3.2 cc.), let stand 45 minutes at room temperature, and brought to isotonicity with triple strength p.Ty.

CHART 9

in gel. Ty. after washing, while another was submitted to water and later washed in an excess of gel. Ty. to restore tonicity. These two specimens, the one of living, the other of killed and somewhat fragmented cells, gave rise to lesions of practically identical size, ruddy projecting growths that appeared in 3 or 4 days, grew vigorously and were soon capped by a broad vesicle. The third cell portion, submitted to immune serum of high potency and then washed, water-killed, and washed again, yielded only slightly smaller lesions, appearing somewhat more slowly. In marked contrast were the findings when the cells had been killed with water prior to contact with serum. This material was not washed so often as that just mentioned and it reached the final suspension in at least as great quantity; yet it gave rise to poor growths that appeared late.

The importance of the bodily condition of the test animal on the development of Shope lesions is illustrated in Chart 8. One of the rabbits (No. 2 of Experiment 9) developed diarrhea shortly after inoculation, and losing weight rapidly, died on the 10th day. Only the more virulent of the inocula caused recognizable lesions in this animal; and they remained so small as to afford few contrasts. Rabbit 1 also afforded few contrasts, but that was because nearly every inoculum caused a large growth.

In Experiment 10, one of several in a group apart, water was used to kill vaccinial culture cells, and isotonicity was restored by adding triple Tyrode prior to the addition of serum. As Chart 9 shows, the material killed with water and washed gave rise to smaller lesions than did that which had merely stood in gel. Ty. The lesions were pronounced and characteristic, however. Material turned with immune serum, washed, killed, and washed twice again yielded lesions nearly as large, whereas that killed prior to rotation with immune serum (with omission of two washings) had but little pathogenic effect.

The individual cells procured by digestion from cultures of the Shope fibroma carried the causative virus. Indeed it was almost wholly localized to the cells, very little being free in the supernatant culture fluid or even in the decelled tryptic digest (Charts 7 and 8). Repeated washing of the living culture cells, followed by exposure to potent immune serum *in vitro*, failed to remove or neutralize the virus associated with them. When killed cells were exposed to the serum, however, the virus was rendered ineffective (Figs. 2 and 3). Repeated washings in gel. Ty. had no evident effect on the virus associated with the killed cells.

The lesions produced by the culture cells and the supernatant fluids respectively were significantly different, although the areas of skin infiltrated by the injection were of the same size. When either living or dead cells had been introduced the tumor developed as a solitary, spherical or discoid mass at the point

of injection, whereas after the introduction of decelled, supernatant fluid the growths were often multiple,—appearing as shotty nodules scattered more especially in the direction of lymph drainage (Figs. 2 and 3). Evidently the injected cells were retained at the injection point, together with the virus fixed upon them, whereas virus free in the supernatant fluid tended to be more widely distributed.

So soon did Shope tumors appear in certain instances after the injection of the living cells as to suggest that some of these had survived and proliferated; but the water-killed cells yielded tumors nearly as promptly (Chart 8), indicating that transplantation had played little part in the results.

In the case of vaccinia the tests do not enable one to say whether the demonstrated association of cells and virus is obligatory for the latter; for during cultivation it may have become fixed secondarily on the cells, as happens when the two are left together briefly *in vitro* (Charts 3 and 4). True, the lesions produced by washed, living, vaccinial culture cells submitted to immune serum and washed again were far larger than those resulting from the injection of rabbit embryo cells exposed but briefly to virus and then to immune serum; and in tests of the latter sort the serum cut down the size of the lesions considerably. But this may have resulted merely from a more abundant or firmer fixation upon the cells derived from vaccinial cultures. The findings with the Shope virus point to an obligate association of it with the cells.

DISCUSSION

The experiments prove that viruses rapidly become fixed upon individual living cells. Can the fixation be due to phagocytosis? In fowl plague and cattle plague the virus present in the blood stream is largely associated with the white corpuscles (12); and a similar association of vaccinia with leukocytes is demonstrable in rabbits at the height of infection (13). In these instances the phenomenon may have resulted from phagocytosis not as yet followed by virus death; for Douglas and Smith (14) and Fairbrother (15) have produced evidence that the blood leukocytes destroy vaccinia. But in our experiments the fixation upon cells was practically immediate at room temperature, and the quantity fixed was large, judging from the lesions caused. The virus-containing suspensions themselves were the only possible source of opsonins, and they had undergone a several thousand-fold dilution with Tyrode solution.

Many workers have supposed that the virus of fowl plague becomes fixed upon the red corpuscles; but Todd's recent work makes this unlikely (16). Some have reported that the virus of foot and mouth

disease can become fixed upon red cells, while others deny this. Several authors have demonstrated that viruses collect out of suspension upon tissue fragments, notably Kraus, von Eisler, and Fukuhara (17), and adsorption upon charcoal, kaolin, aluminum hydroxide, and other substances is known to take place rapidly. In most of the experiments the viruses remained firmly fixed to the cells after the latter had been killed; yet one cannot infer that the conditions of fixation remained the same. Krueger (18), studying the association of phage with bacteria of susceptible sort, has found that when the latter are alive phage is "distributed in a manner typical of numerous materials soluble in both phases of a two-phase system." The fixation upon dead bacteria, on the other hand, is of adsorptive type. The strikingly different findings in our tests with living and dead cells carrying a virus and exposed to neutralizing serum is indicative of a great difference in the conditions. Living cells protected the virus from the influence of the serum whereas dead cells did not.

Andrewes (19) has found that immune serum injected into the skin 5 minutes after an inoculation of vaccine virus into the same spot fails to prevent the development of a lesion. If instead the serum is given 5 minutes beforehand no lesion appears. ("The serum has thus acted in five minutes.") The suggestion has been made by both Andrewes and Fairbrother that in the skin the immune serum acts on the local tissue cells as well as on the virus. In our experiments cells were present. Fairbrother (20) proved by intracerebral inoculations that vaccinia virus is neutralized by standing 4 hours *in vitro* with immune serum and that there is some neutralization in 1 hour. In the tests of the present work an enormous quantity of serum was employed as compared with the amount of virus fixed upon the cells. Antibodies for viruses are readily adsorbed out of serum on collodion particles and diatomaceous earth (21). But certainly no effective fixation occurred on either the living or dead cells of our tests despite excellent opportunities.

In a previous paper from this laboratory (22) the fact has been brought out that living cells protect erythrocytes and bacteria situated within them from hemolysins and the bactericidal principles of homologous serum, as well as from the lethal effects of potassium ferrocyanide. Dead cells are readily penetrated on the other hand by the agents mentioned and these exert their destructive influence. One is tempted to suppose that the viruses used in the present experiments, after becoming attached in some way to living cells, are taken into the

latter, and owe their persistence in active state to an intracellular situation. But the data do not justify this supposition. They prove only that the protection of the viruses is in some way dependent upon cell life. The maintenance of a special state of affairs at or near the cell surface might suffice for protection.

Perdrau and Todd (23) found that tissue freshly procured in suspension from organs infected with viruses, shielded the latter from photodynamic inactivation by methylene blue, whereas after the tissue had been submitted to glycerine no protection was demonstrable. Normal tissues exposed *in vitro* to the viruses and then to the photodynamic influence failed to produce lesions on inoculation,—whence Perdrau and Todd concluded that a penetration of the viruses into the interior of the cells was essential to their protection, and that it had not had time to occur in the tests last mentioned. Our experiments make it seem likely that a fixation of Perdrau and Todd's viruses, one of which was vaccinia, took place on the normal tissues, but that such protection as these may have exerted did not suffice against the photodynamic activity. The authors stated that fragments of tissue as well as separate cells were present in the suspensions tested; and the protection they noted in the case of materials prepared from organs already containing virus may have been due to the situation within tissue bits. The inactivation of viruses by methylene blue is dependent upon the presence of oxygen; and Perdrau and Todd attribute the protection of the viruses within infected tissue to the maintenance by the cells of a special oxidation-reduction potential.

The suggestion has been put forward in a previous paper (24) that a protection of pathogenic bacteria by living cells will explain the obduracy of certain diseases, as *e.g.* tuberculosis, leprosy, and gonorrhea. There is much to indicate that this is the case in some virus diseases as well. Smith (25) has been able to recover vaccine virus from blood leukocytes and those of the peritoneal cavity at intervals up to the 8th or 9th day of infection, although antibodies had been in circulation since the 2nd or 3rd day; and Douglas, Smith, and Price (26) have demonstrated its presence in some of the organs of rabbits recovered from vaccinia inoculated 41 days previously. Olitsky and Long (27) have separated out the virus by electrophoresis from the testicles of immune rabbits so long after inoculation that they believe that it persists indefinitely within the body.

The behavior of growths due to viruses is largely referable to a protection of the latter from circulating immune principles. Two

sorts of resistance can be discriminated in fowls carrying Chicken Tumor I, against cells and causative agent respectively (28). The transplanted cells of this sarcoma will often give rise to tumors in fowls that are completely resistant to the agent itself, and these may progress rapidly and kill despite the presence in the blood of neutralizing antibodies for the agent (29). This is the case with other chicken tumors also (30). The development of the Shope rabbit papilloma, a virus-induced epithelial growth with the characters of a tumor (31), is attended by the appearance in circulation of principles effective against the virus as such (32). Yet the growth continues to proliferate and its cells can be successfully transferred within the host, although the latter is now completely resistant to the virus present in association with the cells and responsible for their multiplication.

When, in our experiments, immune serum was injected together with the virus-carrying, living cells no lesions developed. But vaccinia as a necrotizing virus, doubtless killed almost at once those elements with which it was introduced, thus exposing itself to the action of serum injected with it. Andrewes has shown that the antibodies of serum from animals immune to vaccinia remain localized for as long as 72 hours after intradermal injection, and capable of neutralizing virus introduced into the same spot during this period. The Shope virus induces cell multiplication; but the cells carrying it in our tests had been badly maltreated. That they did not long survive is indicated by the almost equally good lesions produced with water-killed cells.

The prompt fixation of viruses upon living cells, with protection of them afterwards, will go some distance to explain how viruses obtain a foothold in the host, and why serum treatment is so often unsatisfactory once infection is under way. Loss of protection when the virus-affected cells die in the period after antibodies have come into circulation may be the reason for recovery from virus diseases. Local vaccinial lesions evidently develop and progress by a multiplication of the virus in association with cells which, however, are soon killed by it. The resulting exposure of the virus to principles seeping into the lesion from the blood can deter but little the progress of the disease in the days before effective antibodies come into circulation. But when this happens one may suppose that the virus is neutralized

wherever it deprives itself of the protection of cells by killing them. Thus in due course the disease is overcome, though the virus itself may persist in occult form in association with cells that it does not kill. Conditions are somewhat different with the Shope fibroma. Shope reports that the growth continues to enlarge for some time after neutralizing antibodies for the virus have appeared in the blood. Possibly the cells affected by the virus ultimately degenerate and no longer protect the virus from serum antibodies of mounting potency. The histological changes in retrogressing Shope fibromas support this supposition.

Some features of the behavior of the Shope virus have been disclosed incidentally to the work. Like the agent causing Chicken Tumor I, the virus is relatively ineffective in sick or undernourished hosts, causing small growths or none at all. If the condition of the animal improves soon after the inoculation there may be a late appearance of fibromas. Young animals are the most favorable to the growth's enlargement, as in the case of many neoplasms. The virus, like that of Chicken Tumor I, is killed by slight heating, but unlike it is rapidly inactivated by ultraviolet light. In our experience adding kieselguhr to a Shope virus suspension does not increase and may interfere with its effectiveness to cause growths, whereas it greatly aids the agent producing Chicken Tumor I. The latter virus is regularly present in abundance in the supernatant fluid of cultures of the tumor tissue, whereas the fluid from cultures of the Shope fibroma is almost completely innocuous.

The injurious effects of vaccinia on the cells of tissue cultures in which the virus is under propagation seem not to have been observed before. Traub has recently reported that cell damage occurs when pseudorabies virus (32) is cultivated with tissue *in vitro*. The curious bodies appearing in vaccinia cultures merit further scrutiny.

SUMMARY

Methods were developed for a study of the relations existing between viruses and living cells. It was found that vaccinia and the virus causing the infectious fibroma of rabbits (Shope) rapidly become fixed upon tissue cells freed as individuals and submitted to virus in suspension. This happens whether the cells are alive or have been killed

with heat or ultraviolet light. The virus does not come away during agitation of the cells with Tyrode solution and repeated washings with large amounts of it. The exposure to neutralizing antisera of cells carrying virus fails to affect this latter significantly if the cells are alive, whereas if they are dead the activity of the virus is nullified. Cells freed as individuals from tissue cultures of vaccinia and the Shope tumor carry these viruses in abundance through repeated washings, and, if living, protect them from the influence of a neutralizing serum, whereas killed cells exert no such protection.

The findings would appear to throw light on the way in which viruses gain a foothold in the host; and they suggest reasons for the persistence of some viruses in recovered animals and for the unsatisfactory results of serum treatment instituted during the course of virus diseases.

The virus causing the Shope fibroma has been successfully maintained in cultures of the growth. It is closely associated with the cells, almost none being present in the culture fluid. Certain of its other attributes have been determined. Vaccinia greatly damages the cells of cultures of rabbit embryo in which it is under propagation.

BIBLIOGRAPHY

1. Shope, R. E., *J. Exp. Med.*, 1932, **56**, 793, 803.
2. Andrewes, C. H., *Lancet*, 1934, **2**, 63, 117.
3. Rous, Peyton, and Jones, F. S., *J. Exp. Med.*, 1916, **23**, 549.
4. Rous, Peyton, and Turner, J. R., *J. Exp. Med.*, 1916, **23**, 219.
5. Rivers, T. M., *J. Exp. Med.*, 1931, **54**, 453.
6. Rivers, T. M., *J. Exp. Med.*, 1931, **54**, 453. Rivers, T. M., and Ward, S. M., *J. Exp. Med.*, 1933, **58**, 635.
7. Andrewes, C. H., *Lancet*, 1934, **2**, 63, 117.
8. Shope, R. E., *J. Exp. Med.*, 1932, **56**, 793, 903.
9. Smith, W., *Brit. J. Exp. Path.*, 1929, **10**, 93.
10. Woodruff, C. E., and Goodpasture, E. W., *Am. J. Path.*, 1929, **5**, 1. Goodpasture, E. W., and Woodruff, A. M., *Am. J. Path.*, 1930, **6**, 699. Woodruff, C. E., and Goodpasture, E. W., *Am. J. Path.*, 1930, **6**, 713. Goodpasture, E. W., and Woodruff, C. E., *Am. J. Path.*, 1931, **7**, 1.
11. Woodruff, C. E., and Goodpasture, E. W., *Am. J. Path.*, 1929, **5**, 1. Goodpasture, E. W., and Woodruff, A. M., *Am. J. Path.*, 1930, **6**, 699. Woodruff, C. E., and Goodpasture, E. W., *Am. J. Path.*, 1930, **6**, 713. Goodpasture, E. W., and Woodruff, C. E., *Am. J. Path.*, 1931, **7**, 1.
12. Hornby, H. E., *J. Comp. Path. and Therap.*, 1928, **41**, 17. Todd, C., *Brit. J. Exp. Path.*, 1928, **9**, 19.

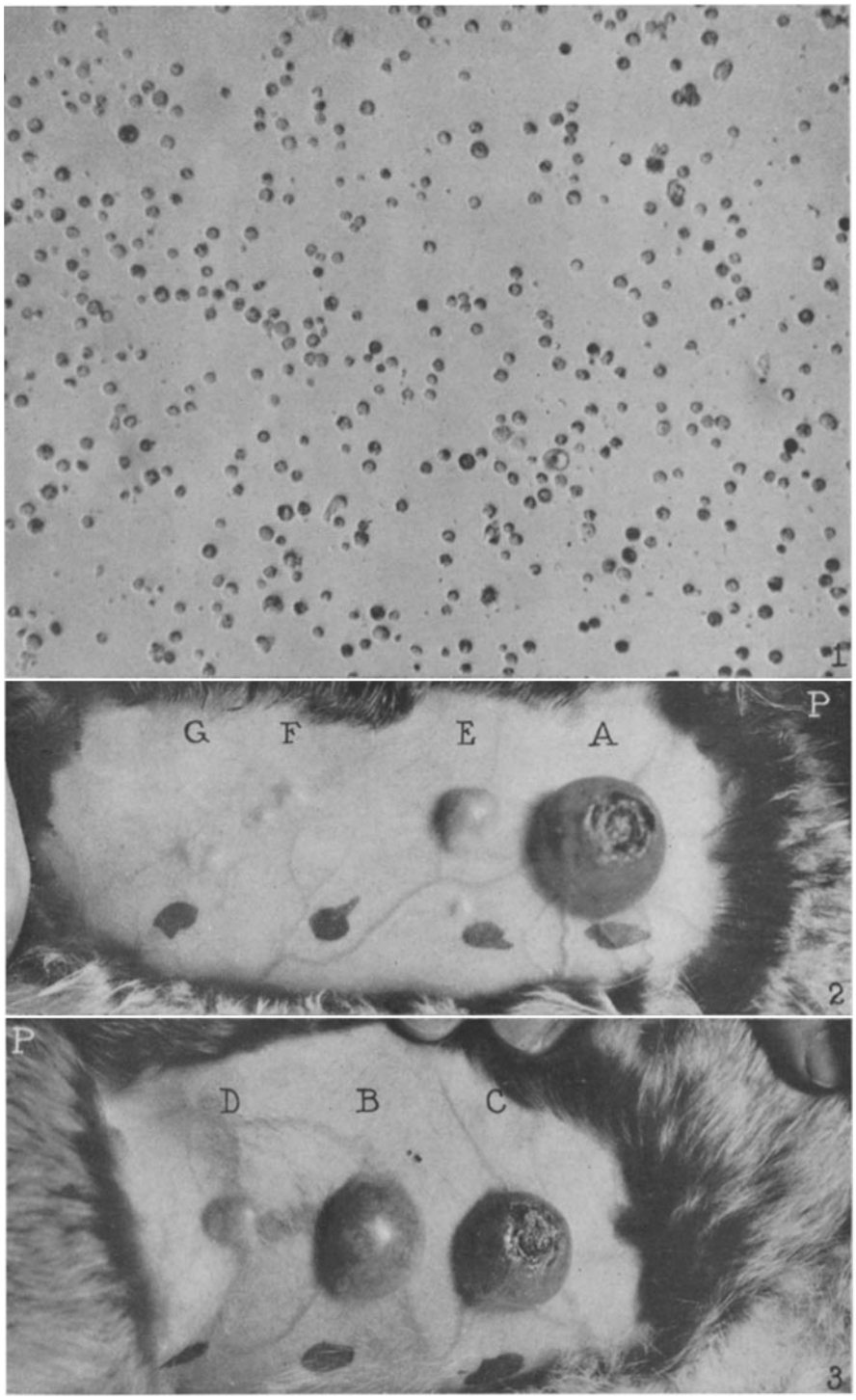
13. Smith, W., *Brit. J. Exp. Path.*, 1929, **10,** 93.
14. Douglas, S. R., and Smith, W., *Brit. J. Exp. Path.*, 1930, **11,** 96.
15. Fairbrother, R. W., *J. Path. and Bact.*, 1932, **35,** 35; 1933, **36,** 55.
16. Todd, C., *Brit. J. Exp. Path.*, 1928, **9,** 19.
17. Kraus, R., von Eisler, and Fukuhara, *Z. Immunitätsforsch., Orig.*, 1908–09, **1,** 307.
18. Krueger, A. P., *J. Gen. Physiol.*, 1930–31, **14,** 493.
19. Andrewes, C. H., *J. Path. and Bact.*, 1928, **31,** 671; 1929, **32,** 265; 1930, **33,** 265.
20. Fairbrother, R. W., *J. Path. and Bact.*, 1932, **35,** 35.
21. Bedson, S. P., *Brit. J. Exp. Path.*, 1929, **10,** 364.
22. Rous, Peyton, and Jones, F. S., *J. Exp. Med.*, 1916, **23,** 601.
23. Perdrau, J. R., and Todd, C., *Proc. Roy. Soc. London, Series B*, 1933, **112,** 288.
24. Rous, Peyton, and Jones, F. S., *J. Exp. Med.*, 1916, **23,** 601.
25. Smith, W., *Brit. J. Exp. Path.*, 1929, **10,** 93.
26. Douglas, S. R., Smith, W., and Price, L. R. W., *J. Path. and Bact.*, 1929, **32,** 99.
27. Olitsky, P. K., and Long, P. H., *J. Exp. Med.*, 1929, **50,** 263.
28. Rous, Peyton, *J. Exp. Med.*, 1913, **18,** 416.
29. Andrewes, C. H., *J. Path. and Bact.*, 1931, **34,** 91; 1932, **35,** 243; 1933, **37,** 27.
30. Rous, Peyton, and Beard, J. W., *J. Exp. Med.*, 1934, **60,** 701. Beard, J. W., and Rous, Peyton, *J. Exp. Med.*, 1934, **60,** 723. Rous, Peyton, and Beard, J. W., *J. Exp. Med.*, 1934, **60,** 741.
31. Shope, R. E., *J. Exp. Med.*, 1933, **58,** 607.
32. Traub, E., *J. Exp. Med.*, 1933, **58,** 663.

EXPLANATION OF PLATE 31

FIG. 1. Suspension of living cells as prepared for exposure to a virus. A few minutes before the photograph was taken a drop of the suspension was placed on a slide coated with neutral red. The cells that appear dark have already segregated the dye. ×130.

FIGS. 2 and 3. Shope lesions on sides of Rabbit 1 of Experiment 8 (Chart 8), photographed on the 17th day. The skin is marked with dye below the site of each injection. *P* = rump of the animal.

The inocula were:—*A*, cells washed in gel. Ty.; *B*, cells killed with water and washed in gel. Ty.; *C*, cells rotated with immune serum, killed with water and washed; *D*, cells killed with water, rotated with immune serum and washed; *E*, decelled fluid from tryptic digest; *F*, decelled supernatant fluid from the cultures; *G*, cells from the supernatant fluid. *F* and *G* gave rise late to scattered, shotty nodules, and one developed several centimeters below the main growth caused by *E*. ×½.



Photographed by Louis Schmidt and Joseph B. Haulenbeek

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