IDENTITY OF "INHIBITOR" AND ANTIBODY IN EXTRACTS OF VIRUS-INDUCED RABBIT PAPILLOMAS

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Extracts of the growths experimentally induced in domestic rabbits with the papilloma virus often contain something that neutralizes or "inhibits" the virus when mixed with it *in vitro*, as Shope first noted (1). The nature of this phenomenon has not been determined. The present paper reports a study of the "inhibitor" and especially of its relationship to the specific antiviral antibody in the blood of animals carrying papillomas. It will be shown that the two are identical.

General Method

To test for inhibitor, the virus-induced papillomas were washed with soap and rinsed well with tap water to remove any adherent blood or virus and were then removed from the animal, using aseptic technique. The connective tissue was carefully trimmed away from the base of the growths and these were cut into small pieces and washed in isotonic saline to remove any adherent blood. Extracts of the freshly procured papillomas thus got were prepared by grinding weighed portions in sand, suspending 1:10 or 1:20 in saline (0.9 per cent), and centrifuging at about 4400 R.P.M. for 20 minutes in an International centrifuge with 51° angle head. The supernatant fluids, which were almost water-clear, were mixed in equal parts with a test virus consisting of a Berkefeld filtrate of naturally occurring cottontail rabbit papillomas. The mixtures, along with appropriate controls containing saline instead of extract, were incubated 2 hours at 37°C. and then rubbed into scarified areas on the abdominal skin of normal domestic rabbits, according to a titration technique already described (2). A number of extracts could be tested concurrently by this method. Three rabbits were used in each titration and the character of the growths was recorded at frequent intervals from the 14th to the 42nd day after inoculation of the test mixtures according to a standard scale: **** = confluent papillomatosis, *** = semiconfluent papillomatosis, ** = many discrete papillomas, * = a few discrete papillomas, ± = 3, 4, or 5 papillomas, ± = one papilloma, 0 = negative (complete neutralization). A comparison of the number of growths produced by the experimental mixtures with those produced by the saline controls denotes the presence or absence of inhibitor and its relative amount. When papilloma extracts containing no inhibitor were mixed with the test virus and inoculated into normal rabbits, the resulting yield of growths was like that produced by the virus-saline control inoculum; whereas fewer or none were got when extracts containing inhibitor had partially or completely neutralized the virus. The inhibitor in a given extract could be titrated by testing the capacity of various dilutions of it to neutralize the test virus.

The *test viruses* were prepared by grinding weighed portions of naturally occurring cottontail rabbit papillomas, which had been preserved in 50 per cent glycerol, suspending the material in 10 or 20 volumes of saline, and centrifuging at about 4400 R.P.M. for 20 minutes in the angle centrifuge. The supernatant fluids were then filtered through Berkefeld V candles.

Blood was obtained from rabbits bearing the papillomas by bleeding from an ear vein or by cardiac puncture. The serum was removed after clotting and tested for antibody by means of the neutralization and complement fixation tests devised in this laboratory, both of which have been described (2, 3). In the *neutralization test* various dilutions of serum were mixed in equal parts with a test virus and put into a water bath at 37°C. for 2 hours. The mixtures were then rubbed into scarified skin areas of normal domestic rabbits, and the character of the growths was recorded by asterisks according to the standard scale described above for the detection of inhibitor in tissue extracts. In the *complement fixation test* various dilutions of serum were tested in mixture with 2 units of complement (titrated immediately beforehand) and an optimal amount of a test virus as the antigen. The mixtures stood at room temperature for 2 hours to allow fixation of the complement and then sensitized red cells were added. Readings were made after 30 minutes in a water bath at 37°C. and again after the tubes had stood overnight in a refrigerator. The latter readings are recorded in the tables as follows, in terms of fixation: ++++ = complete fixation (no hemolysis), +++ = about 75 per cent fixation, ++ = about 50 per cent fixation, + = about 25 per cent fixation, ± = about 10 per cent fixation, 0 = no fixation (complete hemolysis). It has been shown that the virus-neutralizing and complement-fixing capacities of any given serum invariably parallel one another (3, 4). The neutralization test has a slightly lower threshold for the detection of the antibody than the complement fixation test. The latter, however, gives immediate and reliable quantitative results when any considerable quantity of antibody is present, and hence it has been largely used in the present experiments.

It will be noted that the asterisks in all the tables record the number of papillomas produced upon inoculation of the virus in mixture with serum, tissue extracts, or saline. Hence neutralization or inhibition of the virus is demonstrated by fewer papillomas recorded for the experimental mixtures as compared with the controls. The plus signs on the other hand record the effectiveness of the blood antibody, as indicated by its capacity to fix complement in mixture with the virus.

D. R. means domestic rabbit; W. R., wild cottontail rabbit.

Yield of Inhibitor from Papillomas of Rabbits with Different Amounts of Antibody in Their Blood

As a first step toward learning whether there is a relationship between inhibitor and antibody, comparative tests were made of the yield of inhibitor from the papillomas of domestic rabbits and the amount of antibody in the sera of the animals. It was already known that the blood of rabbits carrying such growths acquires in most instances the capacity to neutralize the papilloma virus (2) and to fix complement when mixed with it *in vitro* (3).

Experiment 1.—A 5 per cent virus filtrate of the naturally occurring glycerolated papillomas of W. R. 1-10 was rubbed into several scarified skin areas, each about 4 cm.

square, on the abdomens of six normal domestic rabbits. Scarification had been done with sterilized sandpaper. On the 70th day after inoculation each rabbit had large papillomatous masses covering the areas. They were now bled from an ear vein for serum, and under ether anesthesia a papilloma from each rabbit was removed and a 10 per cent saline extract of it prepared according to the method described in the preceding section. The extracts were then tested for capacity to neutralize a 5 per cent virus filtrate (W. R. 1-10), using the standard technique and inoculating the mixtures into

TABLE I

Yield of Inhibitor from the Papillomas of Domestic Rabbits with Differing Amounts of Blood Antibody

Papillomas from rabbit No.	Tests for inhibitor: Growths resulting from mixture of virus and papilloma extracts†			Serum antibody titer: Complement fixation tests‡ — Dilutions of serum						
	a	b	c	1:4	1:8	1:12	1:16	1:24	1:32	1:48
1	***±	***	***±	++++	0	0	0	0	0	0
6	***±	**±	**	++++	++++	±	0	0	0	0
5	*	±	*	++++	++++	++++	+++±	±	0	0
3	0	0	±	++++	++++	++++	++++	+++	++	0
2	±	±	±	++++	++++	++++	++++	++++	+++±	+±
4	±	*	±	++++	++++	++++	++++	++++	++++	++++
Virus filtrate plus Tyrode (controls).	***±	****	****							

† Inoculum = 5 per cent virus filtrate (W. R. 1-10) and 10 per cent papilloma extracts mixed in equal parts.

**** = confluent papillomatosis.
*** = semiconfluent papillomatosis.
** = many discrete papillomas.
* = a few " "
± = 3, 4, or 5 papillomas.
± = one papilloma.
0 = negative.

‡ Antigen, W. R. 1-10 virus filtrate, 1:60. Complement, 2 units in all tubes.

++++ = complete fixation (no hemolysis).
+++ = about 75 per cent fixation.
++ = " 50 " " "
+ = " 25 " " "
± = " 10 " " "
0 = no fixation (complete hemolysis).

Growths in test rabbits (a, b, c) on the 42nd day after inoculation.

scarified skin areas of three normal domestic rabbits. The serum of each rabbit was tested in various dilutions for antibody by the complement fixation test, using the method described. The W. R. 1-10 virus filtrate diluted 1:60 in saline served as the antigen.

The results of these tests are shown in Table I. The papillomas from D. R. 2, 3, 4, and 5 yielded an inhibitor so potent as to neutralize the test virus almost completely, whereas extracts of the growths of the remaining two rabbits (D. R. 1 and 6) had only a slightly adverse effect on the virus. The antibody titers of the sera of these rabbits varied widely, and on comparison it will be seen that those rabbits in which the titer of serum antibody was most pronounced (D. R. 2, 3, 4, 5) were those with papillomas yielding most inhibitor.

To study further the relation between the inhibitor and serum antibody, papillomas were produced with the virus in another group of domestic rabbits and portions of the growths were removed from time to time and tested for their content of inhibitor, the amount of antibody in the sera of the rabbits being determined concurrently.

Experiment 2.—A 5 per cent virus filtrate of the naturally occurring glycerolated papillomas of W. R. 1-10 was rubbed into a scarified area about 4 cm. square and tattooed as well into eight small areas on the abdomens of six normal domestic rabbits. On the *21st day* after inoculation the rabbits had discrete and semiconfluent papillomatous masses covering the tattoo and scarified inoculation areas, respectively. Each was bled about 10 cc. for serum from an ear vein, and under ether anesthesia a wedge of the semiconfluent papillomatous tissue and several of the discrete growths were removed from each with different sets of sterile instruments.[1] The gaps in the growths were closed with sutures and healing occurred without infection. 10 per cent saline extracts of the excised tissue were prepared in the usual manner and tested for inhibitor in dilutions of 1:10 and 1:20, mixing them with equal parts of a 5 per cent virus filtrate of the infectious papillomas of W. R. 1-10. This virus filtrate was kept frozen at −70°C. and used throughout this experiment. It underwent no detectable decrease in infectivity when inoculated in various dilutions into test rabbits after being frozen 9 months, the longest period employed. After incubating 2 hours at 37°C. the mixtures were rubbed into scarified skin areas of three normal domestic rabbits. The serum of each rabbit was tested in dilutions of 1:10 and 1:20 for capacity to neutralize the virus filtrate and in addition the titer of each serum was determined by complement fixation tests, using another virus filtrate (W. R. 1-56) diluted 1:120 in saline as the antigen.

On the *37th day* another representative portion of the large growth of each rabbit was removed and tested for inhibitor precisely as before. Just before the excision each rabbit was bled from an ear vein and the antibody titers of the sera again determined by the neutralization and complement fixation tests. One rabbit had died and the growths of another had retrogressed during the 4th week after inoculation; hence the results with only four rabbits are available.

On the *70th day* the sera of the rabbits were tested again for antibody content, using

[1] The tests with the discrete papillomas will not be considered in detail. It was found that the yield of inhibitor from discrete and confluent papillomas of the same rabbit was not appreciably different in this experiment and in other tests. In a few instances, however, the discrete papillomas yielded significantly less inhibitor than did the confluent growths, suggesting that the latter afforded a greater opportunity for the extravasation of blood antibody, as Kidd found to be the case regularly in the confluent growths of cottontail rabbits (6). It should be noted that the discrete papillomas of cottontail rabbits usually form orderly, intact, onion-like cones with slightly constricted bases, whereas the discrete growths of domestic rabbits are sessile and become fissured and disorderly early in their development, resembling in this respect the large confluent papillomas of both species. It has been shown that blood antibody is more likely to extravasate in quantity into the fissured, disorderly growths of cottontail rabbits than into intact, discrete papillomas (6).

the complement fixation test. No significant change in antibody titer had taken place, and hence recourse was had to intraperitoneal injections of virus as a means of stimulating a high titer of circulating antibody. Two of the rabbits (D. R. 97 and 100) were given 5 cc. of a 5 per cent freshly prepared virus filtrate (W. R. 1-30) intraperitoneally on the 92nd and again on the 102nd day. The remaining two rabbits (D. R. 96 and 101) were not hyperimmunized.

On the *111th day* the papillomas of three of the rabbits had increased in size, had fleshy bases, and were now fissured and somewhat macerated. The growth of D. R. 100, however, was no larger and a thick connective tissue layer was present beneath it. Again a portion of each was removed, extracted, and tested for inhibitor exactly as on the 21st and 37th days, and serum was obtained just beforehand and tested for antibody as before. Microscopic sections showed that all the growths were still benign papillomas.

The results of the successive tests are summarized in Table II. On the *21st day*, when the first operation was done to procure papillomatous tissue, the serum of every rabbit had a low antibody titer. The serum from D. R. 100 had no antibody detectable either by virus neutralization or complement fixation tests, while the sera of D. R. 96, 97, and 101 partially neutralized the virus in dilutions of 1:10 and 1:20 and fixed complement in low titers. The extracts of the papillomas of these rabbits had no detectable effect upon the ability of the test virus to cause growths. On the *37th day* there were notable differences in the findings. The serum antibody titer of one of the rabbits (D. R. 100) had risen considerably and the extract of the papillomas of this rabbit markedly reduced the pathogenic activity of the virus. Only a slight rise in the antibody titer of the sera of the other rabbits had occurred and the inhibitor content of their papillomas had also increased but slightly. The results of the tests on the *111th day* were quite remarkable in that the sera from three of the rabbits (D. R. 96, 97, 100) now had high antibody titers and their papillomas yielded large amounts of the inhibitor, whereas the serum of the remaining rabbit (D. R. 101) had almost no circulating antibody and no inhibitor could be procured from its papillomas. There had been a spontaneous increase in the blood antibody of rabbit D. R. 96, with result that it now fixed complement in high titer and almost completely neutralized the virus in dilutions of 1:10 and 1:20. The papilloma extract of this rabbit also neutralized the test virus when mixed with it in dilutions of 1:10 and 1:20. Rabbit D. R. 97, which had received hyperimmunizing injections of virus intraperitoneally, now had a high serum antibody titer, and its papilloma extract neutralized the virus markedly. D. R. 100, also hyperimmunized, yielded similar results. Additional tests, not shown in the table, with the materials obtained from D. R. 97 and 100 on the 111th day, revealed that the sera of both when diluted 1:100 almost completely neutralized the virus, while the papilloma extracts, though exerting a pronounced neutralizing effect in dilution of 1:40, did little at 1:80. D. R. 101 provided a remarkable control in that its serum manifested a low titer of circulating antibody throughout these tests and its papillomas at no time yielded any significant amount of inhibitor.

The results of these experiments (Tables I and II) show that the amount of inhibitor in extracts of the papillomas of domestic rabbits and the serum antibody titer of the host vary concurrently not only from animal to animal but in individual rabbits. In an exceptional instance in which

TABLE II

Yield of Inhibitor from the Same Papillomas at Different Times, Compared with the Changes in Circulating Antibody

Time to biopsy of growths	Source of material: Rabbit No.	Yield of inhibitor: Test rabbits	Yield of inhibitor: Growths† resulting from mixture of 5 per cent virus filtrate (W. R. 1-10) and: Saline	Dilutions of papilloma extract: 1:10	Dilutions of papilloma extract: 1:20	Serum antibody titer: Test rabbits	Growths† resulting from mixture of 5 per cent virus filtrate (W. R. 1-10) and: Saline	Dilutions of serum: 1:10	Dilutions of serum: 1:20	Complement fixation tests§: Dilutions of serum: 1:4	1:8	1:16	1:32	1:64
days														
	100	1	****±	****	****	19	****	****	****	0	0	0	0	0
		2	****	***±	***±	20	***±	***	***±					
		3	***±	***	***±	21	****	***±	****					
	97	4	***±	**±	***	19	****	±	*±	++++	±	0	0	0
		5	****	***±	****	20	***±	±	**±					
		6	****	***±	****	21	****	*	*					
21	96	1	***±	***±	****	19	****	±	±	++++	+++±	0	0	0
		2	****	***±	****	20	***±	*	**					
		3	***±	***	****	21	****	±	±					
	101	4	***±	****	****	19	****	***	***	±	0	0	0	0
		5	****	***±	***±	20	***±	*±	**					
		6	****	***	****	21	****	**	**±					
	100	7	***±	*	*±	22	****	±	*	++++	++++	++++	+	0
		8	****	±	*	23	****	0	*					
		9	***±	±	±	24	****	±	*					
	97	10	***±	**	***	22	****	*±	*±	++++	++±	0	0	0
		11	****	***	***±	23	****	±	**±					
		12	***±	**	***	24	****	*	*±					
37	96	7	***±	***	***±	22	****	*	**±	++++	+++±	0	0	0
		8	****	***±	****	23	****	*	±					
		9	***±	***	****	24	****	±	*					
	101	10	***±	***±	****	22	****	***±	***±	+++	0	0	0	0
		11	****	***	***	23	****	**	***±					
		12	***±	***±	***	24	****	***	***					
	100‡	13	***±	±	*	13	***±	0	0	++++	++++	++++	++++	++++
		14	****	±	*±	14	****	±	0					
		15	***±	0	±	15	***±	0	±					
	97‡	16	***±	±	*	16	***±	0	±	++++	++++	++++	++++	±
		17	****	±	*	17	****	0	±					
		18	****	±	±	18	****	0	±					
111	96	13	***±	*	*	13	***±	0	±	++++	++++	++++	+	0
		14	****	±	*±	14	****	0	±					
		15	***±	±	**	15	***±	±	±					
	101	16	***±	****	***±	16	***±	***	****	0	0	0	0	0
		17	****	****	****	17	****	***	****					
		18	****	****	****	18	****	**±	***±					

† Readings made on the 35th day after inoculation of the mixtures, according to the standard scale (see Table I).

‡ Received hyperimmunizing injections of virus intraperitoneally on 92nd and 102nd days.

§ Complement, 2 units in all tubes.

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

antibody was practically lacking in the serum over a long period (D. R. 101 of Experiment 2), inhibitor could not be got from the papillomas.

The findings have been repeatedly confirmed. Papillomas of various size and duration have been tested from 36 rabbits in all. Of these 22 had high titers of serum antibody as determined by virus neutralization and complement fixation tests and their papillomas all yielded large amounts of the inhibitor, whereas little or none could be detected in similar growths from 14 domestic rabbits that had but little circulating antibody. Papillomas that had only recently arisen yielded little or none of the inhibitor, and the sera of the hosts contained little or no antibody. Later on the amount of inhibitor procurable from the growths increased in general proportion to the rise in serum antibody titer. All this indicates the existence of a relationship between the amount of antibody in the blood and the amount of inhibitor in extracts of the papillomas.

The Presence of Inhibitor in Organ Extracts of Rabbits

If the inhibitor in papilloma extracts is blood antibody, as indicated by the preceding experiments, it should also be present in non-papillomatous tissues of rabbits carrying the growths. To test this, organ extracts of normal rabbits and of rabbits carrying papillomas were tested for inhibitor. And to enlarge the observations, extracts of the Brown-Pearce rabbit carcinoma were also tested. It is known that animals carrying this tumor develop no antibodies against the papilloma virus (5).

Experiment 3.—A domestic rabbit (D. R. 1-55), that had carried several large papillomas for 34 days and had received three intraperitoneal injections of a 5 per cent virus filtrate (W. R. 1-28) at weekly intervals to stimulate a high titer of circulating antibody, was bled for serum and killed. Portions of the papillomas, skin, muscle, and liver were immediately removed and 10 per cent saline extracts of them were prepared in the usual way. The extracts and serum diluted 1:10 with saline were tested for capacity to neutralize a freshly prepared 1 per cent virus filtrate (W. R. 1-30). Another domestic rabbit (D. R. 86), with several large papillomas of about 18 weeks' duration, was also bled for serum and killed. A previous complement fixation test had shown that this rabbit had a low serum antibody titer. Portions of papilloma, skin, muscle, and liver were removed and 10 per cent saline extracts prepared. The extracts and serum were tested for capacity to neutralize another 1 per cent virus filtrate (W. R. 1-56).

Experiment 4.—Three normal domestic rabbits (D. R. 1, 2, and 3) were bled for serum under ether anesthesia and then killed. Portions of skin, muscle, and liver were immediately removed from each rabbit and 10 per cent saline extracts were prepared. The extracts and undiluted sera of two of these rabbits (D. R. 1 and 2) were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-56), while the extracts and serum of D. R. 3 were tested with a 5 per cent virus filtrate (W. R. 1-10). In addition 10 per cent saline extracts of the Brown-Pearce tumor prepared from muscle transplants

of two domestic rabbits (D. R. 11 and 8-90), which had been kept frozen, were tested for capacity to neutralize the same virus filtrates.

TABLE III

Yield of Inhibitor from Papillomas, Brown-Pearce Carcinomas, and Certain Organs of Domestic Rabbits

Source of material	Rabbit No.	Test rabbits	Yield of inhibitor: Growths resulting from mixture† of virus filtrate and: Saline (control)	Papilloma extract	Skin extract	Liver extract	Muscle extract	Brown-Pearce tumor extract	Serum antibody titer: Complement fixation tests‡: Dilutions of serum 1:4	1:16	1:64
Rabbits with papillomas	D. R. 1-55	a	****	0	*	*	***±		++++	++++	++++
		b	****	±	±	±	***				
		c	***±	±	*	±	**±				
	86	d	****	**	***	***	****		+++±	0	0
		e	***±	*	**±	**	***				
		f	****	**	**±	**	***				
Normal rabbits	1	g	****		****	****	****		0	0	0
		h	****		****	****	***±				
		i	****		****	****	****				
	2	g	****		****	****	****		0	0	0
		h	****		****	****	****				
		i	****		****	****	****				
	3	g	****		***±	****	***±		0	0	0
		h	****		****	***	****				
		i	****		****	***	****				
Rabbits with Brown-Pearce carcinoma	11	g	****					****	0	0	0
		h	****					****			
		i	****					****			
	8-90	g	****					****	0	0	0
		h	****					****			
		i	****					****			

† Inoculum = 10 per cent extracts and 1 per cent virus filtrate mixed in equal parts, except extracts from D. R. 2 and 8-90 which were tested with a 5 per cent virus filtrate.

Readings made on the 42nd day after inoculation, according to the standard scale.

‡ Complement, 2 units in all tubes.

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

The sera of all of the rabbits used in Experiments 3 and 4 were also tested for antibody by means of the complement fixation test, using the virus filtrate (W. R. 1-56) diluted 1:120 in saline as the antigen.

The results of these tests are summarized in Table III. The serum neutralization tests gave results similar to those obtained with the comple-

ment fixation tests and hence are not included in the table. None of the inhibitor could be detected in saline extracts of the skin, muscle, and liver of the three normal rabbits, nor did extracts of the Brown-Pearce carcinoma, from rabbits uninfected with the papilloma virus, exert any effect upon the latter. The sera of these rabbits contained no antibody detectable either by neutralization or complement fixation tests. In contrast to these findings the inhibitor was present in extracts of the papillomas, skin, muscle, and liver of rabbits carrying the growths. These extracts from rabbit D. R. 1-55, which had a high serum antibody titer, contained large amounts of the inhibitory substance, while similar extracts of rabbit D. R. 86, which had little serum antibody, caused relatively slight neutralization of the virus. Organ extracts of three other rabbits bearing papillomas were also tested, and similar results were obtained. They have not been tabulated. The neutralizing effect of 10 per cent extracts of the papillomas was regularly less than that of a 10 per cent dilution of the sera, but was somewhat greater than that of extracts of the skin, muscle, and liver from the same rabbit. Further tests showed that hemolysin injected intravenously into rabbits carrying papillomas could be detected in extracts of the growths and of normal tissues (skin, muscle, liver). Conditions within papillomas are evidently favorable to the passage of antibody into them.

The Presence of Inhibitor in Cottontail Papillomas

The papilloma virus can usually be procured from the growths of cottontail rabbits, but far from always, and Kidd has shown (6) that the failure to recover it is due to the extravasation of blood antibody into them. Will they on extraction yield the inhibitor, like the papillomas of domestic rabbits? To learn about this, large, vigorously growing papillomas were produced in normal cottontail rabbits by inoculating the virus into broadly scarified areas on the skin of the abdomen. The animals were also injected intraperitoneally with a suspension of the papilloma virus to stimulate a high titer of circulating antibody. For it was known that "masking" of the virus is most likely to occur in the large growths of cottontails having much antibody in their blood (6).

Experiment 5.—A 10 per cent extract of the glycerolated papillomas of W. R. 1-28 was prepared by grinding in sand, suspending in saline, and centrifuging at 2500 R.P.M. for 5 minutes in the angle centrifuge. Some of the turbid supernatant fluid was rubbed into a large scarified area on the abdomen of each of four normal cottontail rabbits. The remainder was again spun at 4400 R.P.M. for 20 minutes, the supernatant fluid was filtered through Berkefeld V candles and diluted in saline to make a 5 per cent suspension; and 6 cc. of the filtrate per kilogram body weight was injected intraperitoneally into

each rabbit on the day after skin inoculation and again on the 8th and 19th days. Complement fixation tests showed that even before the papillomas appeared these rabbits had high serum antibody titers. This state of affairs had no apparent influence on the course of the growths. They enlarged as rapidly as usual.

On the 29th day after skin inoculation, two of the rabbits (W. R. 24 and 27) were bled for serum tests and killed. Each had a large, fleshy papilloma. 10 per cent saline extracts of the growths were immediately made and tested, as were the sera, for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-30). The extracts were also tested for infectiousness by inoculating them into the scarified skin of three normal rabbits.

On the 71st day the remaining rabbits (W. R. 22 and 26) had still larger papillomatous masses, now deeply fissured, with dry, keratinized tops and fleshy bases. Each was bled for serum and killed and 10 per cent extracts were made of the growths. In addition 10 per cent extracts of the skin, muscle, and liver of W. R. 26 were prepared. The extracts and sera were tested for capacity to neutralize the same 1 per cent virus filtrate (W. R. 1-30).

10 per cent saline extracts of the skin, muscle, and liver of a normal cottontail rabbit were also prepared after it had been bled for serum. The materials were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-56).

In additional tests the sera of all the rabbits were tested for complement-fixing antibody.

Table IV shows the results of these tests. (The serum neutralization tests are not included in the table since they only confirmed the results obtained with the complement fixation tests.) Extracts of the growths from rabbits with high antibody titers (W. R. 27, 24, and 22) yielded no virus on inoculation into two rabbits (one of the three test rabbits died 4 days after inoculation). These extracts contained inhibitor in relatively large amount. The serum of W. R. 26 had a low antibody titer and its growth contained but a slight amount of inhibitor and yielded a small amount of virus, as evidenced by the papillomas produced.

The skin, muscle, and liver extracts of W. R. 26 also yielded a small amount of inhibitor. Extracts of the skin, muscle, and liver of the normal cottontail rabbit had no capacity whatever to neutralize the virus, and its serum contained no antibody detectable by neutralization or complement fixation tests.

The tests with cottontails having papillomas that yielded little or no active virus gave findings essentially similar to those in domestic rabbits. Inhibitor proved obtainable from the growths in amount roughly proportional to the serum antibody titer of the host. The amount of inhibitor in extracts of these papillomas was considerably less than that procured from growths in domestic rabbits with comparable blood antibody titers, presumably because of the specific absorption of inhibitor by the virus present in the cottontail growths (4).

Complement Fixation Tests for Inhibitor

The serum antibody that neutralizes the papilloma virus *in vitro* has been shown to be identical with the antibody responsible for the fixation

TABLE IV

Yield of Inhibitor from Papillomas and Certain Organs of Cottontail Rabbits

Source of material	Rabbit No.	Test rabbits	Yield of inhibitor: Growths† resulting from mixture of virus filtrate and: Saline (control)	Papilloma extract	Skin extract	Liver extract	Muscle extract	Test rabbits	Yield of virus: Growths† resulting from inoculation of the papilloma extracts	Serum antibody titer: Complement fixation tests‡: Dilutions of serum: 1:4	1:8	1:16	1:32	1:64	1:128
Rabbits with papillomas	27	a	***±	*				a	0	++++	++++	++++	++++	++++	++++
		b	***±	*				b	0						
	24	a	***±	±				a	0	++++	++++	++++	++±	0	0
		b	***±	*				b	0						
	22	c	****	*				l	0	++++	++++	++++	±	0	0
		d	****	*±				m	0						
		e	****	±											
	26	f	***±	***	**	**±	***	l	*	+++±	0	0	0	0	0
		g	****	***±	**±	**	***±	m	*						
		h	****	****	**	*±	***±								
Normal rabbit	1	i	****		****	****	****			0	0	0	0	0	0
		j	***±		****	****	****								
		k	****		***	****	***								

† Inoculum = 1 per cent virus filtrate (W. R. 1-30) and 10 per cent extracts mixed in equal parts.

Readings made on the 35th day after inoculation according to the standard scale.

‡ Complement, 2 units in all tubes.

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

TABLE V

Antibody in Sera Compared with Inhibitor in Papilloma Extracts

Rabbit No.	Material	Complement fixation tests†: Dilutions of material: 1:4	1:8	1:16	1:32	1:64	1:128	1:256	Neutralization tests: Growths§ resulting from mixture of 5 per cent virus filtrate (W. R. 1-68) and serum or papilloma extract: 1:4: a	b	c
D. R. 10‡	Serum	++++	++++	++++	++++	++++	+++±	±	0	0	0
	Papilloma extract	++++	++++	++++	++++	++			±	0	±
2-47‡	Serum	++++	++++	++++	++++	++++	+++±	0	±	0	0
	Papilloma extract	++++	++++	++++	+	0			±	±	±
24	Serum	+++±	0	0	0	0	0	0	±	±	±
	Papilloma extract	0	0	0	0	0			****	***	***±
2-50	Serum	0	0	0	0	0	0	0	**	***	**
	Papilloma extract	0	0	0	0	0			****	****	****
5 per cent virus filtrate plus saline (controls)									****	****	****

† Complement, 2 units in all tubes.

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

None of the materials was anticomplementary when tested in double amount.

‡ Received two hyperimmunizing injections of papilloma virus intraperitoneally.

§ Growths in test rabbits (a, b, c) on 42nd day after inoculation according to the standard scale.

of complement (3, 4). Will the inhibitor in extracts of papillomas also fix complement in mixture with the virus? To answer this question, extracts of the papillomas of domestic rabbits containing various amounts of inhibitor were tested for capacity to fix complement in mixture with the papilloma virus. The sera of the rabbits furnishing the growths were tested in the same way for comparison.

Experiment 6.—Four rabbits (D. R. 10, 24, 2-47, 2-50) with papillomas of 31 to 45 days' duration were utilized. Two of them (D. R. 10 and 2-47) had received two intraperitoneal injections of a 5 per cent virus filtrate (W. R. 1-28) to raise the circulating antibody to a high titer. All were bled for serum and killed. The papillomas of each rabbit were removed, passed through several changes of saline, and 1:4 suspensions were prepared by grinding in sand and suspending in saline. The extracts were then spun at 4400 R.P.M. for 20 minutes in the angle-head centrifuge. In order to avoid the anticomplementary effect that many papilloma extracts show in suspensions of 1:10 or less, the supernatant fluid was again spun at 20,000 R.P.M. in the air-driven centrifuge and then heated at 60°C. for 30 minutes. A heavy, flocculent precipitate formed which was removed by centrifugation at 4400 R.P.M. for 20 minutes. (It had previously been determined that these procedures do not significantly affect the inhibitor content of the papilloma extracts.) The final supernatant fluids were water-clear with a faint amber color. They were not anticomplementary.

The papilloma extracts thus prepared and the sera of each rabbit were tested in various dilutions for capacity to fix complement in mixture with the papilloma virus. The technique of the test was that previously described (3), using 2 units of complement in each tube and an extract of the highly infectious cottontail rabbit papillomas of W. R. 1-72 diluted 1:120 in saline as the antigen. The papilloma extracts and sera were also tested for capacity to neutralize a 5 per cent virus filtrate (W. R. 1-68) by rubbing the mixtures into scarified skin areas in three normal domestic rabbits according to the usual titration method.

The results of the tests are shown in Table V. It will be seen that the sera from D. R. 10 and 2-47 contained much antibody, as determined by both the complement fixation and virus neutralization tests, and extracts of the papillomas from these animals also fixed complement in high titer and almost completely neutralized the test virus. The serum of D. R. 2-47 had less antibody than that of D. R. 10 and the extract of the papillomas from this rabbit yielded slightly less inhibitor and fixed complement in lower titer than the extracts from D. R. 10. Furthermore, the sera fixed complement in higher titer than the papilloma extracts and, as previously noted (Experiment 2), had a greater neutralizing capacity. The sera of D. R. 24 and 2-50 had low antibody titers and extracts of the papillomas from these rabbits contained little or no inhibitor and did not react in the complement fixation test.

The experiment shows (Table V) that extracts of papillomas from rabbits with much blood antibody neutralize large amounts of the papilloma virus and also fix complement in high titer when mixed with the virus *in vitro*; whereas extracts of growths from rabbits with small amounts of blood

antibody yield little or no inhibitor and have no capacity to react in the complement fixation test. These findings afford further evidence of the identity of inhibitor and antibody, since, as already stated, the antibodies responsible for neutralization of the virus and for the fixation of complement in mixture with it are identical (3, 4). The highest dilution at which the papilloma extracts fixed complement was less than that of the serum of the host bearing the growths, as had been previously noted with the neutralization tests for inhibitor (see Table II).

Tests for Specific Absorption with the Inhibitor and the Virus, Respectively

The virus-neutralizing and complement-fixing antibodies are readily absorbed from the sera of rabbits bearing virus-induced papillomas, when these are mixed with extracts containing the papilloma virus (4). In view of this fact, tests were undertaken to see whether the inhibitor would be specifically absorbed from papilloma extracts when they were mixed with a filtrate containing the virus.

Experiment 7.—The papilloma extracts of rabbits D. R. 10 and 2-47, mentioned in the preceding experiment, were used as the sources of inhibitor. The technique of the absorption test was essentially that employed in the absorption of serum antibody (4). The papilloma extracts in dilution of 1:4 were mixed in equal parts with a highly infectious papilloma virus filtrate (W. R. 1-28) in dilutions of 1:10, 1:20, 1:40, and 1:80 in saline. The mixtures together with appropriate saline controls were incubated for 2 hours at 37°C. and left overnight in the refrigerator. The amount of visible flocculation in each tube was recorded and the mixtures were spun at about 4400 R.P.M. for 20 minutes. The amount of inhibitor remaining in the supernatant fluids was determined by complement fixation and virus neutralization tests.

Table VI shows that the inhibitor was completely removed from the papilloma extract of D. R. 10 when this was mixed with the virus filtrate in dilution of 1:10, as determined by both complement fixation and neutralization tests. Further dilution of the virus filtrate resulted in a corresponding reduction in the absorption of inhibitor. The papilloma extract of D. R. 2-47, which contained less inhibitor than that of D. R. 10, had none that was detectable after absorption with the virus filtrate in dilutions of 1:10 and 1:20, whereas partial absorption occurred with the filtrate diluted 1:40 and 1:80. A visible flocculation was present in those mixtures in which inhibitor was completely removed from the extracts, similar to that noted in optimal mixtures of immune serum and the papilloma virus (4). Subsidiary tests showed that no excess virus was present in any of the mixtures after absorption. The results illustrate incidentally the greater sensitiveness of the neutralization test for inhibitor, a finding which parallels the results of tests by both methods for serum antibody (3, 4). No inhibitor was detectable by the complement fixation test in the mixtures containing the papilloma extract D. R. 10 and virus filtrate 1:20, and extract D. R. 2-47 and virus filtrate 1:40, yet both caused slight neutralization of the test virus.

In the next experiment a constant amount of a virus filtrate was absorbed with papilloma extracts known to contain differing quantities of inhibitor.

Experiment 8.—Two papilloma extracts were utilized containing large amounts of inhibitor (D. R. 10 and 2-47) and two in which there was little or none (D. R. 24 and 2-50). Portions of them had been employed in Experiment 6. They were tested for

TABLE VI

Specific Absorption of Inhibitor with the Papilloma Virus

Source of inhibitor (papilloma extract 1:4)	Dilution of virus filtrate W. R. 1-28 used for absorption	Inhibitor remaining in mixtures after absorption as determined by: Complement fixation tests† — Dilutions of extracts 1:8	1:12	1:16	1:24	1:32	1:48	1:64	Neutralization tests‡ — Test rabbits a	b	c
D. R. 10	1:10	0	0	0	0	0	0	0	****	****	***±
	1:20	0	0	0	0	0	0	0	**	***	**±
	1:40	++++	++++	++++	+	0	0	0	±	±	±
	1:80	++++	++++	++++	++++	+++±	+	0	±	0	0
	Unabsorbed; saline control	++++	++++	++++	++++	++++	+++±	+	0	0	0
D. R. 2-47	1:10	0	0	0	0	0	0	0	****	****	***±
	1:20	0	0	0	0	0	0	0	****	****	***±
	1:40	0	0	0	0	0	0	0	***	***±	**
	1:80	+++±	±	0	0	0	0	0	*	*	±
	Unabsorbed; saline control	++++	++++	++++	+++	±	0	0	±	±	0
5 per cent virus (1-68) plus saline									***±	****	***±

† Complement, 2 units in all tubes.
Antigen, W. R. 1-72 extract, 1:120.
None of the materials was anticomplementary when tested in double amount.

‡ Growths resulting from mixture of 5 per cent virus filtrate (W. R. 1-68) and absorption mixture in three test rabbits on the 42nd day after inoculation.

capacity to absorb virus from a 5 per cent filtrate of the glycerolated cottontail rabbit papillomas of W. R. 1-28. The technique used in the preceding absorption experiment was followed.

It will be seen (Table VII) that the papilloma extracts containing a large amount of inhibitor (D. R. 10 and 2-47) completely absorbed the complement-fixing antigen and removed practically all of the infectivity of the virus suspension. (A single discrete growth occurred in one of the test rabbits.) By contrast the papilloma extract from rabbit D. R. 24, which contained a small amount of inhibitor, absorbed a slight amount of the complement-fixing antigen and the infectivity of the virus filtrate; and the extract of D. R. 2-50, which contained no inhibitor, absorbed no demonstrable amount of the antigen or the infectious virus.

The results of these tests (Tables VI and VII) clearly show that the inhibitor can be specifically absorbed from papilloma extracts with the virus and conversely, that the infectivity and the complement-fixing antigen of a virus filtrate can be absorbed with inhibitor. The findings are precisely like those previously reported for the absorption of serum antiviral antibody (4), and they provide further proof of the identity of inhibitor and antibody.

TABLE VII

Specific Absorption of the Papilloma Virus with Inhibitor

Source of inhibitor used for absorption of virus filtrate (W. R. 1-28)	Rabbit No.	Complement fixation tests† to determine amount of virus antigen remaining after the absorption					Pathogenicity tests‡ to determine amount of infectious virus remaining after the absorption					
		Dilutions of virus filtrate (W. R. 1-28)					17th day			42nd day		
		1:20	1:40	1:80	1:160	1:320	a	b	c	a	b	c
	D. R.											
Rabbits with high serum antibody titer, having papillomas which yielded much inhibitor	10	0	0	0	0	0	0	0	0	0	0	0
	2-47	0	0	0	0	0	0	0	0	0	±	0
Rabbits with low serum antibody titer, having papillomas which yielded little or no inhibitor	24	++++	++++	+++±	++	0	±	**±	**	****	****	****
	2-50	++++	++++	++++	+++±	±	**	***±	***	****	****	****
Virus filtrate plus saline (control)		++++	++++	++++	+++±	±	**	***±	***±	****	****	****

† Complement, 2 units in all tubes.
Immune serum, D. R. 1-65, 1:24.
None of the materials was anticomplementary when tested in double amount.

‡ Growths in test rabbits (a, b, c) according to standard scale.

Effect of Heat on Inhibitor and Antiviral Antibody

In extension of the observations comparative tests were now made of the effect of heat on the inhibitor and serum antiviral antibody.

Experiment 9.—The effect of heat on three immune sera was tested by means of complement fixation tests. The sera were obtained from rabbits carrying papillomas of about 16 weeks' duration (D. R. 1-36, 9-52, 1-53), which had received two intraperitoneal injections of a virus filtrate (W. R. 1-70) to stimulate a high antibody titer. The sera were diluted 1:10 in saline and 3 cc. portions put into sealed glass tubes and submerged in water baths at temperatures varying from 60° to 85°C. for 30 minutes. The sera heated at 60° and 65° showed no visible change, while those heated at 70° and 75°

had a faint bluish opalescence, and this was more pronounced in the tubes heated at 80° and 85°. No gross flocculation was present in any of the heated sera, however. The unheated and heated sera were tested by means of complement fixation tests in the usual way.

The serum antiviral antibody proved to be fairly resistant to heat (Table VIII). In every case heating at 80°C. for 30 minutes inactivated it completely, and there was no detectable antibody in two of the sera after heating at 75°C. Heating at 70°C. inactivated it partially, whereas temperatures of 60° and 65° had no detectable effect. The destruction of the antibody was attended by an increasing opalescence of the sera.

TABLE VIII

The Effect of Heat on the Blood Antibody

Serum	Complement fixation tests†											
Heated 30 min.	Serum D. R. 1-53				Serum D. R. 1-36				Serum D. R. 9-52			
	Dilutions of serum				Dilutions of serum				Dilutions of serum			
	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80
°C.												
Unheated	++++	++++	++++	+++±	++++	++++	+++±	++	++++	++++	+++±	±
60	++++	++++	++++	+++±	++++	++++	+++±	++	++++	++++	+++±	±
65	++++	++++	++++	+++±	++++	++++	+++±	++	++++	++++	+++±	±
70	++++	++++	+++±	+±	++++	+++±	0	0	++++	+++±	±	0
75	++	0	0	0	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0	0	0	0
85	0	0	0	0	0	0	0	0	0	0	0	0

† Complement, 2 units in all tubes.
Antigen, virus extract W. R. 1-70, 1:120.
None of the materials was anticomplementary when tested in double amount.

The experiment shows that the serum antiviral antibody has a considerable resistance to heat. Is this true of the inhibitor in papilloma extracts as well? Comparative tests on the point were undertaken.

Experiment 10.—The papilloma extracts and sera of two of the rabbits of the preceding test (D. R. 1-36 and 9-52) were used in this experiment. 3 cc. portions of each, diluted 1:10 in saline, were heated in water baths at temperatures of 60° to 85°C. for 30 minutes. An abundant flocculent precipitate formed in the heated papilloma extracts. To learn whether it contained the inhibitor, one of the extracts (D. R. 9-52) was spun at 3500 R.P.M. for 10 minutes, and the supernatant fluids were removed and the sediments resuspended in the original volume of saline. Both were tested for inhibitor, as were also whole heated specimens of the extract of D. R. 1-36. None of the sera showed any precipitate after heating, but samples heated above 70° were opalescent, as noted in the preceding experiment. The unheated and heated sera and extracts were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 77).

Table IX shows the results of these tests. The inhibitor and serum antibody were completely inactivated by heating at 80° and 85°C. for 30 minutes, while 75° caused

partial destruction of both. 70°C. caused partial destruction of the inhibitor in the papilloma extract of D. R. 9-52, but there was no detectable decrease in the neutralizing capacity of the extract of D. R. 1-36 when heated at these temperatures. This latter extract, however, contained a larger amount of inhibitor. 60° and 65°C. had no detectable effect on either inhibitor or antibody. The precipitate removed from the heated extracts of D. R. 9-52 by centrifugation contained no detectable inhibitor. This finding has not been included in the table.

The inhibitor in papilloma extracts and the serum neutralizing antibody showed an essentially identical resistance to heat. Exposure to 80°C. for

TABLE IX

The Effect of Heat on Inhibitor and Blood Antibody

Material heated 30 min.	Neutralization tests†											
	Virus filtrate plus											
	Papilloma extract (D. R. 1-36)			Serum (D. R. 1-36)			Papilloma extract (D. R. 9-52)			Serum (D. R. 9-52)		
	a	b	c	a	b	c	d	e	f	d	e	f
°C.												
Unheated	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	±	0
65	±	0	0	0	0	0	0	±	0	±	0	0
70	0	0	0	0	0	0	*	*	±	±	0	0
75	±	±	*	±	±	±	**	**	**	±	±	±
80	****	****	***±	***±	****	****	****	***	****	****	****	****
85	****	****	****	****	****	****	****	****	****	****	****	****
Virus filtrate plus saline.....	****	****	****	****	****	****	****	***±	***±	****	****	***±

† Virus filtrate W. R. 77-E, 1:100.
Papilloma extracts and sera diluted 1:10 in saline.
Growth in test rabbits (a, b, c, d, e, f) on the 35th day after inoculation.

30 minutes proved necessary for their total destruction, whereas 60° and 65°C. were without effect on either. These findings extend those on the destruction of antibody as determined by complement fixation tests (Table VIII).

Precipitation of Inhibitor and Antibody with Ammonium Sulfate

Antibodies can usually be precipitated with the globulin fraction of sera by a variety of methods (7). Tests were now made to see whether the inhibitor and serum antiviral antibody are precipitated by the same concentrations of ammonium sulfate.

Experiment 11.—Portions of the sera and papilloma extracts obtained from rabbits D. R. 1-36 and 9-52 for the purposes of the preceding test were utilized, as were those

from a third rabbit, D. R. 1-35, which had also received intraperitoneal injections of a virus filtrate to stimulate antibody production. After each had been diluted 1:10 with saline, it was mixed with a solution of ammonium sulfate to a final concentration of ⅓ saturation in order to precipitate the *euglobulin fraction.* The mixtures were kept in a refrigerator (4°C.) for 5 hours. A heavy precipitate formed in the bottom of each tube and the visible amount in each was approximately the same. They were then spun at 4400 R.P.M. for 20 minutes, the supernatant fluids were removed, and the sediments resuspended in the original volume of saline (4 cc.). The resuspended serum euglobulin solutions appeared water-clear, whereas those of the papilloma extracts were slightly turbid and a few small particles could be seen. Now more ammonium sulfate,

TABLE X

Precipitation of Inhibitor and Blood Antibody with $(NH_4)_2SO_4$

Material tested		Neutralization tests: Virus filtrate† plus material from rabbits								
		D. R. 1-36			D. R. 9-52			D. R. 1-35		
		a	b	c	a	b	c	d	e	f
Papilloma extract (1:10)	Whole extract	0	0	0	0	0	0	0	0	0
	Euglobulin fraction‡	0	0	0	±	*	*	*	±	±
	Pseudoglobulin " §	0	0	0	±	0	±	±	±	±
Serum (1:10)	Whole serum	0	0	±	0	0	0	0	0	0
	Euglobulin fraction	0	0	±	0	0	±	0	0	±
	Pseudoglobulin "	0	0	0	0	±	0	±	0	0
Virus filtrate plus saline		****±	****±	****	****±	****±	****	****	****±	****

† Virus filtrate W. R. 1-68, 1:100.
Growths in test rabbits (a, b, c, d, e, f) on 35th day after inoculation.
‡ Precipitated with ⅓ saturation ammonium sulfate.
§ Precipitated with ½ saturation ammonium sulfate.

to the extent of ½ saturation, was added to precipitate the *pseudoglobulin fraction.* The mixtures were kept overnight in the refrigerator and all had a heavy, large-flocculent precipitate. They were spun at 4400 R.P.M. for 20 minutes, the supernatant fluids removed, and the sediments resuspended in the original volume of saline. The resuspended pseudoglobulin solutions were all water-clear.

The original papilloma extracts and sera and the euglobulin and pseudoglobulin fractions of each were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-68 E). The extracts and sera that had been precipitated with ½ saturated ammonium sulfate were also tested for neutralizing capacity in dilution of 1:20, as were the original extracts and sera similarly diluted.

Table X shows that the globulin fractions salted out of "inhibiting" extracts and neutralizing sera had nearly as great an ability to neutralize virus as the whole materials. It is evident that inhibitor and antibody

are alike in their precipitation reactions with ammonium sulfate, and the results at ⅓ and ½ saturation indicate that they are distributed between the euglobulin and pseudoglobulin fractions. A small amount of inhibitor and antibody remained in the extracts and sera, respectively, following the precipitation with ½ saturated ammonium sulfate (not shown in the table). Subsidiary tests showed that the inhibitor and serum antibody could be also precipitated with absolute alcohol and with acetone.

Effect of Perfusion on Yield of Inhibitor and Virus from Papillomas

Tests were now undertaken to see whether the inhibitor could be removed from the papillomas by perfusing the rabbit host with saline immediately after it had been killed.

Experiment 12.—A domestic rabbit (D. R. 1-56) and a cottontail rabbit (W. R. 23) each had a large confluent and six small discrete papillomas. Each received two intraperitoneal injections of a virus filtrate at weekly intervals to raise the circulating antibody to a high titer. The rabbits were then bled from the heart for serum and killed. A portion of the large papillomatous mass of each rabbit was immediately isolated by means of a long curved clamp placed across the base and through a portion of the growth. Thus while a portion of the papilloma remained intact the remainder (clamped portion) was isolated from the general circulation. Clamps were also placed at the bases of three of the small discrete papillomas of each rabbit, while the vessels of the remaining three were not blocked. A portion of abdominal skin was similarly isolated. The heart was then cut across through the lower half of the two ventricles, a glass cannula was inserted into the aorta through the left ventricle, and about 3 liters of warm saline (0.9 per cent) was run in from a flask 3 feet above the heart. The blood vessels that had been cut while exposing the heart had been tied to prevent leakage and the saline returned through the right ventricle after passing through the rabbit. Using this same technique in other rabbits, it was found that an India ink-gelatin solution would fill the blood vessels throughout the living portions of the papillomas. The perfused and non-perfused papillomas and skin were removed from each animal with different sets of sterile instruments and 10 per cent saline extracts of them were prepared. The extracts and sera, all in dilution of 1:10, were tested for capacity to neutralize a 1 per cent virus filtrate (W. R. 1-30). The extracts were also tested for infectiousness by means of pathogenicity tests.

The tests disclosed no very great difference in the yield of inhibitor from the perfused and non-perfused papillomas or skin of the domestic or cottontail rabbits (Table XI). Nevertheless the cottontail papillomas that were perfused had a greater infectivity on extraction, notably the small discrete growths, which contained less inhibitor than did the large confluent papillomas. Hence it seems a fair inference that some fraction of the inhibitor had been removed. Most of it, however, doubtless lay outside the vessels as result of extravasation, and hence not likely to be removed when they were flushed.

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

The papilloma virus cannot be recovered ordinarily from the growths induced with it in domestic rabbits; yet the virus is known to be present in them, for the titer of circulating antibody increases as the papillomas enlarge (2). Kidd has shown that little or no virus can be procured from

TABLE XI

Effect of Perfusion on the Yield of Inhibitor and of Virus from Papillomas

Material		Tests for inhibitor†						Pathogenicity tests			
		Growths resulting from the mixture of virus filtrate and extracts from						Extracts from cottontail rabbit (W. R. 23)			
		Domestic rabbit (D. R. 1-56)			Cottontail rabbit (W. R. 23)			17th day after inoculation		35th day after inoculation	
		a	b	c	d	e	f	g	h	g	h
Papilloma extracts from											
(1) Small discrete growths	Not perfused	0	0	0	***	**±	**	0	0	*	*
	Perfused	0	0	0	***	**	***	**±	**±	***±	****
(2) Large confluent growths	Not perfused	0	0	0	**±	*	±	0	0	0	0
	Perfused	0	0	±	*	**	**	0	0	±	±
Skin extract	Not perfused		±	±	*	*	±				
	Perfused	±	*	*	*	**±	±				
Serum		0	0	0	0	0	0				
Controls (virus filtrate plus saline)		***±	***±	****	****	****	****				

† 1 per cent virus filtrate (W. R. 1-28) and 10 per cent extracts in equal parts.
Readings in test rabbits (a–h) on 35th day after inoculation according to the standard scale.

the growths of domestic rabbits, even when only small amounts of antibody are present in the sera of the hosts (6). He concluded therefore that something other than antibody is primarily responsible for the "masking" of the virus in this species. The question arises as to whether the inhibitor has anything to do with the failure to recover virus from extracts of these papillomas. A number of observations made during the course of this work bear upon the problem.

A total of 63 papillomas from 36 rabbits were tested for virus and inhibitor during the course of the experiments reported in this paper. Fifty-one of these papillomas were obtained from 30 rabbits with considerable amounts of antibody in their blood. Extracts

of the papillomas all contained inhibitor and none of them yielded virus upon inoculation into the scarified skin of test rabbits. Twelve papillomas were obtained from 6 rabbits with little or no blood antibody and the results of the tests with this group revealed a striking correlation between the yield of inhibitor and virus from them. The discrete and confluent papillomas resulting from tattoo inoculation and inunction, respectively, of a single virus material into the rabbits of Experiment 2 were tested concurrently for yield of virus and inhibitor. The growths had been washed with soap and thoroughly rinsed with water before removal from the hosts by operation, in order to remove any blood or virus that might be present on their surface. Afterwards they were diced into small pieces and passed through several changes of saline, again for the purpose of removing any adherent blood or virus. Portions of the extracts of the discrete and confluent papillomas, which were obtained on the 21st, 37th, and 111th days after virus inoculation and prepared as described in Experiment 2, were tested for virus by rubbing them into scarified skin areas of normal domestic rabbits. (The confluent growths were large and provided an abundance of material. They were extracted 1:10 in saline. The discrete growths, however, were small and hence had to be extracted 1:20.) The extracts were also tested for inhibitor in the usual way, but with a 1 per cent suspension of the W. R. 1-10 filtrate as the test virus instead of 5 per cent, as used in Experiment 2, in order to render the test somewhat more sensitive. The blood antibody titer of the rabbits was determined by the neutralization and complement fixation tests.

The results of the tests with the extracts and sera obtained on the 21st day after inoculation are summarized in Table XII. (The serum neutralization tests are not included in the table, since they did not differ from those recorded in Table II. One of the three test rabbits used in the pathogenicity tests and one of the group used in the inhibitor tests died soon after inoculation, and hence the results in only two rabbits of each group are available.) It will be seen that the papillomas from D. R. 100, which had no detectable blood antibody, contained no inhibitor and that they yielded a small amount of virus, extracts of the growths producing from 4 to 22 papillomas when inoculated into the test rabbits. Extracts of the papillomas from D. R. 98, 99, and 101 caused just perceptible neutralization of the test virus and the blood antibody titer of these rabbits was quite low. Some of these papillomas yielded virus, namely, the discrete growths from D. R. 98 and 99 and the confluent growths from D. R. 98 and 101, but the amount was less than that from the papillomas of D. R. 100. The blood of D. R. 96 and 97 contained antibody in higher titer than the other four rabbits and extracts of the papillomas contained considerable amounts of inhibitor. These growths yielded no virus.

The results of similar tests with portions of the papillomas of four of the rabbits (D. R. 96, 97, 100, and 101) obtained on the 37th and 111th days can be briefly summarized. (D. R. 98 had died, and the growths of D. R. 99 had retrogressed, so that their papillomas were not available for test.) The serum antibody titers of these rabbits had risen since the 21st day and inhibitor could now be detected in extracts of all of the growths in increased amount (Table II). No virus was obtained from any of the papillomas in these later tests.

The findings indicate that virus cannot be recovered ordinarily from the papillomas of domestic rabbits if they contain any considerable amount of inhibitor. Papillomas obtained from rabbits early in their development,

TABLE XII

Yield of Virus and Inhibitor from the Papillomas of Domestic Rabbits

Rabbit No.	Character of the growths	Test rabbits	Pathogenicity tests with extracts† of the growths			Test rabbits	Tests for inhibitor 1 per cent virus filtrate‡ plus		Serum antibody titer			
									Complement fixation tests			
									Dilutions of serum			
			18th day	28th day	42nd day		Saline (controls)	Papilloma extracts	1:2	1:4	1:8	1:12
100	Discrete	a	±	±	±	c	****±	****±	0	0	0	0
		b	±	±	±	d	****	****				
	Semiconfluent	a	*	**	**	c	****±	****±				
		b	0	0	0	d	****	****				
98	Discrete	a	0	±	±	e	****±	****±	+++±	0	0	0
						f	****±	**±				
		b	0	0	0	g	***	***				
	Semiconfluent	a	0	±	±	e	****±	***				
						f	****±	***				
		b	0	±	±	g	***	**±				
99	Discrete	a	±	*	*	c	****±	***	+++	0	0	0
		b	0	0	0	d	****	***				
	Semiconfluent	a	0	0	0	c	****±	***				
		b	0	0	0	d	****	***				
101	Discrete	a	0	0	0	c	****±	***	+++	±	0	0
		b	0	0	0	d	****	***				
	Semiconfluent	a	0	0	0	c	****±	***				
		b	0	±	±	d	****	***				
97	Discrete	a	0	0	0	e	****±	**±	++++	++++	±	0
						f	****	**				
		b	0	0	0	g	***	**±				
	Semiconfluent	a	0	0	0	e	****±	±				
						f	****±	**				
		b	0	0	0	g	***	±				
96	Discrete	a	0	0	0	e	****±	*	++++	++++	+++±	±
						f	****	**±				
		b	0	0	0	g	***	**				
	Semiconfluent	a	0	0	0	e	****±	**				
						f	****	**				
		b	0	0	0	g	***	**±				

† Discrete papillomas extracted 1:20 in saline.
Semiconfluent papillomas extracted 1:10 in saline.

‡ Virus filtrate W. R. 1-10.
Growths on 35th day after inoculation.

when the blood contains little or no antibody and extracts of the growths contain little or no inhibitor, may yield small amounts of the virus. The amount of virus recovered from the papillomas was never large. 5 to 10 per cent saline extracts of the growths produced from one to 22 discrete papillomas when inoculated into the scarified skin of test rabbits, and the incubation period was prolonged, varying from 18 to 30 days. It is possible that the small amount of virus that was finally recovered had merely persisted on or about the growths from the original inoculum, but the fact that the papillomas were thoroughly washed before extraction makes this possibility seem unlikely.

In contrast to these findings, it is not unusual for the papillomas of cottontail rabbits to yield extracts capable of producing growths after dilution to 1:200,000. Manifestly the inhibitor, like the antiviral antibody, cannot account for the failure to recover virus from the papillomas of domestic rabbits in amount comparable to that procured from wild rabbit growths, for even in its absence only a comparatively small amount of virus could be recovered, as Kidd has also found (6). It appears, however, that the inhibitor when present in considerable amount renders it impossible to recover virus from the papillomas of domestic rabbits with the methods now in use.

DISCUSSION

The prime purpose of the experiments here reported has been to learn whether the virus inhibitor, which is often present in extracts of the virus-induced rabbit papillomas, is identical with antiviral antibody derived from the blood. The evidence may now be briefly recapitulated. There was found to be a quantitative relationship between the inhibitor in papilloma extracts and the serum antiviral antibody. Papillomas tested before the specific antibody could be detected in the serum of the host in any significant amount yielded none of the inhibitor, nor was it present in extracts of normal rabbit tissues or the Brown-Pearce tumor. The inhibitor was not confined to the papillomas but was present in extracts of liver, muscle, and skin from rabbits bearing the papillomas,—and in amounts proportional to the titer of serum antibody. The inhibitor, like the antiviral antibody (2), had no discernible influence on the course of the papillomas, these enlarging progressively or dwindling and vanishing irrespective of their content of inhibitor. Both were completely inactivated by heating at 80°C. for 30 minutes, and they were precipitated together upon treatment with ammonium sulfate. The inhibitor fixed complement in mixture with the papilloma virus in proportion to its neutralizing capacity and it was specifi-

cally absorbed from papilloma extracts when mixed with the papilloma virus, and the infectivity and complement-fixing antigen of a virus filtrate could be completely absorbed with the inhibitor,—findings similar to those previously reported for the blood antibody (3, 4). Taken together these facts prove the inhibitor to be identical with serum antibody.

The inhibitor or antibody—for the terms can be used interchangeably—is readily studied in extracts of the papillomas of domestic rabbits, because the virus, though responsible for the growths and persisting in them (2), is not present in any considerable amount in active form and hence does not act to absorb the inhibitor. The papillomas of cottontail rabbits, on the other hand, usually yield large amounts of the virus, and it would follow that any inhibitor present is doubtless absorbed by virus and hence cannot be demonstrated. In such instances one must suppose the virus to be present in excess, over and above the amount combining with the inhibitor in the extract. Not infrequently, however, so much inhibitor is present, under conditions favorable to the extravasation of antibody in large amount, that the virus is wholly "masked" (6). It is in such instances that inhibitor can be detected in extracts of the cottontail growths, again as representing an excess.

The amount of inhibitor in extracts of freshly procured papillomas from domestic rabbits with high titers of serum antibody is considerable, 0.2 cc. of a 5 per cent suspension neutralizing in some instances an amount of virus equivalent to 2000 or more minimal infectious doses. As already remarked, the amount of detectable antibody (inhibitor) in papilloma extracts was always less than that present in the blood of the host, the titer of the former being roughly one-fourth to one-eighth that of the serum (Tables II and V). Freund (8) found a rather constant numerical relationship between the antibody content of the blood and organs following the intravenous injection of rabbit serum containing typhoid agglutinins into rabbits, the amount in the organs being on the average about one-tenth that in the serum. The greater proportion of antibody in papilloma extracts is probably accounted for by the greater opportunity afforded for extravasation and localization of antibody in the papillomas than in normal tissues. It seems probable, as has been stated, that most of the inhibitor present in extracts of the papillomas represents antibody that has become localized in the tissues, not merely circulating antibody. This is suggested by the finding that extracts of organs containing large amounts of blood (liver) contain no more inhibitor than do extracts of the papillomas (Experiments 3 and 4), and furthermore by the failure of perfusion with saline to effect any large reduction in the inhibitor content of the papillomas (Experiment 12).

The results of Freund's experiments with typhoid agglutinins in normal tissues are quite similar (8).

Certain virus inhibitors have been encountered in other diseases, which may now be considered in relation to the findings with the inhibitor in the rabbit papillomas. Extracts of slowly growing chicken tumors often contain an inhibitor that neutralizes the infectious tumor agent *in vitro* (9, 10, 11). Some of the properties of this inhibitory substance suggest that it is neutralizing antibody of the sort found in the blood. Andrewes has found that the blood of chickens bearing tumors of slow growth usually contains considerable amounts of neutralizing antibody (12). Furthermore, the inhibitor present in extracts of the chicken tumors is a globulin (10, 11), and like the neutralizing antibody for the chicken tumor virus it forms an unstable union in mixture with the latter, which can be readily dissociated by centrifugation or by adsorption on aluminum hydroxide (10, 12). Nasal secretions from human subjects often contain a substance capable of inactivating relatively large amounts of influenza virus (13, 14). Francis found a relationship between the neutralizing effect of the sera and that of the nasal secretions, particularly in patients with blood antibody titers of 1:40 or more. He also noted that the neutralizing antibodies and the agent in the nasal secretions were inactivated at the same temperature (14). The findings as a whole support the view that the specific virus inhibitors of the chicken tumors and of the human nasal secretions may be, like the inhibitor of the papilloma virus, extravasated antiviral antibody. Papilloma virus inhibitor has also been demonstrated in extracts of the cancers deriving from the virus-induced growths of cottontail and domestic rabbits (15), and it is apparently identical with the inhibitor found in the papillomas.

SUMMARY

The "inhibitor" demonstrable in extracts of the virus-induced rabbit papillomas is identical with the antiviral antibody found in the blood of hosts bearing the growths. The conditions in these latter are frequently favorable to its extravasation in considerable amount into them. Its significance and its influence upon the recovery of virus from the papillomas are discussed.

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