THE MASKING EFFECT OF EXTRAVASATED ANTIBODY ON THE RABBIT PAPILLOMA VIRUS (SHOPE)

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Most viruses can be recovered from the lesions they cause, but the one responsible for the cutaneous papillomatosis of rabbits (1) provides some noteworthy exceptions to the rule. This virus passes from infected to susceptible cottontail rabbits under natural conditions, and usually can be obtained readily from the growths it causes on their skin. But when it has been inoculated into an alien host, the domestic rabbit, the virus can be got back only occasionally from the growths induced by it, though these are in general remarkably vigorous; and sometimes the virus-induced papillomas of cottontails fail to yield it on extraction.[1]

Whether virus can be recovered from the papillomas or not, there is convincing evidence that it persists enduringly in them: an antibody specifically directed against the virus and capable of neutralizing it *in vitro* appears in the blood of rabbits carrying the papillomas and increases in titer as the growths enlarge (5); while furthermore extracts of papillomas that yield no infectious virus will nevertheless call forth specific antiviral antibody when injected intraperitoneally into normal rabbits (2). Granting its presence, why can the virus be extracted from some of the papillomas as not from others? Certain observations already recorded have suggested the possibility that the specific antiviral antibody, which appears in quantity in the blood of animals bearing the growth, may be responsible for the "masking" (3). This has proved to be the case in the papillomas

[1] Shope refers to the virus as "masked" in the papillomas from which it cannot be recovered (2), and we have also employed this term (3). But the "masking" of the papilloma virus is distinct in several respects from that of certain strains of the plant viruses commonly referred to as "masked" (4). These latter produce no perceptible lesions but are transmitted from one plant to another under natural conditions and can be readily extracted from infected plants; whereas the papilloma virus may give rise to vigorous and enduring lesions from which, when "masked," it either cannot be extracted at all in infectious form or at most in small amount.

of cottontail rabbits, as will now be made clear, but not in those of the domestic species.

Yield of Virus from Confluent and Discrete Papillomas of Cottontail Rabbits with Various Amounts of Antibody in Their Blood

Previous work had shown that the neutralizing antibody which circulates in the blood of rabbits carrying virus-induced papillomas has no influence upon their course (5),—presumably because the virus is constantly protected by its living cell-hosts from the action of the antibody. But dead cells do not protect viruses in this way (6); and hence it seemed possible that antibody, present in the blood vessels of the growths, or localized in the latter owing to seepage from ruptured or changed capillaries, might neutralize the virus when the cells were killed during extraction or preservation *in vitro*.

In experiments already reported the virus could not be recovered from the large, experimentally induced growths of a number of cottontail rabbits, some of which had high serum-antibody titers (3). To extend the observations cottontail rabbits were inoculated with a single virus material in such a way that small, discrete papillomas were produced in all, and large, confluent growths as well in some. At various times thereafter the amount of antibody in the serum of the animals was determined by means of the complement fixation test,[2] and extracts of their growths were tested for virus.

Twenty-four normal cottontails obtained from Kansas were used. All were carefully examined to make certain that none carried naturally occurring growths. The hair was clipped away from the sides of all, and from the abdomens as well of eight. One drop of a 5 per cent crude suspension of virus in Tyrode solution, made fresh that day by grinding together equal parts of the glycerolated experimental warts of four cottontails (W. R. 35, 36, 54, 56), was then tattooed uniformly into three spots about 2 mm. in diameter on each side of every rabbit, using an electric tattoo machine specially fitted with nine small sewing needles; and in eight of the animals about 0.3 cc. of the same inoculum was rubbed into an area roughly 6 by 8 cm. on the skin of the abdomen, immediately after light scarification with sandpaper. 10 days later, when the inoculated areas had healed but before any growths had appeared, the whole of each inoculated area was washed with soap and rinsed well under the tap, to wash away any

[2] Previous work has shown that the complement fixation test provides a ready way to determine the amount of antiviral antibody (3). In every comparative test,—more than two score have now been made,—the capacity of a given serum to neutralize the papilloma virus has paralleled its ability to fix complement under standard conditions, while furthermore the complement-fixing and virus-neutralizing capacities are absorbed together from immune sera when these are mixed with extracts containing the virus (7).

adherent virus. Between the 14th and 21st days growths appeared in all of the animals except four, which remained negative and were discarded. The tattoo papillomas appeared as fleshy "dew-drops" which rapidly enlarged into characteristic gray or creamy or particolored cones or cauliflowers up to 2 cm. across and 2½ cm. high (Figs. 1 and 2), with dry, keratinized peaks above and more or less fleshy bases, some with rounded, firm, fleshy "pearls" beneath as result of extension of the papilloma into the subcutaneous tissue. The large, scarified areas had contracted markedly during healing, but confluent, semiconfluent, and discrete rugosities soon appeared over most of the inoculated areas, and these grew rapidly into great horny masses of confluent papillomatosis, which were mostly gray but with some creamy "cell-families," and were fleshy at the base, with dry, keratinized peaks 1 to 2 cm. or more high (Figs. 1 and 4).

On the 27th day the outlines of the growths were traced, and serum was obtained from the eight rabbits which carried both discrete and broadcast growths. Then, using aseptic technique, about three-quarters of one of the representative tattoo papillomas of each of these rabbits was excised, as also a small wedge of the confluent or semiconfluent abdominal growth. The connective tissue was carefully trimmed away from the base of the specimens thus got and they were ground thoroughly in separate sterile mortars and suspended in Tyrode, 20 cc. for every gram of material.

The pathogenicity of the extracts was determined by rubbing portions of the whole suspensions into small scarified squares on the abdominal skin of susceptible rabbits, according to a checkerboard titration method used as routine in this laboratory (5). In the test, a fixed amount of each inoculum was rubbed into a shaved area about 3 cm. square on the abdominal skin of each of three normal, domestic test rabbits which had just been scarified with sandpaper. The areas were separated from one another by furry strips and due care was taken to avoid carrying the inoculum from one over into others. After drying the areas with a blast of warm, filtered air, each was covered with a square of gauze moored with adhesive and the whole was included in a many-tailed binder. In this way as many as 22 or 24 inocula could be tested on each animal. Although all domestic rabbits are susceptible to the virus, some are more so than others, owing probably to individual differences in the reactive capacity and thickness of their skins; and hence three animals (labeled for convenience a, b, c in the tables) were invariably used in every test; and the placing of each inoculum was systematically varied from animal to animal to minimize any differences due to situation.

The growths produced by the inocula were recorded from the 15th to the 30th days after inoculation, at intervals of 3 to 5 days, and again about the 35th and 42nd days, according to a standard scale: ++++ = confluent papillomatosis, +++ = semiconfluent papillomatosis, ++ = many discrete papillomas, + = a few discrete papillomas, ± = 3, 4, or 5 discrete papillomas, ± = one papilloma, 0 or − = negative. To conserve space in the charts, only the readings made on or about the 15th, 25th, and 42nd days are recorded. They typify the results.

The complement fixation test utilized to determine the antiviral antibody titer of the sera was applied as previously described (3). Antigen from a single source was used throughout the experiment, and two units of complement (titrated immediately beforehand), and 2 hours at room temperature for fixation. The antigen was freshly prepared for each test and consisted of a 1:20 saline extract of the glycerolated, highly infectious papillomas of a single wild rabbit (W.R. 56 N). (In the light of later experience it

would appear that the 1:20 extract was considerably in excess of the optimal dose of this antigen.) The readings were made after 30 minutes in the water bath at 37°C. and again after the tubes had stood overnight in the refrigerator. The latter findings were recorded in the charts, in terms of fixation, as follows: ++++ = complete fixation (no hemolysis), +++ = about 75 per cent fixation, ++ = about 50 per cent fixation, + = about 25 per cent fixation, ± = about 10 per cent fixation, − = no fixation (complete hemolysis).

The results of the first test (27th day) are shown in Chart 1. All eight rabbits had developed discrete tattoo papillomas on the sides, and while those on any one animal had about the same size there were great differences from host to host. All except one (W.R. 19), which was manifestly resistant, had developed good sized confluent abdominal growths. The serum of two of the rabbits (W.R. 21 and 19), with fairly large and vigorous growths and minute growths, respectively, failed to show antibody on test. The sera of the remaining six rabbits held various amounts of antibody, its titer having no obvious relation to the size of the growths. These findings are in line with observations, already mentioned, which show that the course of the growths is not influenced by circulating antibody.

The results of the tests for virus yield were striking. Tests were made with the materials from six animals. Invariably the extract of the small, discrete, tattoo papilloma contained more virus, as measured by the papillomatosis it produced in the test animals, than did that of the large, confluent abdominal growth of the same rabbit. And the difference was great in every instance but one, that of W.R. 21, an individual with not enough antibody in its blood to fix complement. The confluent abdominal growth of this animal yielded almost but not quite as much virus per unit of mass as did its discrete papillomas. The abdominal growths of all of the other rabbits,—those, that is to say, with appreciable amounts of antibody in their serum,—yielded little or no virus. Moreover, the amount of virus present in the extracts of the tattoo papillomas of the three animals with the highest antibody titers (W.R. 24, 18, 20) was somewhat less than in the extracts from the corresponding growths of the three individuals with lower antibody titers (W.R. 17, 22, 21).

In the first test, made on the 27th day after inoculation of the virus (Chart 1), the large, confluent, abdominal growths of five of the six rabbits tested yielded little or no virus; whereas the small, discrete papillomas produced on their sides by the same inoculum furnished it in greater or less abundance. The only rabbit with a confluent abdominal growth that provided much virus (W.R. 21) had no antibody in its serum detectable by means of the complement fixation test.

The findings support the supposition that antibody plays an important part in the masking of the papilloma virus in the growths caused by it in cottontail rabbits. And they do more. The fact that the confluent abdominal growths of five of the rabbits yielded little or no virus, while the discrete papillomas on the sides of the same animals provided it in quantity, indicates that the local conditions prevailing in confluent papillomatous

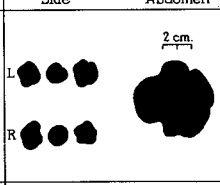
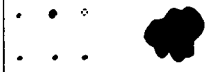
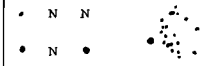
Rab. No.	Size of papillomas 27th day		Pathogenicity tests with extracts of the papillomas from		Complement fixation titer of serum				
	Side	Abdomen	Side	Abdomen	1:4	1:8	1:16	1:32	1:64
24			Test rabs / Day a, b, c — 15: − − − a, b, c — 25: + +± +±; − − − a, b, c — 42: +++ +± ++; − − −		++++	++++	±	−	−
17			++ +± +±; − − − +++± +++ +++±; + ± − +++± ++++ ++++; ++ ± ±		++++	++++	−	−	−
22			++± +++ +++±; − − − ++++ +++± ++++; ± − − ++++ ++++ ++++; ± − −		++++	++++	−	−	−
18			+ + +; − − − ++± +++± +++; ± − ± +++ +++ ++++; ± − ±		++++	++++	++++	++++±	−
21			+++ +++ +++; +± ++± ++ ++++ +++± ++++; ++ ++++ +++ ++++ ++++ ++++; +++ ++++ ++++		−	−	−	−	−
20			− − −; − − − +± + +±; − − − ++ + ++; − − −		++++	++++	++++	−	−
23			Not tested		++++	++++	−	−	−
19			Not tested		−	−	−	−	−

CHART 1. Yield of virus from the discrete and confluent growths of cottontail rabbits with various amounts of antibody in their blood. 27th day. L, R = left and right sides, respectively. N, N = negative. See text for further explanation.

masses also play a part in the phenomenon. Is their part of primary importance, or is it secondary to that of the antibody? The latter would appear to be the case, for in the one animal with no detectable circulating antibody (W.R. 21) the extract of the confluent abdominal growth contained practically as much virus as that of its small, discrete tattoo growths.

In evaluating the results it is well to recall that the virus-induced papillomas may flourish for many months and often as long as the host lives. The growths had been tested very early, on the 27th day after the inoculation causing them. They had been present only 8 to 17 days and the antibody titers of the various sera had not gone as high as previous experience had shown could be expected at a later time, while furthermore the vascular disturbances in the growths might not have reached their maximum. To learn more about the phenomenon of masking—especially about the relative rôles of antibody and local conditions—the rabbits were kept and other tests made at later periods.

A second test was made on the 69th day, with the omission of three animals whose growths had retrogressed. The dimensions of the papillomas were again traced, and portions excised and extracted as before. Blocks of all the specimens thus procured were taken in Zenker's solution for histological study, and the remaining tissue was extracted in the usual way and tested for pathogenicity in three new test rabbits. The antibody titer of the sera was also determined by means of the complement fixation test. Chart 2 deals with the animals bearing confluent abdominal growths as well as discrete papillomas on their sides (animals previously tested on the 27th day), while Chart 3 shows the results in the eleven rabbits bearing tattoo growths only, and not previously tested.

Chart 2, like Chart 1 of the previous test, shows that extracts of the confluent abdominal growths contained much less virus than did the extracts of discrete growths from the same animals. W.R. 21, which manifested no antibody in the preceding test, now had a moderate amount. The extract of its abdominal growth yielded a good deal of virus, though less than the portion of the growth excised on the 27th day; and the extracts of the abdominal growths of two other animals with low antibody titers (W.R. 17 and 22) also contained virus. The extracts of both the discrete and confluent growths of the two rabbits with highest antibody titers (W.R. 20 and 23) contained little virus.

One of the three titration rabbits employed in the test of materials from animals bearing only discrete tattoo papillomas died of diarrhea shortly after inoculation and hence the results in but two individuals are listed (Chart 3). Virus was present in the extracts of the growths of every animal except one, W.R. 15, a rabbit with high serum antibody titer and growths of considerable vigor as indicated by the "pearls" present beneath them. It is noteworthy that virus was recovered from the retrogressing growths of W.R. 12, and that this animal had no demonstrable antibody in its blood. The fact also deserves mention that the three animals with growths yielding little or no virus (W.R. 8, 15, 16) had considerable amounts of antibody in their blood; but it must also be pointed out that the growths of several other animals with high antibody titers (W.R. 1, 11, 14) yielded a good deal of virus.

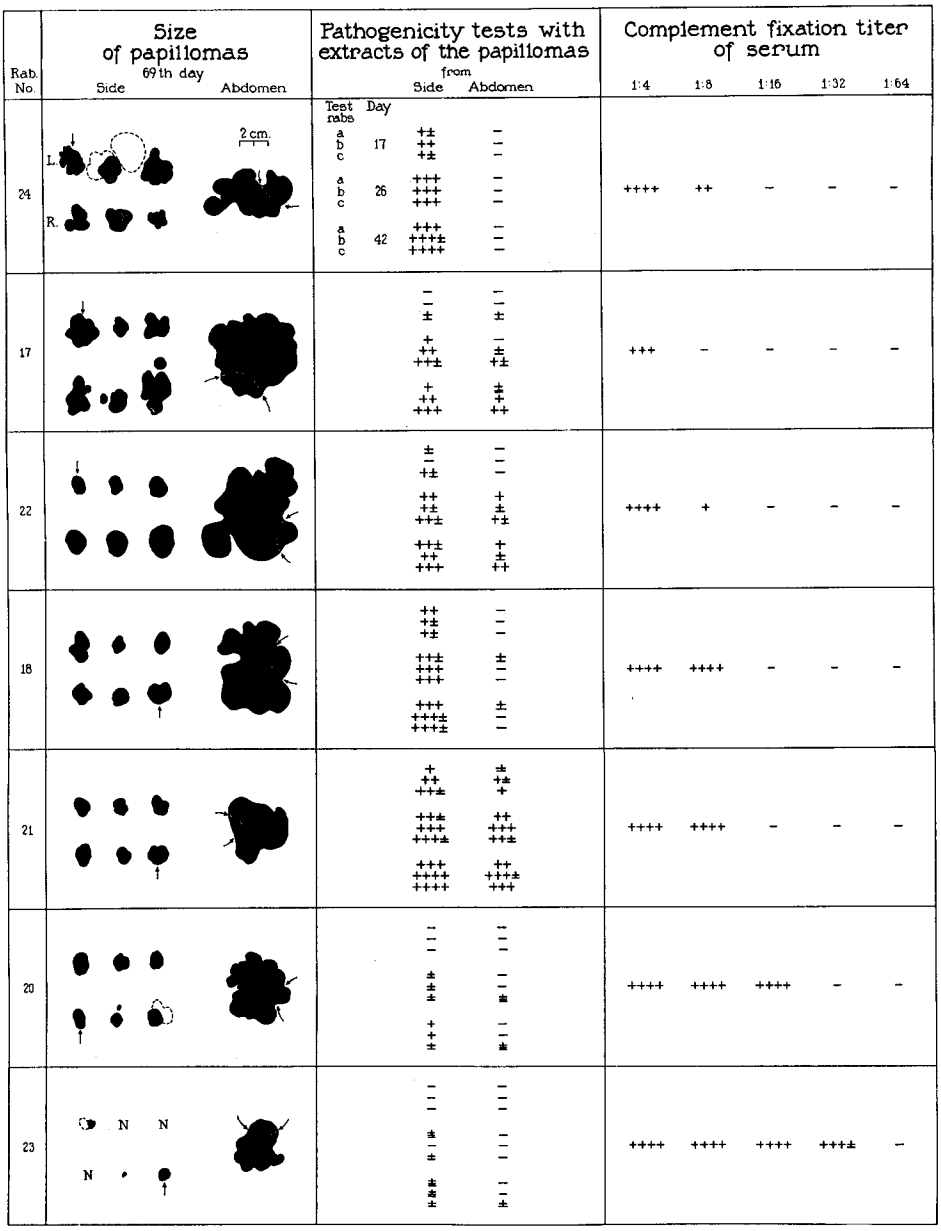


CHART 2. Tests made on the 69th day with materials from the animals of Chart 1. The arrows indicate the discrete papillomas and the portions of the abdominal growths that were excised for test. Broken circles show the outlines of subepidermal "pearls," which are often seen beneath growths of exceptional vigor in cottontails.

Rab No.	Size of papillomas		Pathogenicity tests with extracts of the papillomas			Complement fixation titer of serum				
	27th day	69th day	Test rabs	Day		1:4	1:8	1:16	1:32	1:64
15	2 cm. L R		a b a b a b	18 27 43 	− − − − − −	++++	++++	+++±	−	−
8					− − ± + + +	++++	++++	±	−	−
16					+ − + ± +± ±	++++	+++±	−	−	−
4					± − ++ + ++± +	++++	+++	−	−	−
1					+ − +++ ++ ++++ ++	++++	++++	+++	−	−
9		N			+ ± ++ + +++ ++	++++	++++	±	−	−
14					++ − +++ ++± +++± +++	++++	+++	±	−	−
6					+ + +++ ++± +++± +++	−	−	−	−	−
5					++± + +++± ++± ++++ +++	++++	+++	−	−	−
11					++± − +++± ++ ++++ ++	++++	++++	++++	±	−
12		N N N N			+ − ++ ± ++± ±	−	−	−	−	−
7		N N N N • •			Not tested	−	−	−	−	−

CHART 3. Tests made on the 69th day with materials from the cottontails bearing only discrete papillomas on their sides.

Final tests were made on the 132nd day. Five of the rabbits had died of intercurrent causes or of sepsis associated with the massive abdominal growths, several of which had become macerated and foul. The remaining animals were bled for serum, their growths traced, and portions excised as previously. Tests were made precisely as before. Charts 4 and 5 show the results.

Only four of the rabbits with both discrete and confluent growths had survived (Chart 4). In three the antibody titer proved to be much higher than on the 27th day. The difference was especially noteworthy in the case of W.R. 21, which had shown no

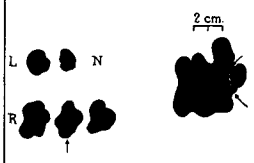
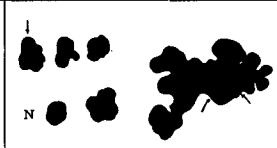
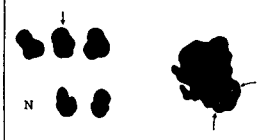
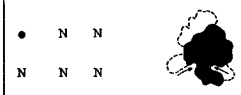
Rab. No.	Size of papillomas 132nd day: Side / Abdomen	Pathogenicity tests with extracts of the papillomas from: Test rabs / Day / Side / Abdomen	Complement fixation titer of serum: 1:4	1:8	1:16	1:32	1:64
22		a, b, c — 16: ±, +, ±; 25: ++, ++, ++; 42: +++±, ++, +++± / ±, −, ±, +++±, ±, +±, +++±, ±, ++	++++	++++	++	−	−
18		−, −, −, +, ±, ±, ++±, ±, + / −, −, −, −, ±, ±, ±, −, ±	++++	++++	+++±	+	−
21		−, −, ±, ±, +, +±, ±, +, ++ / −, −, −, −, −, ±, −, −, ±	++++	++++	++++	+++	−
23		Not tested / ++, ±, +++±, +++, +++, +++, +++±, +++, ++++	++++	++++	++++	+++	−

CHART 4. Tests made on the 132nd day with materials from the surviving cottontails of Charts 1 and 2.

antibody then and only a moderate amount on the 69th day. The abdominal growth of this animal now yielded almost none of the virus instead of a good deal, as previously, while the discrete papillomas yielded very much less than before. The growths of W.R. 22 yielded about as much virus as previously, though the antibody titer of the sera had risen slightly. The confluent growth of W.R. 18, an animal which had had much antibody on both occasions and had yielded little virus, yielded no more now, and the large tattoo papilloma on its side furnished far less than before.

The findings in the case of W.R. 23 deserve special note. The serum-antibody titer of this animal was quite high, as in the preceding test; yet its abdominal growth now

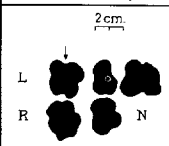
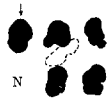
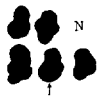
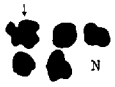
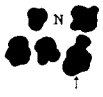
Rab. No.	Size of papillomas 132nd day	Pathogenicity tests with extracts of the papillomas	Complement fixation titer of serum 1:4	1:8	1:16	1:32	1:64
8		Test rabs: a b c — Day 16: − − −; a b c — Day 25: ± ± ±; a b c — Day 42: ± − ±	++++	++++	+++±	−	−
16		− − − ± − ±	++++	++++	++++	++++	++++
4		− − − − − − − ±	++++	++++	++++	++++	+++±
1		− − − − − ±	++++	++++	++++	+++±	−
9		++± + +++ +++ ++± +++ ++++ +++ ++++	++++	++++	+++±	−	−
14		− + +++ ± ++± +++± ± +++± ++++	++++	++++	++++	+++±	−
6		++ − ± +++ ++ ++ ++++ +± +++	+++	−	−	−	−
5		± − ± ++ ++ ++ ++ + ++±	++++	++++	++++	+	−
11		− ± ++ + ++ +++ ± +++ +++±	++++	++++	++++	++++	++

CHART 5. Tests made on the 132nd day with materials from the surviving cottontails of Chart 3.

yielded a good deal of the virus, as had not been the case on the 69th day (Chart 2). Between the 69th and 132nd days the local conditions about the growth had changed greatly, owing to the appearance beneath it of rounded, fleshy, subepidermal "pearls," which had appeared after the 69th day as small, rounded protrusions beneath the growth and had enlarged steadily, some coalescing, and finally raised its base high above the level of the skin with result that dry keratinization took place instead of maceration.[3] It is possible that the alterations in local conditions consequent upon the presence of the pearls may have been responsible for the "unmasking" of the virus in the confluent surface growth of this animal.

Chart 5 shows the results after 132 days in the nine animals that had only discrete, tattoo papillomas. In most of these the antibody titer had risen high as compared with previous findings (Table III); and three animals (W.R. 1, 16, and 4) with greatly increased amounts of antibody in their blood had papillomas that yielded very little virus, —much less than on the 69th day,—though their growths had enlarged steadily from the beginning, in some cases with noteworthy vigor. The growths of W.R. 11 and 14 yielded a good deal of virus in spite of the fact that they were nourished by blood containing much antibody and that of W.R. 9 yielded more virus than previously, though the antibody titer had risen.

In sum, the tests made on the 69th and 132nd days (Charts 2, 3, 4, and 5) confirm and extend that of the 27th day (Chart 1). Little or no virus could be recovered as a rule from the large confluent growths when the rabbits carrying these had had much antibody in their blood for a long time; and frequently the virus was masked in the discrete growths of cottontails with high serum antibody titers. Several instances were encountered, however, in which virus was got in quantity from the discrete tattoo papillomas of cottontails with high serum antibody titers, and a good deal of virus was obtained on one occasion from a confluent growth which had long been nourished by blood containing much antibody. The implications of the findings will be considered in detail further on.

The sequence of events in one animal (W.R. 21) proved especially enlightening. The rabbit had not developed any noteworthy amount of antibody by the 27th day (Chart 1), and its confluent abdominal growth then yielded much virus, as did also the discrete growths removed from its side. On the 69th day there was a moderate amount of antibody in the blood and the abdominal growth yielded much less virus (Chart 2) than previously, while the papilloma from the side provided slightly less. After the 69th day the titer of antibody in the blood rose high, and on the 132nd day the abdominal mass, which had steadily enlarged, contained almost no

[3] Subepidermal growths of this sort have already been described (8). We have encountered many of them beneath the vigorous papillomas of cottontails. Virus is often recoverable from them and the amount may be as large as from the surface growths.

demonstrable virus, while only a little was present in the extract of one of the tattoo growths on its side. The masking of the virus in the growths of this animal manifestly occurred as its titer of circulating antibody increased. In explanation of the findings it might be assumed that an attenuation of the virus took place in the growths of long standing, but much experience indicates that this does not occur in any perceptible measure. The virus has often been recovered in great abundance from

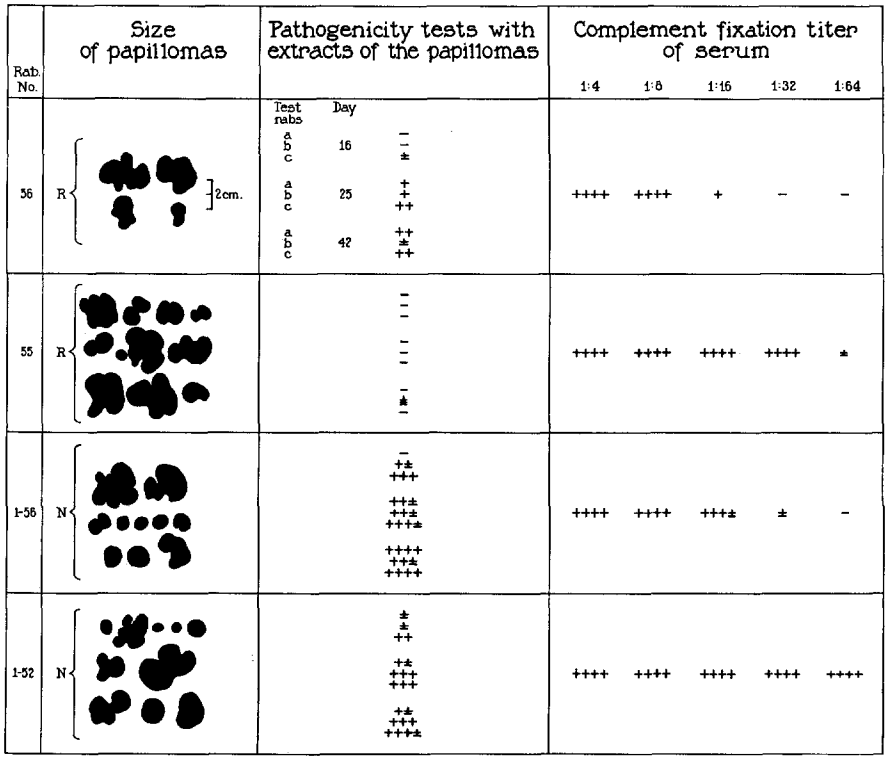


CHART 6. Tests with the growth and sera of cottontails with recurrent and naturally occurring papillomas. See text for further explanation.

the growths of cottontails that had endured for many months, and indeed the papillomas of long duration in some animals of this experiment yielded much of it (W.R. 22, 23, 9, Charts 4 and 5).

Local Conditions Influencing the Masking

In the tests just reported the virus was masked as a rule in the large confluent growths of cottontails when these had noteworthy amounts of

antibody in their blood, though not when this was lacking; whereas often it could be extracted in considerable amounts from the small discrete growths of the same rabbits, as also from growths of this sort from other animals with high antibody titers. The findings point to antibody as responsible for the masking when this occurs, but they indicate also that local conditions must be largely responsible for its effectiveness. What is the nature of these conditions? Certain differences in the characters of growths of the two sorts provide an answer to the question.

The discrete growths resulting from tattoo inoculation of the virus into the skin of cottontail rabbits begin as small, fleshy, dome-shaped "dew-drops," or minute, fimbriated "seed warts," which rapidly enlarge into growths of two general kinds, these being precisely like the ones that occur naturally. The *onion-like cones* (Figs. 2 and 3), one of the forms assumed secondarily, have bases that are slightly constricted, and bulging, fleshy sides with dry, horny tops. They tend to remain discrete, intact, and orderly in their proliferation for long periods; and invariably, in our experience, they have provided much virus upon extraction. The *cauliflower-like, discrete papillomas* (Fig. 1), on the other hand, are frequently cleft to their bases by fissures, which become moist as the growths enlarge and their parts rub against one another. Discrete growths of this sort usually yield the virus, but rather less of it in general than the onion-like cones. The local conditions are very different in the *confluent growths* produced by broadcast inoculation of the virus into scarified skin (Figs. 1, 4, 5). These great papillomatous masses are invariably cleft, and usually they become folded and wrinkled as well when the animals move and crouch, with result in exudation into the many fissures, often followed by widespread maceration (Fig. 5). Confluent growths of this sort yield little or no virus, as a rule, if antibody is present in the blood coursing through them, as the charts of the preceding experiment make clear.

Histological study was made of cross sections of the papillomas taken on the 69th day, and the results of this study provide direct evidence of the presence of extravasated blood in the growths. The living portions of all of the growths looked much alike under the microscope, the cells of the papillomas from which the virus was not recoverable showing no noteworthy differences in number, arrangement, supporting tissue relations, or staining reactions from those of growths that yielded much virus. The keratinized parts of the confluent growths, however, were much more compact and disordered, as a rule, than were the corresponding portions of the discrete papillomas, and they usually stained a deeper pink with eosin. Areas of extravasated blood and of inflammatory exudate were regularly present in them, and often these were numerous and extensive, making up a considerable proportion of the mass (Fig. 6). Such extravasations and exudations were also found in some of the discrete growths, but were much less prevalent and extensive in those than in the confluent ones. A direct comparison of the discrete and confluent growths of the same animals showed that the former contained much less extravasated blood and inflammatory exudate than the latter. The extravasated blood and inflammatory exudate were not sufficient of themselves to bring about the masking, however, for even when these were conspicuous, as was the case in the growths of W.R. 6 and 17 (Charts 3 and 2), the virus was recoverable from the growths if these had been nourished by blood containing little or no antibody.

It seems probable, from what has already been said and from the description just given, that masking of the virus is made possible in the growths of cottontail rabbits by certain local conditions (crowding, folding and wrinkling, inflammation) which favor the escape of antibody from the blood vessels and its accumulation in the growths,—conditions prevailing more or less regularly in the large confluent papillomatous expanses produced by sowing the virus broadcast upon scarified skin, but present only occasionally in the discrete papillomas that occur naturally or as result of tattoo inoculation. Seepage of serum was especially favored in the confluent growths of our experiment, for these were not only large and fissured, but were situated on the abdomens of the cottontails and hence dependent as well. This, while undoubtedly favoring the masking, was not essential to it, however, for masking occurred in several of the large, discrete, fissured cauliflowers on the sides of cottontails with high antibody titers (Chart 5).

The Yield of Virus from Naturally Occurring Papillomas

The virus has never been masked, in our experience, in the benign papillomas that occur under natural conditions. Characteristic growths of this sort from a total of 27 cottontails trapped in Kansas and Texas have been tested in this laboratory during the past several years: invariably these have yielded virus and almost always much of it. Do the local conditions within the natural growths prevent the masking, or do animals bearing growths of this sort fail to develop antibody in high titer? Observations recently made go some distance toward answering this question.

Two cottontails (W.R. 56 and 55), each carrying a number of characteristic, discrete, naturally occurring papillomas, had been received some time previously from Kansas. The growths of both had been "plucked" soon after the animals reached the laboratory,—that is to say, each had been gently pulled off in such a way that a thin, irregular layer of its base remained *in situ*, and from this it rapidly grew again, forming a papilloma like the one previously pulled away, though now more apt to be cleft and cauliflower-like than intact and onion-like. The plucked growths of each animal, which had been cut up and put into separate bottles of 50 per cent glycerol-Locke solution, yielded much virus in standard extracts tested on a number of occasions, those of W.R. 56 regularly providing more than the growths of W.R. 55. 3 months after the growths had first been plucked, when all had long since grown up again to near their original size, all were plucked a second time and cut up separately into glycerol-Locke as before. Again the growths of both animals yielded virus, as manifested upon inoculation into test animals in the routine way, and those of W.R. 56 more than the ones of W.R. 55; but there was a considerable decrease in the yield of virus from the recurrent growths of both animals as compared with those originally plucked.

A third test was made of the recurrent growths plucked after a further interval of 3½ months. By this time two other cottontails with naturally occurring growths (W.R.

1-52 and 1-56) had become available for test. These had recently been sent to us from the same locality in Kansas. Their growths were characteristic: all were discrete, gray, onion-like cones with fleshy bases and dry tops (Fig. 3), some 2 cm. or more across; and, like the generality of natural growths, they were scattered over the bodies of the animals,—some about the pudenda, others on the chest, face, haunches, and elsewhere.

A specimen of serum was procured from each of the four rabbits and the sizes of the original or recurrent growths were traced. Pathogenicity tests were made with extracts of representative samples of the growths, and the amount of antibody in the various sera was determined by complement fixation. Chart 6 shows the results. The recurrent growths of W.R. 56, which had only a moderate amount of antibody in its blood, yielded a moderate amount of the virus; while those of W.R. 55, much more numerous and larger, yielded practically none, the amount of antibody in the serum of this animal being great. The natural growths of W.R. 1-56 and 1-52 yielded much virus, in spite of the fact that the former had a good deal of antibody in its blood, and the latter very much. (In a subsequent test, the complement fixation titer of the serum of W.R. 1-52 was found to be 1:512 in mixture with an optimal dose of antigen; it is by far the most potent antiserum we have come upon.)

The serum antibody titer of cottontails with naturally occurring growths may be high, as the observations just cited prove (Chart 6; see also Table II). But even so, these growths, which are almost always discrete, and usually intact and onion-like (Fig. 3), may yield much virus. These findings, along with those previously cited, make clear the fact that the antibody present in the blood vessels of papillomas is usually insufficient to mask the virus to any very noteworthy extent, this taking place only after antibody has seeped into the growths and perhaps accumulated there. And they make it seem probable that little or no extravasation occurs as a rule in the naturally occurring papillomas, which, like the tattoo growths of the present work, are almost always the result of infection of small, discrete spots by the virus, and which, though often coalescing as they enlarge, usually remain intact and onion-like and without signs of extensive vascular disturbances. The virus may be masked, however, in natural growths that recur after almost complete removal, as the instances of W.R.'s 55 and 56 (Chart 6) make plain. Other instances have been observed more recently in which recurrent growths provided much less virus than had the original tissue. The alteration in local conditions consequent on the plucking away of the growths with later recurrence manifestly made the masking possible.

Relation of Antibody to Recovery of Virus from the Papillomas of Domestic Rabbits

The findings thus far indicate that extravasated antibody is responsible for the masking of the virus when this occurs in the papillomas of cotton-

tails. As is well known, the virus is nearly always masked, and usually completely so, in the growths produced with it in domestic rabbits. Does localized antibody account for the phenomenon in this species also? To learn about this, the actively growing papillomas of a number of domestic rabbits were procured and tested for virus, the amount of antibody in the sera of the same rabbits being determined as well for comparison.

Test rabbits of various titrations, carrying growths produced by a number of different "strains" of the virus, were used as the source for the materials. They were hybrid, gray-brown (agouti) rabbits, which bore characteristic small papillomas at a number of

TABLE I

Yield of Virus from the Papillomas of Domestic Rabbits

Rabbit No.	Time since inoculation with virus	Character of the growths	Total mass of papillomas	Pathogenicity tests with extracts of the growths								
				17 days			27 days			43 days		
				a	b	c	a	b	c	a	b	c
	days		*gm.*									
4-56	28	Discrete, semiconfluent and confluent	36.5	0	0	0	0	±	0	0	±	0
4-57	"	" "	31.2	0	0	0	0	0	0	0	0	0
4-58	"	" "	28.0	0	0	0	0	0	0	0	0	0
2-16	40	One confluent growth		0	0	0	0	±	0	0	±	0
2-17	"	" " "		0	0	0	0	0	0	0	0	0
2-08	42	Semiconfluent	18.5	0	0	0	0	0	0	0	0	0
2-10	"	"	7.0	0	0	0	0	0	0	0	0	0
2-11	"	Discrete and semiconfluent	3.7	0	0	0	0	0	0	0	0	0
4-22	46	Confluent and semiconfluent	39.7	0	0	0	0	0	0	0	0	0
4-23	"	Semiconfluent and discrete	25.2	0	0	0	0	0	0	0	0	0
4-24	"	Confluent and semiconfluent	47.0	0	0	0	±	±	0	±	±	0

a, b, c = test rabbits.

situations, some discrete, others confluent or semiconfluent, all of short duration and vigorously enlarging. (For a photograph of characteristic growths in a titration animal, see Fig. 1 of reference 5.) The rabbits were bled 20 cc. from the heart and the sera stored, along with the growths, which were procured at the same time with aseptic technique and put into individual bottles of 50 per cent glycerol-Locke in the refrigerator. The tests were made after the materials from eleven gray-brown rabbits had accumulated. Some had been stored for periods up to 5 months in the refrigerator at about 4°C.,—treatment that has no harmful effect on the papilloma virus or its antibody, as much experience has shown. The glycerolated materials were washed briefly in Tyrode, then ground in mortars and suspended 1:20 in Tyrode, each being handled individually with a separate set of sterile instruments and glassware. Samples of the crude suspensions were then tested for pathogenicity according to the standard method

(5). The results of the tests are shown in Table I. The growths of only three of the animals (D.R. 2-16, 4-24, 4-56) yielded virus, and this in minimal amount.

Both complement fixation and neutralization tests were done to compare directly the amount of antibody in the sera of the domestic rabbits with papillomas yielding little or no virus and that in the sera of cottontails with growths providing much of it. Included in the complement fixation test (Table II) were sera from five of the cotton-

TABLE II

Comparative Amounts of Antibody in the Sera of Domestic Rabbits with Papillomas Yielding Little or No Virus and Cottontails with Growths Providing Much of It

Source of serum	Rabbit No.	Serum antibody titer as determined by complement fixation tests					
		Dilution of serum					
		1:2	1:4	1:8	1:16	1:32	1:64
Domestic rabbits with papillomas yielding little or no virus	4-56	+++	+	0	0		
	4-57	+++±	+++	0	0		
	4-58	+++	+++±	++++	+++		
	2-16	++	±	0	0		
	2-17	0	0	0	0		
	2-08	0	0	0	0		
	2-10	0	0	0	0		
	2-11	0	0	0	0		
	4-22	++++	+++±	±	0		
	4-23	+++±	++++	+++±	0		
	4-24	0	0	0	0		
Cottontail rabbits with experimental papillomas yielding much virus	1	+++±	++++	++++	++++	++++	++±
	2	++++	++++	++++	++++	++++	+++
	11	++++	++++	++++	++++	++++	++++
	18	++++	++++	++++	++++	+++±	±
	21	++++	++++	++++	++++	++++	++
Cottontail rabbits with natural papillomas yielding much virus	1-28	++++	++++	++++	++++	+++±	0
	1-29	+++	+++±	++++	+++±	±	0
	1-30	+++	+++±	++++	+++±	±	0

2 units of complement in all tubes.
Antigen, W.R. 1-56 N, 1:20 Berkefeld V filtrate.

tails of Charts 4 and 5, all of which had been stored longer than the domestic rabbit sera, and from three recently acquired cottontails with natural growths that were known from repeated tests to provide much virus. Two units of complement were used, and an optimal dose of antigen,—a 1:120 Berkefeld V filtrate of the highly infectious growths of W.R. 1-56 N.

Tables II and III show the results of the tests with the sera. The serum of every one of the cottontails contained more complement-fixing antibody than did that of any

of the domestic rabbits. Indeed, five of the domestic rabbit sera failed to fix complement at all, and the specimens of three others did so in very low titer. Only four of the domestic rabbits (D.R. 4-22, 4-23, 4-57, and 4-58) had sera that fixed complement to any considerable degree and it is noteworthy that these sera were the only ones that manifested any pronounced virus-neutralizing capacity (Table III) when tested according to the routine method (5). The findings were not unexpected, for it was already known from previous work that domestic rabbits carrying virus-induced papillomas generally have little of the antiviral antibody in their blood in comparison with the amount of it usually present in the sera of cottontails with comparable growths (3).

TABLE III

Neutralization Tests with the Sera of Domestic Rabbits with Papillomas Yielding Little or No Virus

Source of serum Rabbit No.	Growths resulting from mixtures of the whole sera in equal parts with 5 per cent virus filtrate W.R. 56								
	17 days			27 days			43 days		
	a	b	c	a	b	c	a	b	c
4-56	++	−	−	++±	++	++	+++	++	++
4-57	−	+	+	++	+++	+++±	++	+++	++++
4-58	−	−	+	±	±	+±	±	±	+±
2-16	++	±	+	+++	++	++	+++	++	+++
2-17	+++	+	++	++++	++	+++±	++++	+++	++++
2-08	+++	±	+++	++++	+±	++++	++++	++	++++
2-10	++++	++	+++±	++++	+++±	++++	++++	++++	+++
2-11	++	−	+++	+++	++	++++	+++±	+++	++++
4-22	−	−	±	±	±	±	±	±	±
4-23	−	−	±	±	±	±	±	±	±
4-24	++±	−	+	++++	++	+++	++++	+++	++++
Tyrode control..	+++±	++	+++±	++++	+++±	++++	++++	++++	++++

a, b, c = test rabbits.

In these tests (Table I), no virus was obtained from the growths of eight of the eleven domestic rabbits employed, and very little of it from the papillomas of the three others,—this in spite of the fact that the growths of all were of brief duration, vigorous, and rapidly enlarging. The finding is representative: growths of many domestic rabbits obtained at various periods after inoculation with the virus have been tested in this laboratory during the past several years; most of them failed to yield the virus when tested under favorable conditions and the remainder provided only small amounts of it.

The results of the serum tests assume large significance in the light of the facts just mentioned. Most of the sera of the eleven domestic rabbits,

which carried growths that yielded little or no virus, had little or no virus-neutralizing capacity (Table III); and none had as much antibody as any one of the sera obtained from eight cottontails with growths that yielded much of the virus (Table II). As bearing on the significance of the findings in relation to the phenomenon of masking, the fact deserves reiteration at this point that cottontails carrying the virus-induced growths have much more antibody in their blood, as a rule, than domestic rabbits with comparable papillomas (3). When considered altogether, the facts at hand make it appear improbable that antibody is primarily responsible for the masking of the virus in the growths produced with it in domestic rabbits.

DISCUSSION

The benign papillomas that occur under natural conditions in the skin of cottontail rabbits have, in our experience, regularly yielded considerable amounts of the virus responsible for them, and so too have many of the growths produced experimentally with the virus in this species. It was noted incidentally, however, in experiments previously reported (3), that the virus could not be recovered from the large, confluent, experimentally induced growths in cottontails that had much antiviral antibody in their blood, and hence the present study was undertaken of the relation of the specific antiviral antibody to the masking of the papilloma virus. The results of this study provide evidence that extravasated antibody is responsible for the "masking" of the virus when this occurs in the papillomas of cottontail rabbits.

Does the extravasated antibody act *in vivo* to mask the virus, or does it merely ooze into the growths during life and perhaps become concentrated in them when drying takes place, exerting its "masking" effect later on when the growths are extracted or preserved *in vitro*? Several facts can be cited that bear upon the question. Firstly, the virus continues to exert its proliferative action on the cells irrespective of the amount of antibody present in the blood vessels of the growths or localized in the latter, as previous experience (5) and the charts of the present paper make clear. It scarcely seems probable that antibody could "mask" the virus *in vivo* without interfering with the proliferation of the virus-infected cells. Secondly, masking may be a transitory phenomenon, as the instance of W.R. 23 showed (Charts 2 and 4). In accounting for this exceptional instance, in which the virus was recovered from the confluent growth on the 132nd day but not on the 69th, it is difficult to imagine that antibody could mask the virus *in vivo* at one time yet not do so at another later on, while it may be readily supposed that less extravasation would take place

into a surface growth after great subepidermal "pearls" had pushed it far out from the abdomen, as happened in this case. And thirdly, viruses are known to be protected from the action of antibody when associated with living cells (6). In this connection the fact deserves mention that the cells of the virus-induced papillomas regularly differentiate and die while the growths are enlarging, and it is quite possible that extravasated antibody may act immediately upon the virus associated with dead cells or liberated from them. When all of the facts are considered together, they are most easily explained on the assumption that antibody merely escapes into the growths during life, acting then perhaps upon the virus associated with or liberated from the differentiated cells but failing to neutralize that associated with the living papilloma cells until these are killed during extraction or preservation *in vitro*.

Does antibody mask the virus in the papillomas of domestic rabbits, as in those of cottontails? Some of the findings of the present study bear upon the point, and they also bring out certain striking differences in the phenomena of "masking" in the two species. The papillomas of cottontails, as has been brought out, yield much virus except in those instances in which antibody localizes in the growths and masks it. But the virus is regularly masked in the papillomas induced therewith in domestic rabbits and usually completely so.[4] At best these growths yield only small amounts of virus, and usually none at all, though tested before any considerable amounts of antibody have appeared in the blood of the animals bearing them (compare Tables I, II, III). Moreover, domestic rabbits carrying virus-induced papillomas that yield no virus generally have much less antibody in their blood than cottontails with growths that provide it in large amounts (Table II). Whether the local conditions within the growths of the two species have anything to do with the differences in yield of virus has not been determined; nor have tests been made to find whether the antibody molecule of domestic rabbits is smaller than that of the wild species and hence capable of seeping more readily into the growths. But considered together, the findings make it appear likely that some factor other than antibody is fundamentally responsible for the masking of the virus in the papillomas of domestic rabbits. When infectious virus happens

[4] Shope has succeeded in propagating several strains of the virus serially in domestic rabbits. But the virus is largely masked even in the growths of domestic rabbits produced with these adapted strains: it is recoverable from them only irregularly and in comparatively small amounts, and it gives rise to growths only after an incubation period much longer than that with virus obtained from the naturally occurring growths of cottontails (9).

to be present in them, however, it seems probable that extravasated antibody may exert its effects, precisely as in the papillomas of cottontails.

SUMMARY

The foregoing experiments have shown that the causative virus is usually "masked" in the large, disorderly, fissured and inflamed papillomas of cottontails when antiviral antibody is present in quantity in their blood, though virus can be recovered as a rule from the smaller, discrete, well ordered papillomas of these rabbits, almost irrespective of the amount of antibody in the blood of the individuals bearing them. Other findings are described which indicate that the masking of the virus in the large fissured growths is due to serum antibody present in them as result of exudation or hemorrhage, which neutralizes the virus when the growths are extracted or preserved *in vitro*. The local conditions that favor extravasation of serum (and the accumulation of antibody) prevail as a rule in the large, confluent growths arising after virus has been sown broadcast on scarified skin, but to lesser extent or not at all in the discrete papillomas that occur naturally or as result of tattoo inoculation.

The state of affairs is notably different in the papillomas of domestic rabbits. The virus is regularly masked in these, and usually masked completely, even when there is little antibody in the blood and the local conditions do not favor its extravasation into the growths. The findings indicate that something other than antibody is primarily responsible for the masking in this species.

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EXPLANATION OF PLATES

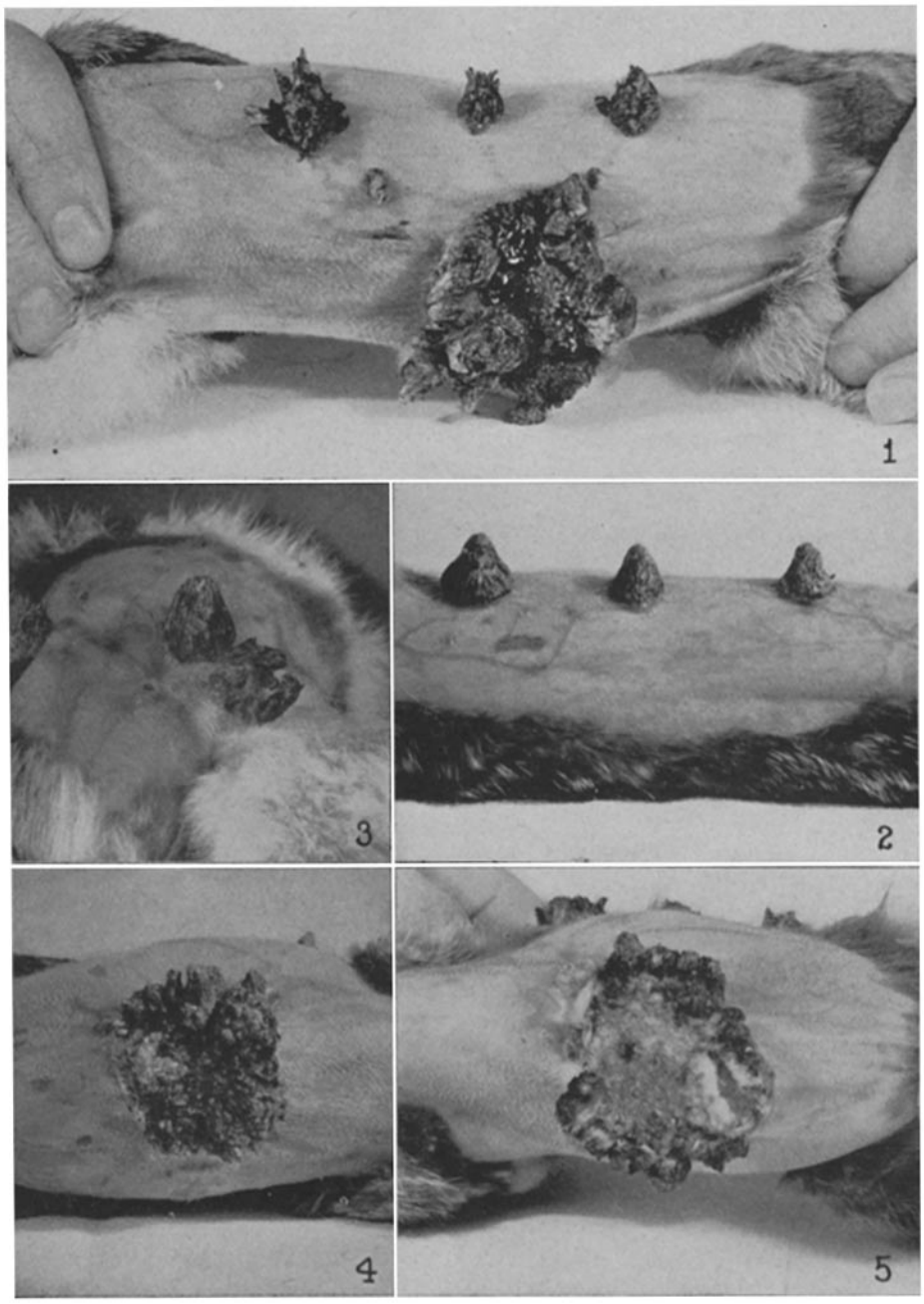
PLATE 46

FIG. 1. A characteristic fissured, confluent growth on the abdomen of a cottontail (W.R. 18) and discrete, cauliflower-like tattoo papillomas on its side. 108 days after virus inoculation. The discrete growths yielded much virus, the abdominal growth almost none (Charts 1, 2, and 4). × ½.

FIG. 2. Discrete, onion-like, tattoo papillomas on the side of W.R. 22. 61 days after virus inoculation. They provided much virus on extraction (Charts 1 and 2). × ½.

FIG. 3. To show the intact, discrete character of naturally occurring papillomas. The two pictured here were situated in the groin of W.R. 1-52. One is a plump gray "onion" intact and fleshy almost to its top, the other a cluster of smaller "onions" and dry slender cones. The growths yielded much virus though nourished by blood with very great antiviral potency (Chart 6). × ½.

FIGS. 4 and 5. To illustrate the gross changes that accompany extravasation of serum into a confluent growth. On the 61st day the abdominal growth of W.R. 20 was still a dry-topped, confluent, papillomatous mass, though cleft by many fissures already moist at their bases (Fig. 4). By the 108th day, however, much of the keratinized material had been rubbed away from its surface owing to the continued wrinkling and folding, and its fleshy base now lay bare and macerated, with purulence in one of its large clefts (Fig. 5). × ½.



Photographed by Joseph B. Haulenbeek

(Kidd: Masking effect of antibody on rabbit papilloma virus)

PLATE 47

FIG. 6. To illustrate the extensive areas of inflammatory exudate and extravasated blood regularly present in the disordered keratinized portions of large confluent growths. W.R. 18. 69th day. B = blood, I = inflammatory exudate, B,S = extravasated blood and plasma.

Photographed by Joseph B. Haulenbeek

(Kidd: Masking effect of antibody on rabbit papilloma virus)