# THE DETECTION OF A "MASKED" VIRUS (THE SHOPE PAPILLOMA VIRUS) BY MEANS OF IMMUNIZATION*

## Results of Immunization with Mixtures Containing Virus and Antibody

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The effects of the rabbit papilloma virus (Shope) are curiously complicated by the fact that the virus is oftentimes "masked:" it cannot be recovered in pathogenic form from the growths produced with it in domestic rabbits (except infrequently and in small amounts) by the methods that invariably extract it in quantity from the natural papillomas of wild cottontails (1). The phenomenon may have implications for the tumor problem. For the Shope virus gives rise to growths which, though benign, have the immediate characters of neoplasms, and, in addition, frequently become cancerous (2).

The general failure of attempts to "unmask" the virus has made necessary recourse to indirect methods for its detection. One of these makes use of serological tests for the specific antiviral antibody. This antibody is never found in the serum of normal domestic rabbits but appears in the blood of animals carrying virus-induced papillomas or transplanted carcinomas derived therefrom; and its titer rises as the growths enlarge,—findings which prove that the virus persists enduringly in the papillomas, as also in masked or altered form in the carcinomas, and that it increases as they proliferate (3). A second indirect method of testing for "masked" papilloma virus makes use of one of Shope's findings, namely that saline suspensions of virus-induced rabbit papillomas may stimulate the production of the specific antiviral antibody when injected intraperitoneally into normal rabbits, even though they contain no pathogenic virus demonstrable by the ordinary test (4). But as a test for the presence of "masked" virus the latter method has certain limitations, which now warrant delineation.

The antigenicity of different papilloma extracts varies widely, as we have repeatedly noted, those containing virus in quantity eliciting antibody in much higher titer when injected intraperitoneally into normal rabbits than others in which little or no infectious virus is present (5). Indeed, certain papilloma extracts devoid of demonstrable virus have failed, in some of our experiments, to elicit detectable amounts of antibody though repeatedly injected into normal

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rabbits,—a phenomenon not encountered by Shope (4). Does the finding indicate that virus may be absent from some papillomas, or merely that its antigenicity as well as its pathogenicity may be somehow "masked" when the growths are extracted? To learn about this we have sought to evaluate the effects of extravasated antiviral antibody upon the antigenicity of papilloma extracts. For studies already reported have shown that the antibody accumulates in the papillomas in various amounts—depending upon the titer of it in the blood of the host and upon the local vascular conditions determining its extravasation—and that it is often present in quantities sufficient to neutralize or "mask" the causative virus when the growths are extracted *in vitro* (6). It seemed possible that the extravasated antibody might also reduce or abolish the antigenicity of the virus; for it had long been known that the antigenicity of other viruses and of bacterial toxins as well may be reduced or abolished upon admixture *in vitro* with antibodies directed specifically against them (7). In the work now to be reported it will become evident that extravasated antibody often influences decisively the outcome of immunization experiments of the sort outlined.

## *Methods and Materials*

The general plan of the immunization experiments was patterned closely after that of Shope (4) in order to procure results comparable with his. Normal adult gray-brown (agouti) rabbits, bought from several sources outside the Institute, and weighing from 2500 to 3200 gm., were used throughout, males and females indiscriminately. Groups of three or more individuals comparable as to weight were used in each experiment. Crude suspensions of papillomas from wild and domestic rabbits were made by grinding the glycerolated tissue in a mortar with sand and suspending the ground paste in 0.9 per cent NaCl solution. Sometimes these extracts were used as such but more often they were spun briefly to remove the sand and gross tissue debris. Occasionally they were spun hard and sometimes filtered through Berkefeld candles in addition. The suspensions or filtrates, sometimes after incubation with immune serum *in vitro*, were then injected intraperitoneally into the experimental animals, with precautions to prevent infection of the skin with virus.[1] After an interval of 7 to 9 days the injections were repeated with freshly prepared extracts, and after a further period of about a week the animals were bled to procure serum for neutralization and complement fixation tests. They were then inoculated with the virus to find whether they had become resistant to it. Also they were felt all over

[1] A modification of Shope's method invariably proved effective: A small slit was made through the skin with a sterile scalpel. The lips of the wound were held open and the needle inserted into the subcutaneous tissue and run forward about 1 inch before it was pushed through the peritoneum. After the injection had been made, slight pressure was put onto the needle with a finger as it was withdrawn, to prevent seepage along the needle track, and afterwards the wound was soaked for a minute in 5 per cent carbolic acid followed by 95 per cent alcohol.

carefully and repeatedly to learn whether any papillomas had developed, as might have happened had the skin become infected with the virus where the injecting needle was thrust through or elsewhere. Growths were never found, and hence the results of the tests can be referred without exception to the effects of the injected materials.

The *resistance tests* were carried out by rubbing various dilutions of virus of known pathogenicity into scarified areas about 6 × 4 cm. on the ventral skin of the injected rabbits, along with suitable normal controls, according to a procedure already described (8). Strips of fur at least 2 cm. across were left between the scarified areas to prevent cross infection, and for the same reason the scarified areas were dried thoroughly by means of a blast of warm air from a small electric hair drier immediately after all of the inoculations on each rabbit had been finished. The lesions were examined as routine every 3 to 5 days from the 15th to the 42nd days, and recorded, as in neutralization and pathogenicity tests (8), according to a standard scale: **** = confluent papillomas; *** = semiconfluent papillomas; ** = many discrete growths; * = 5 to 15 papillomas; ± = 2, 3, or 4 papillomas; ± = 1 papilloma; 0 = negative. In the tables the readings are recorded on three occasions only to conserve space. They typify the findings as a whole.

The *pathogenicity tests* for virus and the *neutralization tests* for antiviral antibody were carried out as already described (8). In the former the sera to be tested were mixed with a virus filtrate of known potency and the mixtures, after incubation at 37°C. for 1 hour, were rubbed into small scarified squares on the skin of three or more titration rabbits (labeled A, B, C, etc., in each experiment), which were then bandaged to prevent cross infection. The presence of neutralizing antibody in any given serum was manifest by a reduction in the number of growths appearing where the mixture of it with virus had been rubbed in as compared with those appearing as result of the normal serum or saline control inoculations.

The *complement fixation test* for antiviral antibody involves the same antigen as the neutralization test, namely, the virus; and many observations now attest to its specificity and reliability as a means of testing for the presence and quantity of the specific antiviral antibody, as also to the fact that neutralization and complement fixation are manifestations of the action of a single antibody (9). In the present work the test was carried out as previously described (9), with 2 units of complement, 2 hours at room temperature being allowed for fixation. Optimal or near optimal amounts of antigen, which consisted of Berkefeld V filtrates or centrifugalized extracts (water-clear) of wild rabbit papillomas containing known amounts of virus, were used to avoid post-zone effects. Preliminary readings were made after 30 minutes at 37°C., and final ones after the tubes had stood overnight in the refrigerator. ++++ = complete fixation (no hemolysis); +++ = about 75 per cent fixation; ++ = about 50 per cent fixation, + = about 25 per cent fixation, 0 = no fixation (complete hemolysis). Control tests for anticomplementary effects were made of the materials in double amounts. These were negative in all of the experiments reported.

The comparative value of the resistance, neutralization, and complement fixation tests as means of assaying the immune responses can best be perceived by the study of actual examples, as *e.g.* Experiments 2 and 3. The resistance test is by far the most delicate of the three, in the sense that it can indicate very minute immune responses. Its value is sharply limited, however, by a "low ceiling,"—meaning thereby

that resistance to the virus is complete or almost complete when a comparatively small antibody response has been elicited. Hence it is far from comprehensive. The complement fixation test, though least delicate of the three in the sense referred to above, has proved most valuable in measuring the degree of antibody response to the materials injected intraperitoneally; for in most of the experiments the serum antibody titers of the immunized animals have exceeded by far the minimum amount detectable by it, and have thus fallen into the range in which the complement fixation test is most useful. It has no "ceiling" and much experience has shown that it provides reliable quantitative results, the complement-fixing and virus-neutralizing capacities of any given serum invariably paralleling one another (9).

### *Effect of Serum Antibody on the Antigenicity of the Papilloma Virus*

As a first step towards determining the effects of extravasated antiviral antibody it seemed well to study the effect of serum antibody on the antigenicity of the virus. Various dilutions of a serum known to contain the antiviral antibody in quantity were mixed with a constant amount of a virus filtrate, and after incubation the mixtures were injected intraperitoneally into normal rabbits. They were tested as well for pathogenicity.

*Experiment 1.*—A 1:20 virus filtrate was made as usual from the natural papillomas of cottontail 1-28, which had been preserved 8 months in 50 per cent glycerol-Locke's. A gram of the glycerolated papillomas was ground with sand, suspended in 20 cc. of 0.9 per cent NaCl solution, centrifuged at 4400 R.P.M. for 15 minutes, and the supernatant fluid filtered through a Berkefeld V candle. Filtrates previously prepared in the same way from the papillomas of this animal had invariably contained much virus. Mixtures were made of the 1:20 virus filtrate in equal parts with saline and with immune serum D. R. F 4 in dilutions of 1:4, 1:32, and 1:128. This serum had come from a domestic rabbit with two large virus-induced papillomas resulting from broadcast virus inoculation 70 days before. Previous tests had shown that it contained the antiviral antibody in large amount (titer 1:96 as determined by complement fixation). The flasks containing the mixtures were sealed with paraffin and put into the water bath at 37°C. for 4 hours and kept overnight in the refrigerator. 20 cc. of each mixture was then injected intraperitoneally as already described into each of three normal gray-brown rabbits, and the remaining portions were tested for pathogenicity in three normal rabbits. Seven days later the injected rabbits were bled from an ear vein and the sera tested for antiviral antibody by means of complement fixation.

The results of the experiment are shown in Table I. The virus-saline mixture was actively pathogenic, giving rise promptly to confluent and semiconfluent lesions in all three test rabbits; and it was highly antigenic also, eliciting antibody in all three of the rabbits injected with it, with result that their sera fixed complement completely or almost so in all dilutions up to 1:64. The mixture of virus and immune serum diluted 1:128 proved slightly less pathogenic, engendering fewer and smaller growths; and it elicited somewhat less antibody, though still much, in the three rabbits injected with it. The mixture of virus and immune serum diluted 1:32 was still less pathogenic and correspondingly less antigenic; and the mixture of virus and immune serum diluted 1:4 engendered very few growths in the pathogenicity tests and elicited com-

TABLE I

*Serum Antibody Titer of Rabbits Injected with Mixtures of Virus and Immune Serum in Various Dilutions*

| Mixtures injected intraperitoneally* | Pathogenicity of injected mixtures (Inoculation growths in test rabbits A, B, C) | | | | | | | | | Rabbit No. | Complement fixation titer of serum of injected rabbits† | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 17th day | | | 26th day | | | 36th day | | | | | | | | | |
| | A | B | C | A | B | C | A | B | C | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
| (*a*) Virus + immune serum 1:4 | 0 | 0 | 0 | 0 | ± | 0 | * | ± | * | 13 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | | 14 | ++ | + | 0 | 0 | 0 | 0 |
| | | | | | | | | | | 15 | + | ± | 0 | 0 | 0 | 0 |
| (*b*) Virus + immune serum 1:32 | 0 | 0 | 0 | ± | ± | ± | ** | * | * | 16 | +++± | +++ | ± | 0 | 0 | 0 |
| | | | | | | | | | | 17 | +++± | ++++ | ++++ | +++ | 0 | 0 |
| | | | | | | | | | | 18 | ++++ | ++++ | ++++ | ++++ | +++± | ± |
| (*c*) Virus + immune serum 1:128 | *** | ± | * | **** | **± | ***± | **** | ** | *** | 19 | ++++ | ++++ | ++++ | +++± | +++ | 0 |
| | | | | | | | | | | 20 | ++++ | ++++ | ++++ | ++++ | +++± | + |
| | | | | | | | | | | 21 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| (*d*) Virus + saline | ***± | **± | ** | **** | *** | ***± | **** | ***± | **** | 22 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | | | | | | | | | | 23 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | | | | | | | | | | 24 | ++++ | ++++ | ++++ | ++++ | ++++ | +++± |

* Virus filtrate W. R. 1-28, 1:20, mixed and incubated with equal parts of immune serum D. R. F 4 in dilutions designated. 20 cc. of whole mixture injected into each rabbit.

†2 units of complement.

Antigen, W. R. 1-56, 1:120.

W. R. = wild cottontail rabbit.

D. R. = domestic rabbit.

paratively small amounts of complement-fixing antibody in the rabbits injected with it,—so little in fact that in one instance (rabbit 13) the serum failed to fix complement at all and in the other two did so only partially, in dilutions of 1:2 and 1:4.

The results of the experiment (Table I) leave no doubt that the antigenicity of the virus filtrate was markedly reduced by admixture *in vitro* with the immune serum. The finding has been repeatedly confirmed in other experiments. Virus filtrates prepared from the papillomas of many wild rabbits have invariably proved antigenic upon intraperitoneal injection into normal rabbits, eliciting antibody in direct proportion to the amount of virus they contained; and the antigenicity of the virus filtrates, though unaffected by normal rabbit serum, has always been reduced or abolished after mixture with serum containing the antiviral antibody.

The following experiment was done to determine whether the antigenicity of the virus can be wholly abolished if the virus is mixed *in vitro* with an excess of antibody.

*Experiment 2.*—A 1:20 virus filtrate was prepared as before of the glycerolated natural papillomas of W. R. 77, which had regularly provided much virus in previous extractions. Mixtures were made: (*a*) with two volumes of saline, and (*b*) with two volumes of the serum 1:4 of W. R. 1-52, a rabbit carrying natural papillomas and having serum with a very high titer of antibody (1:512 in the complement fixation test). A third mixture (*c*) contained no virus but consisted of saline plus two volumes of the immune serum 1:4. All were put into the water bath for 2 hours at 37°C., and then injected intraperitoneally into normal rabbits, 6 cc. of each mixture into each of three rabbits. Eight days later fresh mixtures were made and incubated precisely as before and the injections repeated. Ten days after the second intraperitoneal injection the rabbits were bled for serum along with suitable controls, and all were inoculated with various dilutions of virus (W. R. 1-72), as already described, to test for their resistance.

At the time of the first injections supplemental tests were made to determine roughly the amount of the virus contained in the injected mixtures, and also to learn whether the mixture of virus and immune serum (*b*) contained antibody in excess. Mixture (*a*) was found to contain much virus, engendering confluent growths (****) in each of three test rabbits, the growths appearing before the 14th day. It was found furthermore that 0.2 cc. of the virus filtrate after dilution to 1:10,000 still gave rise to 2 papillomas when inoculated on the standard scarified area of two test animals. Whence it follows that 1 cc. of the 1:20 virus filtrate contained at least 5,000 infectious doses of virus. Since each rabbit in the experiment received a total of 6 cc. at each injection, it is seen that the rabbits injected with the mixture (*a*), which consisted of one part of the virus filtrate 1:20 and two parts of saline, received at least 10,000 infectious doses of virus at each of the two injections. The mixture (*b*), composed of one part of the virus filtrate 1:20, as in (*a*), and two parts of the immune serum 1:4, proved innocuous in the pathogenicity tests,—that is to say, the virus had been completely neutralized; and complement fixation tests of a portion of the mixture that had been

centrifugalized after incubation showed that the supernatant liquid contained practically as much free antiviral antibody as was present in the mixture (*c*), both mixtures fixing 2 units of complement completely in a dilution of 1:64 and incompletely in a dilution of 1:128 upon admixture with an optimal concentration of antigen.

Table II shows the results of the serum and resistance tests of the animals injected intraperitoneally. It will be noted from the complement fixation and neutralization tests that the control mixture of virus and saline (*a*) proved moderately antigenic, the serum of the rabbits injected with it fixing complement completely in dilutions from 1:2 to 1:16 and partially at 1:32, and the antibody was sufficient to cause complete or almost complete neutralization of a potent virus filtrate. Moreover, the rabbits injected with this mixture proved wholly resistant, or almost so, to the virus, no lesions or only a few appearing where the dilutions of the test virus had been inoculated in the resistance tests. The sera of the three rabbits injected with the mixture of virus and immune serum (*b*) failed to fix complement at all and neutralized the virus only slightly; while in the resistance tests the injected rabbits proved almost as susceptible to the virus as the controls. The sera of the rabbits injected with the mixture of saline and immune serum (*c*) also failed to fix complement, but they exerted a considerable neutralizing effect on the test virus, rather more indeed than the sera of the rabbits injected with mixture (*b*) of virus and immune serum; and the rabbits themselves proved slightly more resistant to the virus on inoculation than did the latter group.

The findings are enlightening in several ways. They disclose a fact hitherto unrealized, namely, that resistance to the papilloma virus may be conferred by passively transferred antibody. The passively transferred antibody present in the mixture of virus and immune serum (*b*) could conceivably account for the whole of the slight neutralizing capacity of the sera of the rabbits receiving it, as also for the resistance they manifested to the virus, this also being slight. For the mixture contained a considerable excess of antibody, as supplemental tests proved. That transferred antibody does account for the findings is indicated by the fact that the mixture of virus and immune serum (*b*), which presumably contained somewhat less free antibody than the mixture of saline and serum mixture (*c*), conferred less immunity than the latter, as the results of the neutralization and resistance tests clearly show. The conclusion seems warranted that the antigenicity of the virus, which was considerable as the results with mixture (*a*) proved, was completely abolished by exposure *in vitro* to an excess of immune serum.

*Effects of Extravasated Antibody upon the Antigenicity of Papilloma Extracts*

The findings already given have made plain the fact that serum antibody can reduce or abolish the antigenicity of the papilloma virus when mixed therewith *in vitro*. Is the same effect produced by extravasated antibody, which accumulates in various amounts in the papillomas, depending upon the titer of it in the blood and upon the local conditions influencing its extravasation (9)? There

is every reason to think so, since extravasated and serum antibody are identical (10). The results of several experiments prove the point. In the first of these, two papilloma extracts were compared as to antigenicity, one containing much extravasated antibody, the other little or none. Control tests were done to determine the effect of the antibody transferred passively along with one of the injected materials.

TABL

*Serum Antibody Titer and Resistance of Rabbits Inj*

| Mixtures injected intraperitoneally on 1st and 8th days§ | Rabbit No. | Antibody titer of serum* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Complement fixation tests† | | | | | Neutralization tests‡ | | | | | | |
| | | | | | | | 17th day | | | 27th day | | | |
| | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | D | E | F | D | E | F |
| (*a*) Virus + saline | 1 | ++++ | ++++ | ++++ | ++++ | ± | 0 | 0 | 0 | 0 | 0 | ± |
| | 2 | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | ± |
| | 3 | ++++ | ++++ | ++++ | ++++ | +++ | 0 | 0 | ± | 0 | 0 | ± |
| (*b*) Virus + immune serum | 4 | 0 | 0 | 0 | 0 | 0 | * | * | ****± | ** | ** | **** |
| | 5 | 0 | 0 | 0 | 0 | 0 | ± | * | *** | * | ** | **** |
| | 6 | 0 | 0 | 0 | 0 | 0 | * | * | ***± | ** | **± | **** |
| (*c*) Saline + immune serum | 7 | 0 | 0 | 0 | 0 | 0 | * | 0 | ± | **± | ± | * |
| | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ** | * | 0 | ***± |
| | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | * | ± | * | * |
| (*d*) Nil, controls | 10 | 0 | 0 | 0 | 0 | 0 | ± | ± | ** | * | ± | *** |
| | 11 | 0 | 0 | 0 | 0 | 0 | ***± | ** | ****± | ****± | *** | **** |
| | 12 | 0 | 0 | 0 | 0 | 0 | ** | **± | ****± | ****± | **± | **** |
| Saline control | | | | | | | ** | ***± | ***± | *** | *** | **** |

* Serum procured on the 10th day after the second intraperitoneal injection.

† 2 units of complement. Antigen, W. R. 1-72, 1:20.

‡ Growths resulting from mixtures of 2.5 per cent virus filtrate W. R. 77 E and whole serum in equal parts in

§ The mixtures (*a*) and (*b*) contained at least 10,000 infectious doses of the virus, as supplemental tests prov
The mixture (*b*) contained a considerable excess of antibody, as was shown by supplemental tests with cer

*Experiment 3.*—To procure papillomas containing much extravasated antibody, two domestic rabbits carrying vigorous, confluent papillomas were injected intraperitoneally on the 24th and 31st days after scarification with W. R. 1-28 virus with 10 cc. of a potent 10 per cent virus filtrate (W. R. 77). By the 38th day their serum antibody titers had reached 1:128 and 1:256, respectively, as determined by complement fixation tests. The growths had enlarged steadily in the meantime—no unexpected finding since circulating antibody is known to be ineffective against virus in the living papillomas (8). When harvested on the 38th day the growths were char-

acteristic, vigorous, confluent papillomatous masses 3 to 4 cm. across and raised 1 to 1.5 cm. above the level of the skin. They were washed with soap and rinsed well to reduce the number of surface bacteria and were cut away immediately after the animals had been killed. The blood was blotted off on a sterile sponge and the connective tissue and skin trimmed away. The whole growths, including the dried keratinized parts, which presumably contained much extravasated antibody, were then diced vertically into pieces about 5 mm. across and pooled and put into a single bottle of

E II

*ected with Mixtures of Virus and Immune Serum*

| | | | Resistance to dilutions of papilloma virus | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 38th day | | | 14th day | | | | 24th day | | | | 38th day | | | |
| A | B | C | 1:20 | 1:100 | 1:500 | 1:2500 | 1:20 | 1:100 | 1:500 | 1:2500 | 1:20 | 1:100 | 1:500 | 1:2500 |
| 0 | 0 | ± | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 | ± | 0 | 0 | 0 |
| 0 | 0 | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 0 | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** | ** | **** | ***± | *** | ** | 0 | **** | **** | *** | * | **** | **** | ***± | ± |
| * | ** | **** | **± | ** | ± | 0 | **** | *** | ** | * | **** | ***± | ***± | *** |
| ** | **± | **** | **± | ** | 0 | 0 | *** | **± | ± | ± | ***± | *** | **± | * |
| **± | ± | * | ** | 0 | 0 | 0 | ***± | * | 0 | 0 | ***± | *** | ± | 0 |
| * | 0 | **** | ** | 0 | 0 | 0 | *** | * | 0 | 0 | **** | **± | 0 | 0 |
| ± | * | * | ** | 0 | 0 | 0 | **± | ± | 0 | 0 | **± | 0 | 0 | 0 |
| * | * | *** | *** | ** | * | 0 | **** | *** | *** | ** | **** | **** | ***± | ***± |
| **** | **** | **** | *** | **± | 0 | 0 | **** | **** | *** | **± | **** | **** | **** | ** |
| **** | **** | **** | **± | * | * | 0 | *** | *** | ***± | ** | **** | ****± | ***± | ***± |
| *** | ***± | **** | | | | | | | | | | | | |

.test rabbits A, B, C.
ed,—see text.
galized (absorbed) supernatant fluids.

sterile 50 per cent glycerol-Locke's solution in the refrigerator, only enough of the glycerol solution being added to cover the tissue.

The papillomas of two other rabbits provided material containing little or no extravasated antibody. The growths had been engendered with the same virus inoculum as those of the two hyperimmunized rabbits and at the same time, but were smaller and fewer, discrete and semiconfluent, 1 to 2 cm. across and not over 7 mm. high, though fleshy and vigorous. The sera of the rabbits carrying them failed to fix complement in the standard test in dilutions of 1:2 or more, and it was for this reason

that the two materials were selected. The growths were harvested as in the case of the other animals save that much of the dried material was cut away from their tops to get rid of included blood. With the same aim the diced pieces were pooled and put into about five volumes of Locke's solution for 15 minutes, with occasional stirring, and the procedure repeated with fresh fluid. The diced pieces from both animals were stored together in the refrigerator, in a considerable excess of glycerol-Locke's solution.

After 48 hours in glycerol-Locke's, the two materials were ground in mortars and suspended 1:10 in saline as usual. The suspensions were centrifugalized at very low speed for 1 minute to throw down the sand and largest tissue fragments and the turbid supernatant fluids were removed for use. The material from the hyperimmunized animals, which presumably contained much extravasated antibody, was divided into two portions for injection into normal rabbits. One portion (*a*) was injected as such, as was that from the papillomas presumably containing little or no extravasated antibody (*c*), while the other (*b*) was centrifugalized hard with the object of eliminating from it any virus or other heavy substance that might act as antigen while leaving its content of free antibody largely unaffected. Thus it would provide a control to the effects of antibody present in material (*a*). Previous work had shown that centrifugalization at 25,000 R.P.M. for 60 minutes would throw down the virus but not the antibody (9). Hence material (*b*) was spun at this speed for an hour after a preliminary spinning at 4400 R.P.M. for 15 minutes to remove coarse particles. For comparative purposes a fluid known to contain active virus was utilized. It was a 1:10 Berkefeld V filtrate (*d*) made from the glycerolated natural papillomas of W. R. 56, which were known to contain an abundance of virus. The four materials were injected intraperitoneally into comparable groups of normal rabbits, 5 cc. in each animal. Eight days later the injections were repeated, with materials prepared precisely as before. The pathogenicity of the injected materials was also tested and the capacity of (*a*), (*b*), and (*c*) to neutralize virus *in vitro*. Sera were drawn from the injected and control animals on the 16th day for antibody tests, and inoculations with various dilutions of virus were done on the 18th day to test for resistance.

The results of the tests are set down in Table III. The papilloma suspension (*a*) proved non-pathogenic, and it rendered almost innocuous the potent 1 per cent virus filtrate with which it was mixed for the neutralization test. It elicited no antibody, however, upon injection into three normal rabbits—or, at any rate, too little to be detected by the complement fixation test—though the rabbits injected with it proved partially resistant to the virus. The results were practically identical with material (*b*), which presumably had been rid of virus or other immunizing material by centrifugation but contained as much extravasated antibody as (*a*). It follows that the partial resistance to the virus manifested by the rabbits injected with the two materials can be attributed to the effects of passively transferred antibody. Material (*c*) contained no detectable free antibody, as was shown by the fact that it had no more effect on the virus than so much saline in the neutralization tests. Indeed it even proved slightly pathogenic, giving rise to 1 and 3 papillomas, respectively, on the expanses of scarified skin of two of the three rabbits of the pathogenicity test. It was notably antigenic, eliciting considerable amounts of the complement-fixing antibody in all of the six rabbits injected with it. The amount of antibody elicited was decidedly less,

however, than that called forth by the much more highly pathogenic W. R. filtrate (*d*) though the resistance to the virus was complete or almost so in all of the animals of both groups.

The results of the experiment (Table III) may be briefly summarized. The suspension of domestic rabbit papillomas that contained much extravasated antibody (*a*) proved non-antigenic upon injection into normal rabbits, and it conferred no more resistance than could be accounted for by the passive transfer of the antibody contained in it, as the findings with material (*b*) make clear. The suspension of domestic rabbit papillomas (*c*), which contained little or no extravasated antibody but, by contrast, a small amount of infectious virus, proved notably antigenic, though not so much so as a filtrate of wild rabbit papillomas that contained virus in abundance (*d*).

The findings would seem to indicate that the extravasated antibody present in quantity in one of the materials had abolished the antigenicity of any "masked" virus associated with it. It is conceivable, however, that the antigenic principle—be it "masked" virus or something else—could have been absent from this material as not from the other. A comprehensive experiment was undertaken therefore to broaden the findings. This time papilloma suspensions procured from a number of rabbits, some containing much extravasated antibody and others little, were compared as to antigenicity, as in the preceding experiment.

*Experiment 4.*—Papillomas were harvested from two groups of rabbits. One group of six rabbits carried growths engendered 40 days before with a single virus material (W. R. 56). Three of them carried confluent growths 3 to 4 cm. across and up to 1.0 cm. high and had very high serum antibody titers in consequence of two hyperimmunizing injections on the 24th and 33rd days of potent virus filtrates prepared from the pooled natural papillomas of five wild rabbits; while three others had smaller discrete and semiconfluent papillomas and smaller serum antibody titers, less than 1:2 in two instances. Another group of three rabbits carried confluent papillomatous masses 4 to 6 cm. across and up to 2.0 cm. high, which had been engendered 142 days before with a different virus inoculum (W. R. 1-28). Their sera contained considerable amounts of antibody but nowhere near as much as was present in the sera of the hyperimmunized rabbits. The growths were harvested and stored in glycerol-Locke's solution precisely as in Experiment 3. Those of the two rabbits with least amounts of serum antibody as determined by complement fixation were washed twice in Locke's solution further to remove any serum antibody.

The glycerolated materials were ground and suspended 1:20 in saline as usual and spun lightly to throw out sand and coarse fragments. The turbid supernatant suspensions were used for intraperitoneal injection into comparable groups of rabbits. A 1:20 Berkefeld V filtrate of the highly infectious papillomas of cottontail 1-68 was injected into another group for comparison. The rabbits to be immunized received 5 cc. of the respective materials on 2 successive days and 10 cc. of freshly prepared suspensions on the 8th day. Sera were procured for antibody tests on the 16th day, and virus was inoculated immediately afterwards to test for resistance.

Table IV shows the results of the experiment. It will be seen that the papilloma suspensions procured from the hyperimmunized rabbits (63, 62, 61) contained no pathogenic virus but on the contrary had the capacity to neutralize almost completely a potent 1 per cent virus filtrate when mixed with it *in vitro*. The sera of the animals receiving these suspensions failed to fix complement in the antibody tests and the animals manifested a relatively slight resistance to the virus on inoculation as com-

TABLE

*Serum Antibody Titer and Resistance of Rabbits Injected with Suspensions of Domestic*

| Materials injected intraperitoneally on 1st and 9th days* | Pathogenicity of injected suspensions 24th day | | | Neutralizing capacity of injected suspensions† 24th day | | | Rabbit No. | Complement fixation | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | A | B | C | | 1:2 | 1:4 | 1:8 |
| (*a*) Whole suspension of D. R. papillomas containing much extravasated antibody | 0 | 0 | 0 | 0 | 0 | ± | 52 | 0 | 0 | 0 |
| | | | | | | | 53 | 0 | 0 | 0 |
| | | | | | | | 54 | 0 | 0 | 0 |
| (*b*) Same as (*a*) except supernatant of suspension spun at 25,000 R.P.M. for 1 hr. | 0 | 0 | 0 | 0 | 0 | ± | 55 | 0 | 0 | 0 |
| | | | | | | | 56 | 0 | 0 | 0 |
| | | | | | | | 57 | 0 | 0 | 0 |
| (*c*) Whole suspension of D. R. papillomas containing little or no extravasated antibody | 0 | ± | ± | ***± | ***± | **** | 46 | +++± | + | 0 |
| | | | | | | | 47 | +++± | +++ | 0 |
| | | | | | | | 48 | ++++ | ++++ | +± |
| | | | | | | | 49 | ++++ | ++++ | ++++ |
| | | | | | | | 50 | ++ | ± | 0 |
| | | | | | | | 51 | +++± | + | 0 |
| (*d*) Filtrate of W. R. papillomas containing much infectious virus | **** | *** | **** | | | | 58 | ++++ | ++++ | ++++ |
| | | | | | | | 59 | ++++ | ++++ | ++++ |
| | | | | | | | 60 | ++++ | ++++ | ++++ |
| (*e*) Nil, controls | | | | | | | 61 | 0 | 0 | 0 |
| | | | | | | | 62 | 0 | 0 | 0 |
| | | | | | | | 63 | 0 | 0 | 0 |

* 10 cc. of a 1:10 saline extract injected twice into each rabbit as indicated.

† Growths resulting from mixtures of the extracts indicated (centrifugalized) and 1 per cent virus filtrate W.

‡ Procured on the 18th day.

pared with the normal controls. The papilloma suspension of rabbit 11-15, which had long carried large growths and had a considerable amount of serum antibody (titer 1:32), also failed to elicit complement-fixing antibody in the injected rabbits; and these latter proved very slightly resistant to the virus, even less so than did the rabbits injected with the papilloma suspensions of the hyperimmunized rabbits. Since the neutralization tests showed that the amount of antibody passively transferred with the papilloma suspension of rabbit 11-15 was less than that in the materials of the hyperimmunized rabbits 63, 62, and 61, and since the resistance manifested by

the rabbits injected with it was less than that conferred by the materials of the hyperimmunized rabbits, it seems likely that the slight degree of resistance manifested by all four groups of injected rabbits may have been due to passively transferred antibody. This is rendered probable also by the fact that the degree of resistance was comparable to that conferred by the passively transferred antibody of material (*b*) in Experiment 3 (see Table III).

III

*Rabbit Papillomas Containing (a, b) Much and (c) Little Extravasated Antibody*

| titer of serum‡ | | | Resistance to dilutions of papilloma virus inoculated on 18th day | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 15th day | | | | 25th day | | | | 35th day | | | |
| 1:16 | 1:32 | 1:64 | 1:20 | 1:100 | 1:500 | 1:2500 | 1:20 | 1:100 | 1:500 | 1:2500 | 1:20 | 1:100 | 1:500 | 1:2500 |
| 0 | | | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | ± | ± | 0 | 0 |
| 0 | | | * | 0 | 0 | 0 | **± | ± | 0 | 0 | *** | ± | 0 | 0 |
| 0 | | | * | ± | ± | 0 | **± | **± | ** | ± | *** | **± | ** | ± |
| 0 | | | * | 0 | 0 | 0 | *** | * | ± | 0 | **** | * | ± | 0 |
| 0 | | | ** | ± | 0 | 0 | *** | * | ± | 0 | ****± | ** | ± | 0 |
| 0 | | | 0 | 0 | 0 | 0 | *± | 0 | 0 | 0 | *** | ± | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 | ± | 0 | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 | * | 0 | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | ± | ± | 0 | 0 |
| ++++ | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 | ± | 0 | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ++++ | ++++ | ++± | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 | ± | 0 | 0 | 0 |
| ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | ± | ± | 0 | 0 |
| ++++ | ++++ | ++± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 |
| 0 | | | ** | * | 0 | 0 | ***± | *** | **± | **± | **** | **** | ***± | **± |
| 0 | | | *** | **± | **± | 0 | ***± | ***± | ***± | ** | **** | **** | ***± | ** |
| 0 | | | **± | ** | ± | 0 | ***± | ***± | ***± | ** | ***± | ***± | ***± | ** |

R. 77 E. Saline control = same as (*c*). A, B, C = test rabbits.

The papilloma suspensions coming from the rabbits with little or no serum antibody (55 and 68) had no neutralizing effect on the test virus; they elicited detectable amounts of complement-fixing antibody in the injected rabbits, and these proved largely resistant to the virus. Similar results were got with the materials of rabbits 11-17, 69, and 10-76. The suspensions of these growths contained moderate or small amounts of neutralizing antibody and they elicited various though in general small amounts of complement-fixing antibody and induced but slight resistance in the rabbits injected with them.

| Source of suspensions injected intraperitoneally | | | Pathogenicity of suspensions | Neutralizing capacity of injected suspensions§ 28th day | | | Rabbits injected | Complement fixation titer | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Papillomas procured from | Antibody titer of serum | Duration of growths | | A | B | C | | 1:2 | 1:4 | 1:8 | 1:16 | |
| | | *days* | | | | | *No.* | | | | | |
| D. R. 63‡ | 1:256 | 40 | 0 | 0 | ± | 0 | 1-20 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-21 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-22 | 0 | 0 | 0 | 0 | |
| 62‡ | 1:128 | " | 0 | 0 | ± | 0 | 1-23 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-24 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-25 | 0 | 0 | 0 | 0 | |
| 61‡ | 1:128 | " | 0 | 0 | ± | ± | 1-26 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-27 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-28 | 0 | 0 | 0 | 0 | |
| 11-15 | 1:32 | 142 | 0 | ± | * | * | 1-29 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-30 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-31 | 0 | 0 | 0 | 0 | |
| 11-17 | 1:16 | 142 | 0 | ± | * | ± | 1-36 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-35 | + | 0 | 0 | 0 | |
| | | | | | | | 1-37 | +± | ± | 0 | 0 | |
| 69 | 1:16 | 40 | 0 | ** | *** | ** | 1-42 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-43 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-41 | ± | 0 | 0 | 0 | |
| 10-76 | 1:32 | 142 | 0 | ±± | ** | ±± | 1-32 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-33 | +± | 0 | 0 | 0 | |
| | | | | | | | 1-34 | +++ | ++++ | ++++ | ++++ | + |
| 55 | <1:2 | 40 | 0 | ** | *** | *** | 1-46 | ± | 0 | 0 | 0 | |
| | | | | | | | 1-45 | +++± | ++ | ± | 0 | |
| | | | | | | | 1-44 | +++± | ++++ | ++ | 0 | |
| 68 | <1:2 | " | 0 | ** | *** | *** | 1-39 | + | ± | 0 | 0 | |
| | | | | | | | 1-40 | ++ | ++ | ± | 0 | |
| | | | | | | | 1-38 | +++± | ++++ | ++++ | +++± | |
| W. R. 1-68 | | | **** | | | | 1-47 | ++++ | ++++ | ++++ | ++++ | + |
| | | | | | | | 1-48 | ++++ | ++++ | ++++ | ++++ | + |
| | | | | | | | 1-49 | ++++ | ++++ | ++++ | ++++ | + |
| Normal controls | | | | | | | 1-68 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-69 | 0 | 0 | 0 | 0 | |
| | | | | | | | 1-70 | 0 | 0 | 0 | 0 | |

10 cc. of 1:20 suspensions of the papillomas injected on the 1st and 9th days. Sera procured for test on the 2 units of complement; antigen, W. R. 1-28 E, 1:160.

‡ Hyperimmunized.

§ Growths resulting from mixtures of centrifugalized extracts and 1 per cent virus filtrate 1:100. Saline

BLE IV

*Injected with Extracts of Domestic Rabbit Papillomas*

| of serum | | | Resistance to dilutions of papilloma virus inoculated on 16th day | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 15th day | | | | 25th day | | | | 35th day | | | |
| 1:32 | 1:64 | 1:128 | 1:20 | 1:100 | 1:500 | 1:2500 | 1:20 | 1:100 | 1:500 | 1:2500 | 1:20 | 1:100 | 1:500 | 1:2500 |
| [illegible] | | | ***± | * | 0 | 0 | **** | *** | ± | 0 | **** | ***± | ± | 0 |
| [illegible] | | | * | * | 0 | 0 | *** | *** | 0 | 0 | *** | *** | 0 | 0 |
| [illegible] | | | † | | | | | | | | | | | |
| 0 | | | **** | *** | 0 | 0 | **** | **** | ** | ± | **** | **** | ** | ± |
| 0 | | | *** | ** | 0 | 0 | **** | ***± | ± | 0 | **** | **** | ± | 0 |
| 0 | | | *** | 0 | 0 | 0 | **** | ***± | ± | 0 | **** | **** | * | 0 |
| [illegible] | | | | | | | | | | | | | | |
| 0 | | | ***± | ***± | * | 0 | **** | **** | ** | ± | **** | **** | *** | * |
| 0 | | | 0 | 0 | 0 | 0 | ** | * | 0 | 0 | ***± | **± | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | ± | ± | ± | 0 | ± | ± | ± | 0 |
| 0 | | | **** | *** | **± | * | **** | **** | *** | **± | **** | **** | ****± | ***± |
| 0 | | | ***± | * | 0 | 0 | **** | ***± | ** | 0 | **** | ***± | *** | ± |
| 0 | | | *** | *** | 0 | 0 | ***± | ***± | * | 0 | **** | **** | ** | ± |
| 0 | | | ***± | * | 0 | 0 | **** | ***± | *** | * | **** | ***± | *** | **± |
| 0 | | | † | | | | | | | | | | | |
| 0 | | | † | | | | | | | | | | | |
| 0 | | | 0 | 0 | 0 | 0 | **± | * | 0 | 0 | ***± | * | ± | 0 |
| 0 | | | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | * | * | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| [illegible] | | | * | 0 | 0 | 0 | ***± | **± | 0 | 0 | **** | **± | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | ± | ± | 0 | 0 |
| [illegible]++ | | | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 | ± | ± | ± | 0 |
| 0 | | | 0 | 0 | 0 | 0 | * | ± | 0 | 0 | **± | ± | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | * | * | 0 | 0 | * | * | 0 | 0 |
| 0 | | | 0 | 0 | 0 | 0 | * | ± | 0 | 0 | **± | * | ± | 0 |
| [illegible] | | | | | | | | | | | | | | |
| 0 | | | 0 | 0 | 0 | 0 | **± | ± | ± | 0 | **± | ± | 0 | 0 |
| [illegible] | | | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | * | ± | 0 | 0 |
| [illegible] | | | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | ± | ± | 0 | 0 |
| [illegible] | | | | | | | | | | | | | | |
| +++ | +++ | 0 | 0 | 0 | 0 | 0 | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| +++ | ++++ | +± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| +++ | ++++ | +++± | 0 | 0 | 0 | 0 | ± | ± | 0 | 0 | ± | ± | ± | 0 |
| [illegible] | | | ****± | *** | ** | * | **** | **** | **** | *** | **** | **** | **** | ***± |
| 0 | | | ***± | ** | * | 0 | ****± | ****± | *** | **± | **** | **** | ****± | *** |
| [illegible] | | | ****± | *** | ** | * | **** | ****± | ****± | ** | **** | ***± | ****± | ** |

16th day.

control same as materials 68 and 55.

The material from cottontail 1-68, which contained much virus, elicited far more antibody and far more solid resistance to the virus than any of the papilloma suspensions procured from domestic rabbits.

In sum, the results of the experiment (Table IV) confirm and extend those of Experiment 3. Suspensions of the papillomas of rabbits with high serum antibody titers (D. R. 63, 62, 61, 11-15), which contained much extravasated antibody, proved wholly or almost wholly non-antigenic, as was shown by the fact that they elicited no antibody detectable by the complement fixation test. By contrast papilloma suspensions containing little or no extravasated antibody (D. R. 55, 68, 10-76, 69) proved slightly or moderately antigenic, as indicated by the amount of complement-fixing antibody present in the sera of rabbits injected with them. The various degrees of resistance to the virus manifested by the injected rabbits on test inoculation provide confirmatory findings when due allowance is made for the effects of passively transferred antibody and for the limitations of the resistance test as a measure of immunity.

The findings of Experiments 2, 3, and 4 make clear the fact that passively transferred antibody will often account for much or all of the resistance to the virus manifested by rabbits injected with papilloma extracts. And they also demonstrate the fact that the test for resistance is inferior to the serum antibody tests as a measure of the immune response to the injected materials. Enough antibody was often transferred passively to confer perceptible resistance to the virus, but under the circumstances of the experiments it was never sufficient to give complement fixation when the blood of the recipient was tested. And yet the complement fixation test is sufficiently delicate to detect comparatively small quantities of the antiviral antibody. For most practical purposes it would seem that papilloma extracts may be deemed non-antigenic if they fail to stimulate sufficient antibody to react in the complement fixation test.

### *Effect of Extravasated Antibody on the Antigenicity of Extracts of Cottontail Papillomas*

In the following experiment the effect of extravasated antibody on the antigenicity of the virus was tested directly by mixing the two *in vitro* prior to inoculation.

*Experiment 5.*—The glycerolated papillomas of D. R. 63 and 11-15 were known from Experiment 4 to contain extravasated antibody. 1:10 saline suspensions were made of them and spun at 4400 R.P.M. for 20 minutes with removal of the clear supernatant liquids for use. Mixtures were made as indicated in Table V with a freshly prepared 1:2500 Berkefeld V filtrate of the highly infectious papillomas of W. R. 1-28. For comparison, two mixtures were made also with the immune serum of D. R. F 4 (Experiment 1). The mixtures were not incubated, but were injected intraperitoneally

into five lots of normal rabbits after they had stood at room temperature for from 5 to 15 minutes, the time required to complete the manipulations. Eight days later the injections were repeated with fresh mixtures made in precisely the same way. On the 16th day the injected animals were bled and the sera tested.

From Table V it will be seen that the control mixture containing virus and saline proved moderately pathogenic, as evidenced by the papillomas produced in the test animals; and it elicited considerable amounts of antibody, as the complement fixation

TABLE V

*Tests with Serums of Rabbits Injected with Virus Mixed with (a) Extracts of Domestic Rabbit Papillomas Containing Extravasated Antibody and (b) with Immune Serum*

| Mixture injected intraperitoneally on 1st and 8th days | Pathogenicity of injected materials | | | | | | Rabbit No. | Complement fixation titer of serum‡ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 24th day | | | 38th day | | | | 16th day | | | | |
| | A | B | C | A | B | C | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| Virus W. R. 1-28, 1:2500, 5.0 cc. + saline, 5.0 cc. | ± | ± | 0 | *± | * | ± | 2-04 | ++++ | +++± | 0 | 0 | 0 |
| | | | | | | | 2-05 | ++++ | ++++ | 0 | 0 | 0 |
| | | | | | | | 2-06 | ++++ | ++++ | ++++ | ++++ | +++ |
| Virus W. R. 1-28, 1:2500, 5.0 cc. + D. R. 63 papilloma extract, 5.0 cc. | 0 | 0 | 0 | 0 | 0 | 0 | 2-07 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-08 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-09 | 0 | 0 | 0 | 0 | 0 |
| Virus W. R. 1-28, 1:2500, 5.0 cc. + D. R. 11-15 papilloma extract, 5.0 cc. | 0 | 0 | 0 | 0 | 0 | 0 | 2-10 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-11 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-12 | 0 | 0 | 0 | 0 | 0 |
| Virus W. R. 1-28, 1:2500, 5.0 cc. + immune serum, D. R. F 4, 1:32, 5.0 cc. | 0 | 0 | 0 | 0 | 0 | 0 | 2-13 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-14 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-15 | 0 | 0 | 0 | 0 | 0 |
| Virus W. R. 1-28, 1:2500, 5.0 cc. + immune serum, D. R. F 4, 1:128, 5.0 cc. | 0 | 0 | 0 | 0 | 0 | ± | 2-16 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-17 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | 2-18 | 0 | 0 | 0 | 0 | 0 |

‡ 2 units of complement.
Antigen, W. R. 1–72, 1:120.

tests proved. The mixtures containing virus plus the papilloma extracts of D. R. 63 and 11-15, which contained extravasated antibody, proved non-pathogenic and failed to elicit detectable amounts of antibody upon injection. The mixtures containing virus plus the immune serum D. R. F 4 gave similar results.

The findings of Experiment 5 show that extravasated antiviral antibody, as procured from papillomas, reduces or abolishes the antigenicity of the virus precisely as does serum antibody. Tests were next made to determine the antigenicity of extracts of cottontail papillomas containing extravasated antibody in quantity.

*Experiments 6 and 7.*—Previous work had shown that antibody extravasates in quantity into the large confluent papillomas produced by sowing virus broadcast on the scarified skin of wild cottontail rabbits (6). To procure such growths now a 10 per cent suspension of the highly infectious W. R. 1-28 virus was rubbed into scarified areas about 8 × 10 cm. on the bellies of ten normal cottontails recently trapped in Kansas. Two weeks later, three of the animals with the largest papilloma masses were selected for hyperimmunization. 10 cc. of a 1:20 Berkefeld V filtrate of the infectious papillomas of W. R. 56 was given intraperitoneally to each, on the 14th and 21st days. On the 30th day the large, fleshy, confluent growths of two of these animals (W. R. 2-70 and 2-71), which were found to have serum antibody titers of 1:64 and 1:128 respectively, were harvested, diced, and placed separately in glycerol-Locke's, only enough of this being present to cover the tissue.

In *Experiment 6* a 1:20 saline suspension was made of the glycerolated papilloma tissue of W. R. 2-71, after it had been kept 29 days in glycerol. The suspension was spun at 2800 R.P.M. for 10 minutes and the supernatant liquid, opalescent but free from gross particles, removed for use. For comparison a 1:20 saline suspension was made in the same way of the glycerolated papillomas of W. R. 1-28, which were known to yield much virus. 2.0 cc. of each material was injected intraperitoneally into four normal Kansas cottontails and five normal domestic rabbits, and supplemental tests were made to determine the pathogenicity of the injected materials. The rabbits were bled for serum tests on the 7th day.

Table VI shows the results of the experiment. It will be seen that the injection of the W. R. 2-71 material,—which proved non-pathogenic in the supplemental tests,—elicited no demonstrable antibody in either the wild or the domestic rabbits; whereas the W. R. 1-28 material, which contained virus in quantity, proved notably antigenic, eliciting high titers of antibody in the blood of all of the animals injected with it, roughly the same in both species.

In *Experiment 7* two intraperitoneal injections were made of the materials, which at the time of the first injection had stood 6 days in glycerol, and supplemental tests were done to determine their pathogenicity as also their capacity to neutralize added virus. A Berkefeld V filtrate of the naturally occurring discrete growths of W. R. 1-21 was included for comparison. Table VII shows the findings. The 1:20 crude extract of the confluent growth of W. R. 2-71 proved non-pathogenic, engendering no growths whatever where inoculated into three test animals. Indeed it contained an excess of extravasated antibody, as shown by the fact that it neutralized almost completely an equal volume of potent 1 per cent virus filtrate. Yet it proved antigenic, though not powerfully so. Of three rabbits injected with 2.0 cc. of the material on the 1st and 8th days of the experiment, one (89) had no antibody detectable by the complement fixation test, another (90) had the barest minimum, and the third (91) had a considerable amount. Repeated injections of 5.0 and 10.0 cc. of the same material elicited correspondingly greater amounts of antibody. The 1:20 crude extract of the W. R. 2-70 material exerted some effect on the virus in the neutralization test, though less than the W. R. 2-71 material.[2] Nevertheless it gave rise to a

[2] "Neutral" or near-neutral mixtures of virus and antibody often prove slightly pathogenic, as previous observations have shown. For a discussion of the phenomenon, see *The Journal of Experimental Medicine*, 1940, **72**, 531.

few discrete papillomas where rubbed into the skin of the most susceptible test animal (A) though it proved non-pathogenic in the other two. Injections of it elicited antibody in all of the rabbits, and the antibody titers were in general somewhat higher than those elicited by corresponding amounts of the W. R. 2-71 material. The W. R. 1-21 material, which contained much virus, was much more highly antigenic than either of the two just considered.

TABLE VI

*Tests with Serum of Rabbits Injected with Extracts of Cottontail Papillomas Yielding (a) No Virus and (b) Much Virus*

| Source of material injected intraperitoneally | Rabbit No. | Complement fixation titer of serum | | | | |
|---|---|---|---|---|---|---|
| | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| (*a*) Confluent experimental papillomas of hyperimmunized W. R. 2-71 (yielded no virus on test) | W. R. 1 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 0 | 0 | 0 | 0 | 0 |
| | D. R. 1-50 | 0 | 0 | 0 | 0 | 0 |
| | 1-51 | 0 | 0 | 0 | 0 | 0 |
| | 1-52 | 0 | 0 | 0 | 0 | 0 |
| | 1-53 | 0 | 0 | 0 | 0 | 0 |
| | 1-54 | 0 | 0 | 0 | 0 | 0 |
| (*b*) Discrete natural papillomas of W. R. 1-28 (yielded much virus on test) | W. R. 6 | +++± | +++± | ++++ | ++++ | +++± |
| | 7 | +++ | +++ | ++++ | ++++ | ++++ |
| | 8 | +± | +± | +++± | ++++ | ++++ |
| | 10 | ++++ | ++++ | ++++ | ++++ | ++++ |
| | D. R. 1-55 | +++± | ++++ | ++++ | ++++ | +++± |
| | 1-56 | ++++ | ++++ | ++++ | ++++ | ++++ |
| | 1-57 | +++ | +++ | ++++ | +++ | +± |
| | 1-58 | +++ | +++± | ++++ | ++++ | ++++ |
| | 1-59 | +++± | ++++ | ++++ | +++± | ++ |

2 cc. of a 1:20 suspension of the papillomas was injected into each rabbit. Serum procured 7 days later for test.

2 units of complement in all tubes.

None of the sera was anticomplementary when tested concurrently in double amounts.

Antigen, W. R. 1-72, 1:120.

The findings of Experiments 6 and 7 are representative of the results of several experiments made with extracts of wild rabbit papillomas that proved non-pathogenic. A single injection of 2.0 cc. of the papilloma extract of W. R. 2-71,—which contained no infectious virus but on the contrary a considerable excess of extravasated antibody, as supplemental tests proved,—elicited no antibody detectable by the complement fixation test when injected into normal cottontail and domestic rabbits (Table VI). The same material, however, elicited antibody in a subsequent experiment (Table VII) when crude suspensions of it were injected repeatedly, and so too did suspensions of another ma-

TABLE VII

*Tests with Serums of Rabbits Injected with Extracts of Cottontail Rabbit Papillomas Containing Little or No Demonstrable Virus*

| Material injected intraperitoneally on 1st and 8th days | Pathogenicity of injected materials | | | | | | Neutralizing capacity of injected materials† | | | | | | Amount injected | Rabbit No. | Complement fixation titer of serum | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 19th day | | | 38th day | | | 19th day | | | 38th day | | | | | 16th day | | | | | | | |
| | A | B | C | A | B | C | A | B | C | A | B | C | | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
| | | | | | | | | | | | | | *cc.* | | | | | | | | | |
| Crude extract 1:20 of the confluent growth of hyperimmunized W. R. 2-71 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ± | 0 | 2.0 | 89 | 0 | 0 | 0 | 0 | 0 | | | |
| | | | | | | | | | | | | | | 90 | + | 0 | 0 | 0 | 0 | | | |
| | | | | | | | | | | | | | | 91 | +++± | +++± | +++± | ± | 0 | | | |
| | | | | | | | | | | | | | 5.0 | 93 | + | 0 | 0 | 0 | 0 | | | |
| | | | | | | | | | | | | | | 92 | +++± | +++± | +++ | 0 | 0 | | | |
| | | | | | | | | | | | | | | 94 | ++++ | ++++ | ++++ | +++± | ± | | | |
| | | | | | | | | | | | | | 10.0 | 96 | ++++ | +++± | ++± | 0 | 0 | | | |
| | | | | | | | | | | | | | | 97 | ++++ | ++++ | ++++ | 0 | 0 | | | |
| | | | | | | | | | | | | | | 95 | ++++ | ++++ | ++++ | ++++ | ++++ | | | |
| Crude extract 1:20 of the confluent growth of hyperimmunized W. R. 2-70 | 0 | 0 | 0 | * | 0 | 0 | ± | ± | ± | ** | ** | * | 2.0 | 82 | +++ | ++± | ± | 0 | 0 | | | |
| | | | | | | | | | | | | | | 80 | +++ | ++++ | ++++ | +++± | 0 | | | |
| | | | | | | | | | | | | | | 81 | +++± | ++++ | ++++ | ++++ | +++ | | | |
| | | | | | | | | | | | | | 5.0 | 83 | + | ++ | +± | +± | 0 | | | |
| | | | | | | | | | | | | | | 85 | ++++ | ++++ | ++++ | +++ | 0 | | | |
| | | | | | | | | | | | | | | 84 | ++++ | ++++ | ++++ | +++ | 0 | | | |
| | | | | | | | | | | | | | 10.0 | 86 | ++ | +++± | +++ | ± | 0 | | | |
| | | | | | | | | | | | | | | 87 | ++++ | ++++ | +++ | + | 0 | | | |
| | | | | | | | | | | | | | | 88 | +++± | ++++ | ++++ | ++++ | ++++ | | | |
| Saline control | | | | | | | ** | * | ** | *** | *** | *** | | | | | | | | | | |
| Berkefeld V filtrate of the naturally occurring, discrete growths of W. R. 1-21 | **** | *** | *** | **** | **** | **** | | | | | | | 10.0 | 105 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | 0 | 0 |
| | | | | | | | | | | | | | | 104 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++± | 0 |
| | | | | | | | | | | | | | | 106 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +± |

2 units of complement.

Antigen, W. R. 1-28, 1:120.

† Growths resulting from mixtures of papilloma suspensions and 1 per cent virus filtrate W. R. 1-21 in equal parts.

terial of similar sort. But both materials proved far less antigenic than cottontail extracts containing virus in quantity.

It is noteworthy that extracts of wild rabbit papillomas, even when they contained little or no infectious virus as in Experiments 6 and 7, have invariably proved more highly antigenic than those of domestic rabbit papillomas.

## COMMENT

The experiments here reported were undertaken to learn why extracts or suspensions of some domestic rabbit papillomas fail to elicit antiviral antibody upon intraperitoneal injection into normal rabbits. The finding had been exceptional in our experience and Shope had not encountered it (4). Yet it seemed to bear upon the phenomenon of the "masking" of the virus in domestic rabbit growths, and to mean either that the virus is absent from some papillomas as not from others, or that something is present in certain growths that renders extracts of them non-antigenic. Extravasated antiviral antibody is often present in the papillomas and cancers of wild and domestic rabbits in amounts sufficient to neutralize and effectively "mask" any virus that might be liberated when the growths are extracted *in vitro* (6); and it seemed likely that extravasated antibody might reduce or abolish the antigenicity of the virus. This proved to be the case. Saline suspensions of domestic rabbit papillomas that contained much extravasated antibody proved completely or almost completely non-antigenic upon injection into normal rabbits; while suspensions or extracts of comparable growths that contained little or no extravasated antibody were invariably antigenic in concurrent tests.

The findings disclose certain limitations in the value of immunization experiments as a means of demonstrating "masked" virus. Extravasated antibody may complicate the outcome of such experiments in either or both of two ways. Firstly, antibody transferred passively with the injected material often confers resistance to the virus (Experiments 3 and 4), and this may be confused with an immune response on the part of the injected animal. For this reason the positive results of such experiments require careful interpretation. Secondly, negative results are often due to the fact that extravasated antibody has reduced or abolished the antigenicity of the virus present in the injected material (Experiments 3, 4, and 5). Since the antiviral antibody is almost invariably present in greater or lesser amounts in extracts of virus-induced papillomas, as also in extracts of the cancers derived from them (6), the value of immunization experiments as a means of demonstrating "masked" virus in such growths would appear to be restricted to the instances in which positive results are got, and then only when the effects of passively transferred antibody can be ruled out. This can be done when considerable amounts of serum antibody are called forth. Obviously, a negative result can have no certain meaning if extravasated antibody was present in undetermined quantity in the injected material. For it may mean either that the antigenicity of the virus had been abolished

by extravasated antibody, or that the virus was present in amounts too small, or in a form which did not stimulate detectable amounts of antiviral antibody, or that it was actually absent.

Mention should be made here of the negative results of immunization experiments with extracts of cancers deriving from the papillomas of cottontail rabbits. Syverton reported that he had failed to elicit detectable amounts of antibody upon injection of cottontail cancer extracts into normal rabbits (11), and Dr. Peyton Rous and I got the same result in similar, unpublished experiments. Our further tests showed that antibody extravasates in great quantity into cottontail cancers (12). Whether this was the sole reason for failure of the immunization experiments cannot now be said, since the amount of antibody which had come out into the growths was not determined, though it was noted to be present in such quantity in some cases that the cancer extracts proved capable of neutralizing added virus. Certainly the latter persists in masked or altered form in the cancers deriving from the papillomas of domestic rabbits (3), and much evidence indicates that a variant of it is responsible for the cancers of cottontails (12).

A fact discerned in previous work (5) and strikingly illustrated in the experiments of the present paper deserves special emphasis here, namely, that extracts of domestic rabbit papillomas are in general much less antigenic than extracts of wild rabbit growths. For example, crude suspensions of the most potent domestic rabbit materials (Experiments 3 and 4) elicited scarcely as much antibody as did a Berkefeld filtrate of a cottontail growth diluted 1:2500 (Experiment 5). The finding fits in with the observation that the antibody titer attained in the blood of domestic rabbits carrying virus-induced papillomas is in general much lower than that in cottontails with growths of comparable size and duration (5). Does it provide a clue to the phenomenon of the "masking" of the virus in domestic rabbit papillomas? Can the comparatively slight antigenicity of suspensions of domestic rabbit papillomas mean that they contain a relatively small amount of virus,—an amount too small in most instances to be detected by the inoculation methods now in use? Or does the finding indicate that the virus is somehow altered, both antigenically and pathogenically, in domestic rabbits, a species to which it is foreign? As concerns the state of the "masked" virus in the papillomas of domestic rabbits, the fact may be mentioned that the antigenic principle present in them appears to elicit antibody of precisely the same sort as that called forth by the naturally occurring virus (9), though in much lower titer, as already mentioned. Furthermore, it would seem to be acted upon by the antibody in precisely the same way: the results of Experiments 3 and 4 make plain the fact that the antigenic principle in domestic rabbit papillomas is rendered non-antigenic by extravasated antibody precisely as is the virus. At first sight this finding would seem to conflict with two facts previously noted, namely, that extracts of domestic

rabbit papillomas fail as a rule to fix complement perceptibly upon admixture with the antiviral antibody, and that they fail also to absorb detectable amounts of the latter *in vitro* (9). But considerable quantities of the virus are required to consummate the *in vitro* serum reactions,—we have never observed complement fixation or antibody absorption with virus filtrates diluted more than 1:1280,—and the amount present in domestic rabbit papilloma extracts may be too small. All of the facts are compatible with the assumption that much less virus is present in the papillomas of domestic rabbits than in those of cottontails, its natural host.

## SUMMARY

A study has been made of the immunization procedure described by Shope, with particular reference to the detection of "masked" papilloma virus by means of it. Papilloma extracts were frequently encountered which, though non-pathogenic, elicited the specific antiviral antibody and induced resistance to the virus upon injection intraperitoneally into normal rabbits. The results of the immunization experiments were often complicated, however, by the effects of extravasated antibody, which had accumulated in various amounts in many of the papillomas and was consequently present in extracts of them together with "masked" virus. The extravasated antibody was often sufficient to render extracts of domestic rabbit papillomas non-antigenic; and sometimes, when present in excess, its passive transfer conferred resistance to reinfection with the virus. The conclusion seems warranted that only positive immunization findings can be interpreted with certainty. Negative results provide no decisive evidence as to whether "masked" virus is or is not present in the injected material, unless the amount of extravasated antibody also present is known. The findings may have a bearing on the negative outcome of immunization experiments with extracts of the cancers deriving from the natural papillomas of cottontails.

Crude suspensions of domestic rabbit papillomas, which contain little or no virus demonstrable by ordinary methods, are far less antigenic than extracts of the natural growths of wild rabbits, which contain virus in quantity. In explanation of the finding the possibility seems worthy of attention that domestic rabbit papillomas may contain much less virus than the growths of cotton tails, the natural hosts of the virus.

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