UNION IN VITRO OF THE PAPILLOMA VIRUS AND ITS ANTIBODY

By WILLIAM F. FRIEDEWALD, M.D., and JOHN G. KIDD, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The rabbit papilloma virus (Shope) provides a notably favorable material for immunological investigation, as previous studies have shown (1). The virus itself appears to be antigenic, eliciting an antibody that is capable both of neutralizing the virus and of fixing complement in mixture with it, and its immunological reactions are not complicated by any associated "soluble antigen."[1] To learn more, we have now inquired whether the virus can be absorbed by its specific antibody, and *vice versa*;[2] and if so whether the virus-antibody union can be dissociated. The question is more than academic: it bears directly on the problem of the recovery of virus from the papillomas and cancers caused primarily by it, for these often contain extravasated antibody in amounts sufficient to render impossible the recovery of virus from them (16). The findings bear upon the general problems of virus-antibody reactions and will be discussed in this relation.

The Single Antibody Elicited by the Rabbit Papilloma Virus

The immunological responses to viruses in general are rendered complex by the fact that two distinct types of antibodies may be called forth in a single virus disease. In one category are antibodies responsible for the neutralization of specific viruses and presumably for protection against them. Antibodies of this sort may be present for long periods in the blood of individuals that have recovered from virus diseases; they fail, as a rule, to manifest flocculation or complement fixation reactions of noteworthy titer when mixed with the viruses *in vitro*. Antibodies of another type are called forth during the acute phases of virus diseases by "soluble antigens,"

[1] Hoyle (*J. Path. and Bact.*, 1940, **50**, 169) has confirmed the essential findings of the immunological study of the papilloma virus. Using a variety of methods, he sought evidence of a "soluble antigen" in extracts of the papillomas but found none and concluded, as we had, that the virus itself is antigenic.

[2] Preliminary note in the *Proceedings of the Society for Experimental Biology and Medicine*, 1939, **41**, 218.

—specific components of the various viruses and separable from them or products of the interaction of the viruses and the cells they infect. These antibodies have no apparent affinity for the viruses *per se*, yet they react strongly *in vitro* in mixture with the "soluble antigens." Antibodies of both types have been found in most of the virus diseases extensively studied heretofore,—for example, in vaccinia, yellow fever, psittacosis, influenza, myxomatosis, and lymphocytic choriomeningitis (2),—and it has proved difficult to evaluate the part played by the viruses themselves in eliciting the immunological responses since the "soluble antigens" have been dominant in all. In exception to such findings the papilloma virus appears to elicit a specific antibody of one sort only, which is responsible for neutralization of the virus *in vitro* as also for the fixation of complement in mixture with it (1).

In three comparative tests already recorded the ability of any given serum to neutralize the papilloma virus was observed to vary more or less directly with its capacity to react in the complement fixation test (1). A number of later tests, which will not be given in detail, have provided similar findings. More than fifty immune sera in all have been examined, some procured from rabbits bearing natural and experimental growths of various durations and others from animals carrying transplanted cancers derived from virus-induced papillomas (3). All were tested primarily to find whether antibody was present in them rather than to determine its precise titer, and it was feasible to employ only one or two dilutions of serum in the neutralization tests. For this reason as well as for another to be discussed later on,—namely, that neutralization of the virus is often incomplete under the conditions of the tests,—the results of the neutralization tests are difficult to interpret quantitatively. In every instance, however, in which the neutralizing power of a serum was great, its complement-fixing titer was also great; and whenever a serum had little or no neutralizing capacity it failed to fix complement in the tests or did so only poorly. There were many intermediate instances, though, in which no very strict correlation could be made between the neutralizing and complement-fixing capacities of a serum. Hence it became necessary to find out whether the discrepancies, which were never large, were merely apparent and due to the quantitative limitations of the neutralization tests or whether they were real, that is to say, were consequent upon actual disproportions in the neutralizing and complement-fixing capacities of the sera. The distinction can be made, since quantitative complement fixation tests are readily carried out and since it is possible to determine the neutralization titer of a serum quite accurately by mixing a considerable number of serum dilu-

tions with a carefully standardized dose of virus and rubbing the mixtures into the scarified skin of test rabbits (4). To decide the matter, a large number of immune sera, some procured from rabbits with naturally occurring or experimentally induced papillomas and others from rabbits immunized artificially against the papilloma virus, were compared directly in these ways.

The *neutralization test* has already been described (4). Briefly, it consists in mixing the serum, either as such or in dilutions with 0.9 per cent saline, with a freshly prepared virus filtrate of known potency, made by grinding glycerolated cottontail papillomas in a mortar, suspending the ground material in saline, centrifugalizing in the 51° angle-head centrifuge at 4400 R.P.M. for 20 minutes, and filtering the supernatant through a Berkefeld V candle. The mixtures of serum and virus are incubated for 2 hours at 37°C., then rubbed into small, scarified squares on the abdomens of three normal domestic test rabbits, with precautions to prevent cross-infection. The character of the growths arising is recorded every 2 or 3 days from about the 15th to the 25th day after inoculation, and at intervals of 5 to 7 days thereafter until about the 42nd day, using a standard scale: **** = confluent papillomatosis, *** = semiconfluent papillomatosis, ** = many discrete papillomas, * = a few discrete papillomas, ± = 2, 3, or 4 papillomas, ± = one papilloma, 0 = negative (complete neutralization). (In the tables the asterisks will discriminate the neutralization readings from the results of the complement fixation tests.) To conserve space, the readings of only 1 day or of a few days are given in the tables. They are representative of the findings as a whole.

The *complement fixation test*, also described previously (1), was carried out with 2 units of complement, 2 hours at room temperature being allowed for fixation. In the present work an optimal dose of antigen (a Berkefeld V filtrate of the infectious papillomas prepared as described above) was used in each test to avoid the post-zone effects noted in the preceding work. Readings were recorded as usual in terms of fixation after the tubes had been kept at 37°C. for 30 minutes, and again after standing overnight in the refrigerator: ++++ = 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). Control tests for anticomplementary effect were made in every experiment, using double amounts of each serum and antigen. No such effect was encountered in any of the experiments that follow.

Experiment 1.—Sera were obtained from the following: (*a*) two cottontail rabbits bearing naturally occurring growths (duration unknown); (*b*) two domestic rabbits and one cottontail with experimental growths of 21 to 149 days' duration; (*c*) two rabbits of each species with experimentally induced growths of 37 days' duration, which had received intraperitoneal injections of 5 cc. of a 1:10 Berkefeld V filtrate of the highly infectious glycerolated papillomas of W. R. 1-28, on the 2nd, 8th, and 19th days to heighten their antibody titer; (*d*) two normal rabbits of each species which had received intraperitoneal injections of a suspension of washed papilloma virus prepared as follows: A 1:10 Berkefeld V filtrate of the glycerolated papillomas of W. R. 1-30, known from subsidiary tests to contain virus in high titer, was spun for 90 minutes at 30,000 R.P.M. in 7 cc. lusteroid

tubes in the air-driven centrifuge. The supernatant fluid was removed and discarded, and the small pellet of sediment resuspended in the original volume of dilute phosphate buffer (approximately M/200), pH 7.2. This was spun at 4400 R.P.M. for 20 minutes in the 51° angle-head centrifuge to remove aggregated material, and the supernatant fluid spun again at 30,000 R.P.M. for 90 minutes. The pellet, a small button of transparent, jelly-like material, was again resuspended in dilute phosphate buffer and the suspension spun at 4400 R.P.M. for 20 minutes to remove aggregated particles and colloidal material. The final supernatant fluid was removed for use; it was only slightly opalescent but contained infectious virus in large amount, as subsidiary tests showed. The suspension of washed virus was injected intraperitoneally into two normal domestic rabbits and two normal Kansas cottontails, 6 cc. per kilo of body weight in each. To preclude infection with the virus, a small slit was made in the skin, which was held open while the injecting needle was run forward about an inch in the subcutaneous tissue before puncturing the peritoneum. Afterwards the wound was soaked for a moment with carbolic acid and then with 95 per cent alcohol. The procedure proved effective, for no papillomas appeared in the healed wounds. On the 7th day the injections were repeated, using the same dose of a fresh suspension of washed papilloma virus prepared from the same material precisely as before.

In this experiment the sera were tested in various dilutions for capacity to fix complement and to neutralize a potent 5 per cent virus filtrate. Table I shows the results.[3] It will be seen that the complement-fixing and virus-neutralizing capacities of the sera varied in close parallel, though they were of widely different origins and potentialities. The serum of W. R. 1-52, a rabbit with naturally occurring growths, had the greatest complement-fixing capacity of all, and it neutralized the virus best, few or no growths appearing where the mixtures of virus with this serum had been inoculated. The serum of D. R. 96, a rabbit with experimental growths, fixed complement least well, and it was poorest in virus-neutralizing capacity, exerting only a slight effect upon the virus when mixed with it in a dilution of 1:4, and no discernible effect in dilutions of 1:40 or 1:120. The other sera fell in between in ability to fix complement, and invariably their neutralizing potency paralleled their capacity to fix complement, as a study of the table makes clear. The fact is noteworthy that considerable amounts of antibody were present in the sera of the rabbits injected intraperitoneally with virus washed by centrifugation (W. R. 30 and 28, and D. R. 1-66 and 1-67)—about as much in fact as in the serum of the animals

[3] In scrutinizing the tables it will be well to keep in mind that the plus signs and asterisks have opposite implications as concerns amount of serum antibody. The more the asterisks (as connoting ability of the mixtures to produce growths) the less there was of this antibody, while the more the plus signs the stronger was complement fixation.

carrying papillomas—and these sera, too, had parallel complement-fixing and virus-neutralizing titers.

The table exemplifies certain findings already reported, namely, that the neutralization test is capable of detecting a somewhat smaller amount of antibody than the complement fixation test, though the latter is quite as sensitive when the antibody is present in any considerable quantity. The serum of W. R. 1-31, for example, failed to fix complement at a dilution of 1:32, yet it partially neutralized the virus at a dilution of 1:40, and so too did the serum of W. R. 30, which had also failed to fix complement at a dilution of 1:32; and the sera of D. R. 1-55, 1-66, and 1-67, which failed to fix complement in the test at a dilution of 1:128, partially neutralized the virus at a dilution of 1:120. Within its limits, the complement fixation test accurately reflects small differences in neutralizing capacity,—as, *e.g.*, in the case of the sera of D. R. 4 and W. R. 16, and D. R. 1-66 and 1-67. The end points in the complement fixation tests are very much sharper than those in the neutralization tests,—a finding noted repeatedly in previous comparative tests.

Simultaneous Absorption of the Complement-Fixing and Virus-Neutralizing Antibodies

The findings of Experiment 1, together with those previously got in similar tests, make clear the fact that the virus-neutralizing and complement-fixing capacities of papilloma virus immune sera invariably parallel one another closely, and the fact suggests that the complement-fixing and virus-neutralizing antibodies are identical. Absorption of the antibody, if it could be effected,would provide a further means of testing whether this is true. Hence experiments to this end were undertaken.

The procedure for absorption of antibody (or antigen) was as follows: The immune serum, diluted as seemed fit with 0.9 per cent saline, was mixed with an equal volume of the absorbing material, usually a Berkefeld V filtrate of the infectious papillomas, which will be referred to as *virus filtrate*.[4] The mixtures, along with appropriate saline controls, were put into the water bath at 37°C. for 2 hours, then overnight in the refrigerator, and centrifugalized at 4400 R.P.M. for 20 minutes in the 51° angle-head centrifuge. (This amount of centrifugation, while insufficient to throw down any considerable amount of the virus or its antibody, proved adequate to deposit the antigen-antibody aggregate, as will become evident. The results of tests on the supernatant liquids after centrifugation at this speed were identical with those obtained in two experiments when duplicate

[4] In all of the absorption experiments a sufficient concentration of immune serum was used in each mixture to render the absorbing dose of virus filtrate completely non-infectious, or almost completely so, except in two of the mixtures of Experiment 9.

TABLE I

Comparative Complement Fixation and Neutralization Tests with Various Immune Sera

Source of immune serum	Rabbit No.	Complement fixation tests‡									Neutralization tests§							
		Dilutions of serum									21st day				42nd day			
											Dilutions of serum			Saline control	Dilutions of serum			Saline control
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		1:4	1:40	1:120		1:4	1:40	1:120	
(*a*) Rabbits bearing naturally occurring papillomas	W. R. 1-52	++++	++++	++++	++++	++++	++++	++++	+±	a	0	0	0	****±	0	0	±	****
										b	0	0	0	****	0	±	0	****
										c	0	0	0	****±	±	0	±	****
	W. R. 1-31	++++	++++	+++	0	0	0	0	0	a	*±	***	***±	****±	*±	****	****	****
										b	±	*	**	****	±	***±	***	****
										c	*	**	****	****±	*	***	****	****
(*b*) Rabbits bearing experimentally induced papillomas	W. R. 16	++++	++++	++++	++++	++++	+++±	0	0	a	0	±	±	***±	±	*	*	****
										b	0	0	*	****	0	0	*	****
										c	0	0	**	****±	±	±	**	****
	D. R. 4	++++	++++	++++	++++	+++±	0	0	0	d	0	0	***±	***	±	±	***	****
										e	0	0	*±	****±	±	*	***±	****
										f	0	±	**	****	±	*±	***	****
	D. R. 96	+++±	0	0	0	0	0	0	0	d	±	***±	***±	***	*	****±	****	****
										e	***±	****±	****±	****±	***	****	****	****
										f	*±	****±	****	****	**	****	****	****

(*c*) Rabbits bearing papillomas and injected intraperitoneally with whole virus filtrate	W. R. 27	++++	++++	++++	++++	++++	++++	+++±	0	a	0	0	*	***±	±	0	*	****
										b	0	0	0	****	0	0	0	****
										c	0	0	*	***±	±	±	**±	****
	W. R. 24	++++	++++	++++	++±	0	0	0	0	a	±	**±	***	***±	±	**	***	****
										b	±	*	**±	****	±	**	****	****
										c	±	***	***±	***±	±	***±	****	****
	D. R. 1-53	++++	++++	++++	++++	++++	++++	++±	0	d	0	0	±	***	0	±	±	****
										e	0	0	±	***±	±	±	±	****
										f	0	±	*	****	±	±	**±	****
	D. R. 1-55	++++	++++	++++	++++	++++	±	0	0	d	0	±	*	***	±	±	**	****
										e	0	*	**	***±	±	*	**±	****
										f	±	**±	***	****	±	**	***	****
(*d*) Rabbits injected intraperitoneally with papilloma virus purified by differential centrifugations	W. R. *30*	++++	++++	+++±	0	0	0	0	0	a	±	**	***±	***±	±	***±	****	****
										b	±	**	***±	****	±	***	****	****
										c	±	***±	***±	***±	±	***	****	****
	W. R. 28	++++	++++	++++	±	0	0	0	0	a	±	**	***±	***±	*	***±	****	****
										b	±	**	***±	****	±	***	****	****
										c	±	***	****	***±	*	****	****	****
	D. R. 1-66	++++	++++	++++	++++	+++±	0	0	0	d	0	±	**	***	±	*	**	****
										e	0	*	**	***±	0	*	***	****
										f	0	*	**	****	±	*	***	****
	D. R. 1-67	++++	++++	++++	++++	±	0	0	0	d	0	*	**	***	0	**	***±	****
										e	0	±	**±	***±	±	*	***	****
										f	0	***±	***±	****	*	***	****	****

D. R. = domestic rabbit, W. R. = wild cottontail rabbit.

‡ Antigen, Berkefeld V filtrate of the infectious papillomas of W. R. 1-56, 1:120.

Complement, 2 units in all tubes.

++++ = complete fixation of complement (no hemolysis)—see scale in text.

0 = no fixation (complete hemolysis).

§ Growths resulting from mixture of 5 per cent virus filtrate (W. R. 1-56) and dilutions of serum or saline in equal parts in test rabbits (a, b, c, d, e, f).

**** = confluent papillomatosis.

*** = semiconfluent papillomatosis.

** = many discrete papillomas.

* = few discrete papillomas.

± = 2, 3, or 4 papillomas.

± = one papilloma.

0 = negative (complete neutralization).

mixtures were spun at 20,000 R.P.M. for 1 hour, irrespective of the relative proportions of antigen and antibody.) The supernatant liquids of the control and test mixtures were then removed and tested for residual antibody (or antigen) by means of the standard complement fixation, neutralization, and pathogenicity tests.

Experiment 2.—Immune sera obtained from a cottontail rabbit (W. R. 4) with naturally occurring growths and a domestic rabbit (D. R. 2) with experimentally induced growths of 149 days' duration were diluted 1:4 with saline and absorbed with various dilutions of a Berkefeld V filtrate of the glycerolated infectious papillomas of W. R. 1-56, known from previous tests to yield much virus. The amount of antibody remaining in the supernatant liquids after absorption and centrifugation was then determined by means of the complement fixation and neutralization tests.

Table II shows the results of the absorption experiment. The virus filtrate diluted 1:10 absorbed all of the detectable complement-fixing antibody from the cottontail rabbit serum, and most of it when diluted 1:20, while relatively little or no detectable absorption was brought about by the filtrate diluted 1:40 and 1:80. With the serum of the domestic rabbit, which contained less antibody at the start than the serum of the cottontail, all of the detectable complement-fixing antibody was absorbed by the filtrate at dilutions of 1:10, 1:20, and 1:40, and most of it at a dilution of 1:80. The neutralization tests gave parallel results, those mixtures that had manifested no complement-fixing ability having no neutralizing capacity either.

A number of tests were made in which virus filtrates from different sources were used and immune sera obtained from other domestic and cottontail rabbits carrying naturally occurring and experimentally induced papillomas. Table III shows the findings of one such experiment, which may be regarded as typical. In all of the various mixtures of this experiment the complement-fixing and virus-neutralizing capacities were directly proportional, and the amount of antibody remaining in them after absorption varied inversely as the concentration of the virus filtrate used for absorption.

Relation of Visible Flocculation to Absorption of the Antibody

In the experiment of Table III, a small amount of rather coarse, flaky flocculation was observed after incubation of the mixture of serum and virus filtrate 1:40, which had no detectable amount of residual antibody. Flocculations of this sort have often been noted in mixtures of suspensions containing the papilloma virus and its specific immune sera, though never in control mixtures with saline or normal serum (5). Flocculations are sometimes perceptible after incubation at 37°C. for 2 hours, and fairly regularly after incubation and overnight storage in the refrigerator. Their amount

TABLE II

Complement Fixation and Neutralization Tests with Immune Sera after Absorption with Various Amounts of a Virus Filtrate

Source of immune serum	Dilutions of virus filtrate W. R. 1-56 used for absorption	Antibody remaining in mixtures after absorption, as determined by: Complement fixation tests‡ — Dilutions of serum 1:8	1:12	1:16	1:24	1:32	1:48	1:64	1:96		Neutralization tests§ Virus filtrate W. R. 1-10, 1:100 plus — Dilutions of serum 1:8	1:16	1:32	Saline
Cottontail rabbit with experimental growths (W. R. 4)	1:10	0	0	0	0	0	0	0	0	a	***	****±	****±	****
										b	****±	***	****±	***
										c	**±	***	****±	***
	1:20	++++	±	0	0	0	0	0	0	a	0	±	0	****
										b	0	0	0	***
										c	0	0	0	***
	1:40	++++	++++	++++	++++	++++	+++	++	0	a	0	0	0	****
										b	±	0	0	***
										c	±	±	0	***
	1:80	++++	++++	++++	++++	++++	++++	+++±	±	a	±	0	0	****
										b	0	0	0	***
										c	0	±	0	***
	Unabsorbed, saline control	++++	++++	++++	++++	++++	++++	++++	±	a	0	±	0	***
										b	0	0	0	***
										c	±	±	0	***
Domestic rabbit with experimental growths (D. R. 2)	1:10	0	0	0	0	0	0	0	0	e	****±	****	****	****
										f	***	***	***	***
										g	***±	**±	***	***
	1:20	0	0	0	0	0	0	0	0	e	***	****	****±	****
										f	***	**	***±	***
										g	***	**	***	***
	1:40	0	0	0	0	0	0	0	0	e	****±	****±	****±	****
										f	***±	***	**±	***
										g	**	***	***	***
	1:80	++++	++	0	0	0	0	0	0	e	±	0	±	****
										f	0	0	0	***
										g	0	0	0	***
	Unabsorbed, saline control	++++	++++	++++	++++	+++	0	0	0	e	±	0	0	****
										f	±	0	±	***
										g	0	0	0	***

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

§ Lesions in test rabbits a, b, c, e, f, g on 31st day.

has never been great, in our experience, and often it was only barely perceptible.

We have recorded the relative amount of flocculation in the experiments to follow according to the following scale: ++ = small amount of rather coarse flocculation visible in bottom of tube, with supernatant liquid less opalescent than saline controls; + = finely granular flocculation rising from bottom of tube on shaking, with supernatant liquid about like saline controls; ± = no sediment but perceptible increase in opalescence of liquid. In several experiments a small amount of finely granular material was thrown down upon centrifugation of some of the mixtures, as not in the controls, though no flocculation or increase in opalescence had been perceptible.

TABLE III

Complement Fixation and Neutralization Tests with an Immune Serum after Absorption with Various Amounts of a Papilloma Virus Filtrate

Dilutions of virus filtrate W. R. 1-28 used for absorption of immune serum D. R. F4 (1:8)	Visible flocculation in mixtures	Antibody remaining in mixtures after absorption, as determined by											
		Complement fixation tests‡						Neutralization tests§ Virus filtrate W. R. 1-56, 1:100 plus					
		Dilutions of serum							Dilutions of serum				Saline
		1:16	1:24	1:32	1:48	1:64	1:96		1:16	1:32	1:64	1:96	
1:40	++	0	0	0	0	0	0	a	***±	***±	***±	***±	***±
								b	***	***	***	***	***
								c	***±	***±	***±	***±	***±
1:80	0	++++	+++	0	0	0	0	a	±	±±	**	***±	***±
								b	0	±	±	**	***
								c	±	±	**	***±	***±
1:120	0	++++	++++	++++	+++±	++±	0	a	0	0	±	±±	***±
								b	0	±	±	*	***
								c	±	±	±	±	***±
1:160	0	++++	++++	++++	++++	++++	++	a	0	0	±	±	***±
								b	0	0	0	±	***
								c	0	0	±	0	****
Unabsorbed, saline control	0	++++	++++	++++	++++	++++	++++	a	0	0	0	±	***±
								b	0	0	0	±	***
								c	±	±	±	0	***±

‡ Antigen, W. R. 1-56 virus filtrate, 1:120. Complement, 2 units in all tubes.

§ Lesions in test rabbits a, b, c on 27th day.

As will become evident from the tables to follow, visible flocculations were regularly noted in mixtures containing optimal proportions of virus filtrate and immune serum. In mixtures containing an excess of either virus or antibody, there was less or no flocculation.

Specificity of the Absorption

In the experiments thus far given, absorption of the antibody took place when sera containing it were mixed with extracts of the infectious papil-

lomas. Was the absorption specific, and due to the virus? To learn about this a test was made using extracts of the virus-induced papillomas of cottontail and domestic rabbits which were known from previous tests to yield no infectious virus, and an extract of the Brown-Pearce rabbit tumor as well.

Experiment 4.—The virus-induced papillomas of five cottontail and four domestic rabbits had been preserved in glycerol for 3 to 12 weeks. These were ground as usual and extracted 1:10 in saline, and a similar crude saline extract was made of the healthy portions of the Brown-Pearce tumors, which were growing in the muscles of D. R. 8-33, 16 days after the implantation of the tumor cells. The tumor tissue had been kept frozen at −22°C. for 74 days. All of the domestic rabbit papillomas, and those of three of the cottontails, had yielded no virus or but very little in every one of a number of preceding tests, while the growths of two of the cottontails (W. R. 36 and 11-83) had always provided much of it upon several previous extractions. To provide an index of the antigenicity of the various materials—which in other tests has always been directly proportional to the pathogenicity (1)—portions of the crude suspensions were centrifugalized at 4400 R.P.M. for 20 minutes and the supernatant fluids tested for complement-fixing capacity in mixture with an optimal dose of immune serum (D. R. 4, 1:32), while 1 cc. of each was used as such in absorption tests with an equal volume of immune serum D. R. 4, 1:16.

Table IV shows the results. The crude extracts of the papillomas of cottontails 36 and 11-83, which contained much active virus as proven in concurrent tests, were the only ones that had any ability to fix complement in mixture with the immune serum, and these alone absorbed antibody to any noteworthy degree. The crude suspension of the Brown-Pearce tumor failed completely to absorb the antibody, and so too did the suspensions of papillomas that yielded little or no virus, except that of the growths of W. R. 19, which absorbed a slight amount.

The findings were confirmed by other, similar experiments. Crude saline extracts of the virus-induced papillomas of a number of domestic rabbits, which yielded no infectious virus on repeated trials, have been tested for capacity to absorb the antibody. None of these had any such ability, nor had extracts of normal rabbit skin, liver, or muscle; while extracts containing the papilloma virus extracted from the natural growths of cottontails invariably absorbed antibody in the tests, their capacity to do so varying directly with the amount of virus they contained.

Is the Virus Responsible for Absorption of the Antibody?

It would appear, from the results just given, that the papilloma virus itself may be responsible for the absorption of antibody; for only when it was present in quantity did absorption occur. To obtain further informa-

tion on this point a test was now made to determine the absorbing capacity of the two fractions of a virus filtrate separated by means of high-speed centrifugation, one of which contained practically no virus whereas the other contained much.

Experiment 5.—A 1:40 Berkefeld V filtrate of the glycerolated papillomas of W. R. 1-28 was prepared as usual. Part of it was saved for use as such and the remainder centrifugalized at 30,000 R.P.M. for 90 minutes. The water-clear supernatant was removed and the pellet of sediment resuspended in the original volume of saline. The

TABLE IV

Absorption Tests with Extracts of Infectious and Non-Infectious Papillomas and Extracts of the Brown-Pearce Tumor

Source of materials used in absorption tests with immune serum D. R. 4, 1:16	Rabbit No.	Complement fixation tests								
		Antigenicity of extracts used for absorption*				Antibody remaining in mixtures after absorption‡				
		Dilutions of serum				Dilutions of serum				
		1:10	1:20	1:40	1:80	1:32	1:48	1:64	1:96	1:128
Papillomas yielding much virus	W. R. 36	++++	++++	++++	+	0	0	0	0	0
	11-83	++++	++++	+++±	0	0	0	0	0	0
Papillomas yielding little or no virus	19	0	0	0	0	++++	++++	++++	++	0
	15	0	0	0	0	++++	++++	++++	+++±	±
	37	0	0	0	0	++++	++++	++++	+++±	+
	D. R. 4-58	0	0	0	0	++++	++++	++++	+++±	+
	4-56	0	0	0	0	++++	++++	++++	++++	+
	4-24	0	0	0	0	++++	++++	++++	+++±	+
	4-22	0	0	0	0	++++	++++	++++	++++	+
Brown-Pearce tumor	8-33	0	0	0	0	++++	++++	++++	+++±	+
Unabsorbed, saline control						++++	++++	++++	+++±	+

* Capacity to fix 2 units of complement in mixture with immune serum D. R. 4, 1:32.

‡ Antigen, W. R. 1-68 virus filtrate, 1:120. Complement, 2 units in all tubes.

various fluids were then tested for infectiousness, for capacity to fix complement in mixture with immune serum, and for capacity to absorb antibody. The whole filtrate and resuspended sediment were highly infectious, both giving rise to large confluent papillomas 21 days after inoculation into three test rabbits, and both fixed complement in high titer (1:320) in mixture with an optimal dose of immune serum. The supernatant fluid, on the other hand, contained practically no virus (two of the rabbits inoculated with it remained negative; the third developed a solitary growth) and had no capacity to react in the complement-fixing test. The sulfosalicylic acid test showed that the whole filtrate and the supernatant fluid contained much protein, roughly the same amount of heavy precipitate forming in each upon addition of the reagent, while the resuspended sediment contained very much less, as was indicated by the slight precipitation resulting in the test.

The results of the absorption tests are shown in Table V. The whole filtrate, and the suspension of the pellet that had been sedimented at 30,000 R.P.M., both of which contained much virus, absorbed the antibody completely, a small amount of coarse flocculation forming in both mixtures. The supernatant fluid after the high-speed centrifugation, which contained much protein but practically no virus, absorbed no detectable amount of

TABLE V

Tests with an Immune Serum Absorbed with Fractions of a Virus Filtrate

Fraction of virus filtrate W. R. 1-28 used for absorption of immune serum D. R. F4 (1:8)	Visible flocculation in mixtures	Antibody remaining in mixtures after absorption, as determined by: Complement fixation tests‡ — Dilutions of serum 1:16	1:24	1:32	1:48	1:64	1:96		Neutralization tests§ Virus filtrate W. R. 1-56, 1:100 plus — Dilutions of serum 1:16	1:32	1:64	1:96	Saline
Whole filtrate (1:40)	++	0	0	0	0	0	0	a	****±	****±	****	****	****±
								b	***	***	***	****±	****
								c	****±	****	****±	****±	****
(*a*) Resuspended sediment after centrifugation at 30,000 R.P.M. for 90 min. (1:40)	++	0	0	0	0	0	0	a	**	****±	****	****	****±
								b	****±	***	***	***	****
								c	****±	****±	****±	****	****
(*b*) Supernatant of (*a*) (1:40)	0	++++	++++	++++	++++	++++	++++	a	0	0	0	±	****±
								b	*	0	±	±	****
								c	±	±	*	*	****
Unabsorbed, saline control	0	++++	++++	++++	++++	++++	++++	a	0	0	0	±	****±
								b	0	0	±	*	****
								c	±	0	±	*	****

‡ Antigen, W. R. 1-56 virus filtrate, 1:120. Complement, 2 units in all tubes.

§ Lesions in three test rabbits (a, b, c) on 27th day.

antibody, and no flocculation appeared in the mixture containing it and immune serum.

Only a limited number of sera were used in the absorption tests just described. Some had been obtained from cottontails with natural growths and others from domestic rabbits with experimental papillomas. To broaden the findings, a variety of immune sera were now selected from amongst those utilized in Experiment 1, and absorbed, as in the preceding test, with the two fractions of a virus filtrate obtained by means of high-speed centrifugation, one of which contained much virus and relatively little

or no "soluble protein," and the other very little virus though much "soluble protein."

Experiment 6.—A suspension was made of the highly infectious glycerolated papillomas of W. R. 1-70 in the dilute phosphate buffer, as already described, filtered through a Berkefeld V filter, and the filtrate spun at 30,000 R.P.M. for 90 minutes in the air-driven centrifuge. The supernatant fluid was pipetted off with care so as not to disturb the sedimented pellet, which was then resuspended in the original volume of dilute phosphate buffer. The supernatant fluid failed to fix complement in mixture with an optimal dose of immune serum while the resuspended sediment did so completely in all dilutions up to 1:80 and partially at 1:160, being only slightly inferior in this respect to the whole filtrate. The addition of sulfosalicylic acid reagent to the supernatant fluid resulted in a heavy precipitate, while similar treatment of the resuspended sediment gave no precipitate and only a moderate increase in opalescence. Nitrogen determinations made by Dr. Lee Farr showed that the supernatant fluid contained 61 mg. per 100 cc., while the resuspended sediment had 4.44 mg. per cent.

It will be seen from Table VI, in which the results of the absorption tests are set down, that the resuspended sediment, which contained much virus, absorbed the antibody from every serum; whereas the supernatant fluid, which contained very little virus though much "soluble protein," failed to do so. It will be noted further that there was visible flocculation in all of the mixtures in which antibody absorption took place but not in the others.

Absorption Tests with Virus Suspensions Heated at Various Temperatures

As bearing further on the share of the virus in the absorption of antibody, a test was next made with virus suspensions that had been heated at temperatures known to be close to that which is critical for the virus (1).

Experiment 7.—The virus suspension was prepared as follows: The glycerolated papillomas of W. R. 1-30, which were known from previous tests to yield much virus, were ground in a mortar as usual and suspended 1:10 in dilute phosphate buffer pH 7.2, centrifugalized at 4400 R.P.M. for 20 minutes, and the supernatant fluid passed through a Berkefeld V filter. To obtain a suspension of virus fairly free from "soluble protein," the filtrate was then spun at 30,000 R.P.M. for 90 minutes and the pellet resuspended in buffer after the supernatant fluid had been discarded. The resuspended sediment was spun at 4400 R.P.M. for 20 minutes to throw down aggregated material and the supernatant fluid, which was only slightly opalescent, was removed for use. 6 cc. portions of this were sealed in three ampules and these were submerged for 30 minutes in water baths at 56°, 60°, and 66°C., respectively. The heating caused no visible change in the specimens, which were now tested, along with a control portion that had not been heated, for pathogenicity, capacity to fix complement in mixture with a standard dose of immune serum, and for ability to absorb antibody. One of the test rabbits inoculated in the pathogenicity tests died of diarrhea on the 21st day, and hence the lesions of only two are recorded.

The unheated suspension, which contained much virus and much of the complement-fixing antigen, absorbed all of the antibody detectable in the

TABLE VI

Tests with Immune Sera Obtained from Various Sources Absorbed with Two Fractions of a Virus Filtrate

Source of immune serum	Rabbit No.	Fraction of centrifugalized virus filtrate W. R. 1-70 (1:10) used for absorption*	Visible flocculation	Amount of antibody remaining after absorption Complement fixation tests‡ Dilutions of serum 1:8	1:12	1:16	1:24	1:32	1:48	1:64
(*a*) Rabbit bearing naturally occurring papillomas	W. R. 1-31	Sediment	++	0	0	0	0	0	0	0
		Supernatant	0	++++	++++	+++±	+	0	0	0
		Unabsorbed, saline control	0	++++	++++	+++±	±	0	0	0
(*b*) Rabbits bearing experimentally induced papillomas	W. R. 23	Sediment	+±	0	0	0	0	0	0	0
		Supernatant	0	++++	++++	++++	++++	++++	++++	+++±
		Unabsorbed, saline control	0	++++	++++	++++	++++	++++	++++	+++±
	D. R. 4	Sediment	++			0	0	0	0	0
		Supernatant	0			++++	++++	++++	++++	+++±
		Unabsorbed, saline control	0			++++	++++	++++	++++	+++±
(*c*) Rabbits bearing papillomas and injected intraperitoneally with whole virus filtrate	W. R. 24	Sediment	++	0	0	0	0	0	0	0
		Supernatant	0	++++	++++	++++	+++±	+	0	0
		Unabsorbed, saline control	0	++++	++++	++++	+++±	±	0	0
	D. R. 1-55	Sediment	++			0	0	0	0	0
		Supernatant	0			++++	++++	++++	++++	+++±
		Unabsorbed, saline control	0			++++	++++	++++	++++	+++±
(*d*) Rabbits injected intraperitoneally with papilloma virus purified by differential centrifugations	W. R. 28	Sediment	++	0	0	0	0	0	0	0
		Supernatant	0	++++	++++	++++	+++±	+	0	0
		Unabsorbed, saline control	0	++++	++++	++++	+++±	+	0	0
	D. R. 1-67	Sediment	++			0	0	0	0	0
		Supernatant	0			++++	++++	++++	++++	++±
		Unabsorbed, saline control	0			++++	++++	++++	++++	+++

* The Berkefeld V filtrate had been spun at 30,000 R.P.M. for 90 minutes with removal of the supernatant fraction for use as such, and resuspension of the sedimented pellet in the original volume of saline. The resuspended sediment contained much virus but little protein, while the supernatant fluid contained practically no virus though much "soluble protein,"—see text.

‡ Antigen, W. R. 1-56, 1:120.

Complement, 2 units in all tubes.

test (Table VII); and the suspension heated at 56°, which contained a little less virus and a little less of the complement-fixing antigen, absorbed almost all of it. The preparation heated at 60° had lost most of its infectivity

TABLE VII

Tests with an Immune Serum Absorbed with Heated Virus Suspensions

Suspension of centrifugalized virus W. R. 1-30 (1:10) Heated 30 min.		Pathogenicity tests‡			Complement fixation tests												Visible flocculation in absorption mixtures
					Antigenicity of the suspension§					Amount of antibody remaining after absorption of serum W. R. 1-55 (1:4) with the suspension‖							
		Day			Dilutions of virus suspension					Dilutions of serum							
		17	27	35	1:10	1:20	1:40	1:80	1:160	1:8	1:12	1:16	1:24	1:32	1:48	1:64	
°C.																	
Unheated	a	**	****	****	++++	++++	++++	±	0	0	0	0	0	0	0	0	++
	b	**±	****	****													
56	a	**	****±	****	++++	++++	++++	0	0	+++±	0	0	0	0	0	0	±
	b	*	***	****													
60	a	0	±	±	++++	+++	0	0	0	++++	++++	++++	++++	+++±	0	0	0
	b	0	0	0													
66	a	0	0	0	+±	0	0	0	0	++++	++++	++++	++++	++++	+++	±	0
	b	0	0	0													
Control (immune serum W. R. 1-55 plus saline)										++++	++++	++++	++++	++++	+++	+±	0

‡ Lesions in test rabbits a and b.

§ Capacity to fix 2 units of complement in mixture with immune serum D. R. 4, 1:32.

‖ Antigen, W. R. 1-30 virus filtrate, 1:120.

Complement, 2 units in all tubes.

though retaining some part of its ability to absorb antibody and to fix complement when mixed with it; while the suspension heated at 66° contained no demonstrable active virus and had only slight power to absorb antibody and to fix complement.

It is interesting to note that the infectivity of the virus suspension was destroyed somewhat before the complement-fixing capacity, the findings in this respect being similar to those obtained when suspensions of the virus are treated with alkali or ultraviolet light (1). The use of a virus suspension that had been freed from much extraneous protein by differential centrifugation probably accounts for the fact that the virus was inactivated by heat more readily in the experiment just given than in tests previously reported (1, 6).

Tests with Virus Suspensions Irradiated with Ultraviolet Light

Since it was known that ultraviolet light will destroy the pathogenicity of a papilloma virus filtrate without necessarily reducing markedly its capacity to react with immune serum in the complement fixation test (3), it was of interest to learn whether suspensions of the virus would still absorb antibody after irradiation.

Experiment 8.—26 cc. of a purified virus suspension, prepared by high-speed centrifugation as in the preceding experiment from the glycerolated papillomas of W. R. 1-30, was irradiated in Lavin's apparatus under the standard conditions described by him (7). 6 cc. samples were removed from the quartz flask, which oscillated continuously in an arc 15 cm. from the carbon light, after 10, 30, 60, and 120 minutes. These, and a control portion not irradiated, were tested for pathogenicity, complement-binding antigenicity, and absorbing capacity.

Irradiation for 10 minutes caused a slight reduction in the pathogenicity of the suspension and a very slight reduction in its capacity to fix complement and to absorb antibody, as Table VIII shows. Irradiation for 30 minutes rendered the material non-infectious without destroying much of its ability to absorb antibody or to react with immune serum in the complement fixation test. After 60 minutes' irradiation the suspension still absorbed most of the antibody, though the pathogenicity of the material had been completely abolished and its complement-fixing capacity considerably reduced. Even after 120 minutes the suspension still absorbed some antibody though all of its infectiousness and most of its complement-fixing ability had been abolished.

Quantitative Relations in the Absorption of Antibody

Salaman found it essential to use massive doses of the elementary bodies of vaccinia in order to absorb the specific neutralizing antibody—1500 to

100,000 times more elementary bodies being required to absorb the antibody from a given quantity of immune serum than the same amount of serum would render non-infectious (8). In contrast to these findings, a relatively small amount of papilloma virus will absorb much antibody—an observation that finds illustration in the next experiment, in which constant

TABLE VIII

Tests with an Immune Serum Absorbed with Papilloma Virus Irradiated with Ultraviolet Light

Irradiation of centrifugalized virus W. R. 1-30 with ultraviolet light		Pathogenicity tests‡			Complement fixation tests											
					Antigenicity of the filtrate§				Amount of antibody remaining after absorption of serum W. R. 1-55 (1:4) with the filtrate‖							
		Day			Dilutions of virus filtrate				Dilutions of serum							Visible flocculation in absorption mixtures
		17	27	35	1:10	1:20	1:40	1:80	1:8	1:12	1:16	1:24	1:32	1:48	1:64	
min.																
0	a	**	****	****	++++	++++	++++	±	0	0	0	0	0	0	0	++
	b	**±	****	****												
10	a	**	****±	****	++++	++++	++++	0	+	0	0	0	0	0	0	++
	b	±	**±	****±												
30	a	0	0	0	++++	++++	++++	0	+±	0	0	0	0	0	0	+
	b	0	0	0												
60	a	0	0	0	++++	++++	±	0	+++	0	0	0	0	0	0	±
	b	0	0	0												
120	a	0	0	0	++	0	0	0	++++	++++	++++	±	0	0	0	0
	b	0	0	0												
Control (immune serum W. R. 1-55 plus saline).....									++++	++++	++++	++++	++++	+++	+±	0

‡ Lesions in test rabbits a and b.

§ Capacity to fix 2 units of complement in mixture with immune serum D. R. 4, 1:32.

‖ Antigen, W. R. 1-30 virus filtrate, 1:120.
Complement, 2 units in all tubes.

amounts of an immune serum were absorbed with different amounts of a virus filtrate.

Experiment 9.—A 1:10 Berkefeld V filtrate of the glycerolated papillomas of W. R. 1-28 was made with isotonic saline as usual. In a subsidiary test the filtrate gave rise to lesions in all dilutions up to 1:100,000, and it fixed 2 units of complement completely in dilutions up to 1:320 in mixture with an optimal dose of immune serum. Various amounts of it were tested for capacity to absorb the antibody from a constant amount of immune serum (D. R. 4, diluted 1:24 with saline).

The experiment yielded comprehensive results, as Table IX shows. There was an excess of antibody after absorption in the mixtures containing immune serum and virus filtrate diluted 1:640 and 1:320, as determined by both the complement fixation and neutralization tests, and an excess of complement-fixing antigen and virus in the mixtures where 2 and 4 volumes of virus filtrate 1:10 had been added to the immune serum. The other mixtures were "neutral,"—that is, they contained no great excess of antigen or antibody after absorption. It is manifest that relatively little of the virus was required to absorb detectable amounts of the antibody, for the mixture containing virus filtrate in a dilution of 1:640—an amount of virus too small to fix completely 2 units of complement in mixture with an optimal dose of immune serum—absorbed a considerable amount of antibody, as the table shows. All or practically all of the antibody was absorbed in the mixture containing virus filtrate 1:160, though the same amount of antibody was capable of neutralizing the virus filtrate in all dilutions up to 1:10. Thus, in contrast to the findings of Salaman with vaccine virus, a given amount of antibody can be completely absorbed by even less of the papilloma virus than it is capable of neutralizing.

It will be noted further in Table IX that the supernatant fluids were "neutral" (that is, contained no great excess of antibody or antigen) after absorption with the virus filtrate in all dilutions from 1:10 to 1:160, though enough virus escaped neutralization in all of these mixtures to produce a few papillomas, as the pathogenicity tests showed,—a point that will be referred to again further on. The virus filtrate absorbed antibody in every one of the dilutions employed, and practically all of it in a dilution of 1:160, which contained, as a parallel test showed, at least 600 infectious doses of virus per unit volume; yet the same amount of antibody neutralized the virus almost completely in all dilutions up to 1:10, which contained more than 10,000 infectious doses per unit volume. The ratio of combined antibody:antigen was hence greater in the mixture of serum and virus filtrate 1:160 than in the mixture of serum and virus filtrate 1:10. In other experiments, which will not be given in detail, similarly broad "equivalence zones" were obtained when various dilutions of an immune serum were mixed with a constant amount of a virus filtrate.

Although the mixtures containing serum and virus filtrate in dilutions of 1:10 to 1:160 may be referred to as "neutral" in the sense that none contained any detectable amounts of complement-fixing antigen or antibody after absorption, scrutiny of the results of the neutralization and pathogenicity tests shows that, strictly speaking, none of the mixtures was "neutral." From the neutralization tests of Table IX it will be seen that

TABLE IX

Tests of Mixtures of an Immune Serum with Various Amounts of a Papilloma Virus Filtrate

Dilutions of virus filtrate W. R. 1-28 used in absorption of immune serum D. R. 4 (1:24)	Visible flocculation in mixtures	Antibody remaining in mixtures after absorption, as determined by: Complement fixation tests‡ — Dilutions of serum D. R. 4				Neutralization tests§						Antigen remaining in mixtures after absorption, as determined by: Complement fixation tests‖ — Dilutions of virus filtrate W. R. 1-28						Pathogenicity tests‡‡ Infectiousness of mixtures in test rabbits a, b, c					
						21st day			42nd day									21st day			42nd day		
		1:48	1:64	1:96	1:128	a	b	c	a	b	c	1:20	1:40	1:80	1:160	1:320	1:640	a	b	c	a	b	c
Unabsorbed serum	0	++++	++++	+++±	0	0	0	0	0	±	0												
1:640	0	++++	+±	±	0	0	0	0	0	±	±	0	0	0	0	0	0	0	0	0	0	0	0
1:320	0	+	0	0	0	0	0	0	0	±	±	0	0	0	0	0	0	0	0	0	0	0	0
1:160	±	0	0	0	0	*	**	**	***	***	***±	0	0	0	0	0	0	0	0	0	0	0	0
1:80	+	0	0	0	0	**	***	***	****	****	***±	0	0	0	0	0	0	0	0	0	0	±	±
1:40	+±	0	0	0	0	**	***	***±	****	****	***±	0	0	0	0	0	0	0	0	0	0	±	0
1:20	+±	0	0	0	0	***	***	**	****	***±	***±	0	0	0	0	0	0	0	0	0	±	*	0
1:10	++	0	0	0	0	***	***	**	****±	***±	***±	0	0	0	0	0	0	0	±	0	0	±	±
1:10 (2 volumes)	0											++++	+++±	0	0	0	0	**	***	**	****	****	***±
1:10 (4 volumes)	0											++++	++++	++++	++++	0	0	***±	****	***	****	****	****
Virus-saline controls						***	***±	***±	****	****	****	++++	++++	++++	++++	++++	+	***±	****	****	****	****	****

‡ Antigen, W. R. 1-56 virus filtrate, 1:120.
Complement, 2 units in all tubes.

§ Lesions in test rabbits a, b, c from inoculation of virus filtrate W. R. 1-56 (1:100) plus absorption mixtures.

‖ Immune serum D. R. F4, 1:32.

‡‡ The control mixture contained virus filtrate 1:10 and saline in equal parts.

the mixture containing virus filtrate in dilution 1:160, though containing no antibody detectable by means of the complement fixation tests, had some, though very little, capacity to neutralize the added test virus, lesions resulting from inoculation of the mixture that were slightly smaller than those resulting from the virus-saline control fluid. From the results of the pathogenicity tests it will be seen that one or a few lesions appeared where the supernatant liquids after absorption of the mixtures of immune serum and virus filtrate 1:10 to 1:80 had been rubbed into the test animals. It is plain, therefore, that a little virus, and perhaps a few antibody molecules as well, may remain "unsaturated" throughout the whole broad equivalence zone. It should be noted, however, that no infectious virus was detected in the mixtures containing antibody in considerable excess (*i.e.*, those containing the virus filtrate in dilutions 1:320 and 1:640), and that the number of papillomas produced by inoculation of the "neutral" mixtures was very small in comparison to the number elicited by the control inocula. It is interesting, too, that the amount of virus escaping neutralization, as manifest by the number of papillomas arising after inoculation, was no greater in the mixture containing the virus filtrate 1:10 than in the one containing it at 1:80, and on the average was about the same as in the other "neutral" mixtures. The implications of these findings will be considered in the discussion.

Stability of the Virus-Antibody Union

The findings given thus far demonstrate that a single antibody is elicited in rabbits by the papilloma virus, and that this antibody can be absorbed by the virus in the test tube, a union of the two taking place. The question remains, however, as to the stability of the virus-antibody union. Is it dissociable by dilution or centrifugation, as are many combinations of virus and antibody *in vitro*? A number of experiments have been done to learn about this. It was first necessary to determine the effect of incubation on a mixture of the papilloma virus and its antibody, for others have found that mixtures of various viruses and their neutralizing antibodies fail to manifest the "dilution phenomenon" if they have been incubated for considerable periods beforehand (9).

Table X shows the results of an experiment to compare the results with various mixtures of a virus filtrate and an immune serum, some of which had been incubated 2 hours at 37°C. and then kept in the refrigerator at 4°C. for 40 hours, while identical mixtures made individually for each test rabbit were inoculated immediately, without incubation. (The time elapsing between the mixture of virus and immune serum and the inoculation of the mixture onto scarified skin was not more than 30 seconds.) It

will be seen from the table that neutralization of the virus was immediate, for the mixtures inoculated immediately were completely or almost completely "neutral," giving rise only occasionally to solitary lesions in the test rabbits, as did the incubated mixtures.[5]

TABLE X

Does Incubation Affect Neutralization of the Papilloma Virus?

Mixtures‡ of virus filtrate (1:80) and dilutions of serum	Period of incubation of virus-serum mixtures	Pathogenicity tests								
		17th day			24th day			35th day		
		a	b	c	a	b	c	a	b	c
1:16	None	0	0	0	0	0	0	0	0	0
	2 hrs. at 37°C. and 40 " " 4°"	0	0	0	0	±	0	±	±	0
1:32	None	0	0	0	0	±	0	0	±	0
	2 hrs. at 37°C. and 40 " " 4°"	0	0	0	0	±	0	0	±	0
1:64	None	0	0	0	0	0	0	0	0	±
	2 hrs. at 37°C. and 40 " " 4°"	0	0	0	0	0	0	0	±	±
1:128	None	0	0	0	0	±	0	0	±	0
	2 hrs. at 37°C. and 40 " " 4°"	0	0	0	*	±	±	*	±	±
Virus plus normal serum (controls).....		**	**	**	***±	***±	***±	****	***±	****

‡ Virus filtrate W. R. 1-30 and immune serum W. R. 1-52 mixed in equal parts.

Having determined that the papilloma virus is neutralized immediately by its antibody, it became necessary, in order to test for the "dilution phenomenon" under conditions designed to give it every chance to manifest

[5] The effect of incubation on the capacity of mixtures of the papilloma virus and its antibody to fix complement was tested in several subsidiary experiments. Various dilutions of virus and immune serum were set up in the usual way in mixture with 2 units of complement. Sensitized red cells were then added to identical sets of mixtures after different periods of incubation, ranging from 1 minute at room temperature to 2 hours at room temperature plus overnight in the refrigerator. The results of the tests can be briefly summarized. In none of the mixtures left 1 minute and 5 minutes at room temperature did complete fixation take place. There was complete fixation, however, in fairly high dilutions of the mixtures left 1 hour at room temperature, and in even higher dilutions in the mixtures allowed to stand 2 hours. The complement-fixing capacity of the mixtures kept 2 hours at room temperature and overnight in the ice box was still greater, the titers being two to four times higher than those observed in identical mixtures that had stood only 2 hours at room temperature. It seems likely from the findings that the fixation of complement occurs during a secondary stage in the antigen-antibody reaction, along with flocculation; whereas neutralization, which takes place immediately (Table X), would appear to be consequent upon a primary union of virus and antibody.

itself, to mix together a potent virus filtrate with those dilutions of an immune serum that would only just neutralize the virus completely or would fail to do so by a slight margin, and to dilute some of the mixtures and centrifuge others as rapidly as possible thereafter. An experiment of the sort was thus done.

A 5 per cent Berkefeld V filtrate of the highly infectious glycerolated papillomas of W. R. 1-30 was prepared in the usual way. Immune serum procured from a cottontail rabbit (W. R. 1-28) bearing naturally occurring papillomas was used. (Subsidiary tests showed that the virus filtrate fixed complement in a dilution of 1:160 and the serum in

TABLE XI

Effect of Dilution and Centrifugation on Infectivity of Virus-Immune Serum Mixtures

Mixtures‡ of virus filtrate (1:20) and dilutions of immune serum	Infectivity§ of diluted virus-immune serum mixtures						Infectivity of centrifugalized virus-immune serum mixtures								
	Dilution of mixtures with 0.9 per cent saline						Before centrifugation (controls)			After centrifugation at 20,000 R.P.M. for 60 min.					
	Undiluted (controls)		1:10		1:100					Resuspended sediment			Supernatant liquid		
	a	b	a	b	a	b	d	e	f	d	e	f	d	e	f
1:1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1:4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1:16	±	0	0	0	0	0	±	±	±	0	0	±	0	0	0
1:48	*	±	±	0	0	0	*	*±	*	*	*	±	0	0	0
1:96	***±	***	**	**	±	0	***±	**	***±	***±	**	**	0	±	0
Virus plus normal serum (controls)	****	****	***±	***	*	±	****	****	***±	***±	***	***	0	±	±

a, b, d, e, f = test rabbits.

‡ Virus filtrate W. R. 1-30 and immune serum W. R. 1-28 mixed in equal parts and incubated 5 minutes at room temperature. Portions of the mixtures were then immediately diluted with saline or centrifugalized as indicated before inoculation onto the scarified skin of the test rabbits.

§ Growths on the 42nd day after inoculation, according to the standard scale.

dilution of 1:48 in mixture with optimal amounts of an immune serum and a virus filtrate, respectively.) The virus filtrate was mixed in equal parts with the immune serum in dilutions varying from 1:1 to 1:96, and a control mixture was made of the virus and undiluted serum from a normal cottontail rabbit. The mixtures were allowed to stand 5 minutes at room temperature. A portion of each was then diluted in two tenfold dilutions in 0.9 per cent saline, and another portion spun at 20,000 R.P.M. for 1 hour in the air-driven centrifuge. The supernatant fluids of the latter were carefully removed and the small pellet in the bottom of each tube resuspended in the original volume of saline. The diluted and whole virus-serum mixtures were then tested for infectiousness by rubbing them into scarified skin areas of three normal domestic rabbits, and the resuspended sediments and supernatant fluids and the whole mixtures as well were tested in another group of three normal rabbits. The inoculations were made immediately after dilution and after centrifugation of the mixtures.

Table XI shows the results of the tests. The growths in one of the test rabbits of

the first group retrogressed and hence the results with only five are available. The whole mixtures containing the immune serum undiluted and in the dilution 1:4 were completely non-pathogenic, and the one containing immune serum in the dilution 1:16 was almost so; whereas the virus had been only partially neutralized in the mixtures containing serum diluted 1:48 and 1:96. It will be seen that none of the neutralized mixtures was rendered more pathogenic by dilution or centrifugation; nor, for that matter, were the underneutralized mixtures (those containing the serum in dilutions 1:48 and 1:96), the diluted and centrifugalized specimens of all being no more pathogenic than the controls. Indeed, as the table shows, there was actually a decrease in the pathogenicity of the diluted portions of the mixtures containing immune serum 1:48 and 1:96 as well as in the control mixture containing normal serum,—owing doubtless to the dilution of pathogenic entities. The slight pathogenicity of the supernatant liquids after centrifugation of the control mixture and the mixture containing immune serum 1:96 can be explained by the probability that a little virus had clung to the sides of the centrifuge tubes or had got stirred up from the sedimented button when the supernatant liquids were pipetted off.

In the experiment of Table XI there was no evidence of any dissociation of the virus-antibody union upon dilution or centrifugation of mixtures that had stood for only 5 minutes at room temperature, though the conditions of the test were such as to favor this and to demonstrate it. Other tests along similar lines using other virus-serum mixtures yielded identical results.[6]

COMMENT

The experiments reported here provide further evidence that only one antibody is called forth by the papilloma virus. The complement-fixing and virus-neutralizing potencies of immune sera were found to parallel one another invariably, irrespective of whether the sera were procured from rabbits bearing naturally occurring or experimentally induced papillomas, and this held true also of sera from other rabbits immunized artificially with extracts of the growths or with partially purified papilloma virus. The findings also demonstrate that the antibody can be readily absorbed from an immune serum when this is mixed with an extract containing the papilloma virus, and that the complement-fixing and virus-neutralizing capacities of a serum are proportionately reduced or abolished upon absorption.

[6] Chester (10) was able to recover large proportions of the tobacco mosaic virus present in "neutralized" virus-serum mixtures by digesting the mixtures with pepsin, which destroyed the antibody but left the virus effective. In an extensive series of experiments of similar sort, done in collaboration with Dr. Peyton Rous, we have failed thus far in attempts to recover the papilloma virus from "neutral" virus-serum mixtures by digestion with various enzymes.

A number of the findings make it seem likely that the virus itself, or an integral part of it, is responsible for the absorption of antibody. Heating a virus suspension at temperatures sufficient to abolish the pathogenicity of the virus destroys its capacity to absorb antibody; and so too does irradiation with ultraviolet light, though there is an amount of this latter which will destroy the pathogenicity of a virus suspension without markedly reducing its capacity to absorb antibody or to fix complement in mixture with immune serum. The supernatant liquid procured after high-speed centrifugation of an extract of the papillomas, which contains much "soluble protein" though very little of the virus, has no capacity to absorb the antibody; whereas a suspension of the pellet of sediment, which contains much virus and but little or no "soluble protein," absorbs antibody practically as well as the whole extract. Extracts of normal rabbit tissue and of the Brown-Pearce tumor have no capacity to absorb the antibody, while finally extracts of virus-induced papillomas containing no infectious virus have no capacity to absorb the antibody. This holds true whether they are derived from the growths of cottontail or domestic rabbits. Taken together the findings render it unlikely that a "soluble antigen" plays any part in the absorption.

The observations, which show that the specific neutralizing antibody is readily and irreversibly absorbed *in vitro* by the papilloma virus, parallel those made by Chester with tobacco mosaic and ring spot viruses (10). Both sets of findings contrast sharply with those of the many workers who have found it difficult or impossible to absorb specific neutralizing antibodies with other animal viruses and certain bacteriophages. Salaman, for example, found that the elementary bodies of vaccinia would absorb only 1/1500th to 1/100,000th the amount of antibody required to neutralize them (8). The papilloma virus, on the other hand, will absorb much more antibody than is required to neutralize it (Experiment 9). Similar findings are the rule in the absorption of agglutinins by red blood cells, as is well known, and of antibody by the potato ring spot virus (10). Future work must determine whether the failure of some viruses readily to absorb their specific neutralizing antibodies *in vitro* is due to fundamental peculiarities in their antigenic structure, or to a "protective screening" provided by "soluble antigens," a possibility suggested by Burnet (11), or to some cause as yet unformulated.

Visible flocculations were noted in mixtures containing optimal proportions of the papilloma virus and its specific antiserum but not when an excess of either reagent was present after absorption. The occurrence of visible flocculations in mixtures of a virus and its neutralizing antibody

appears to be unique in virus immunology, except in the case of certain plant viruses and the antibodies produced by injecting them into rabbits (10). The objection might be raised in the case of the papilloma virus-antibody flocculation that the complement-fixing antibody (reacting with an antigen distinct from the virus) is responsible for the flocculation, and that the neutralizing antibody is merely adsorbed secondarily to it. But the objection scarcely seems to stand in view of the evidence already presented of the identity of the complement-fixing and virus-neutralizing antibodies, and in the light of the demonstration that the complement-fixing and virus-neutralizing capacities of a serum are proportionately reduced upon absorption; while additional facts already cited point to the virus itself, or an integral part of it, as the complement-fixing antigen and the agent responsible for the absorption.

The neutralization tests of the present paper confirm a finding repeatedly obtained previously, namely, that a little virus often escapes neutralization except when the antiserum is exceptionally strong, and sometimes even then. Other workers have reported similar findings in neutralization tests with other viruses—Andrewes and Elford, for example, with a bacteriophage (12) and, as already mentioned, Chester with the virus of tobacco mosaic (10). It is noteworthy, too, that the amount of virus remaining infectious may be about the same in "neutral" mixtures containing widely various amounts of the virus (Experiment 9). The findings accentuate the limitations of the neutralization test in quantitative experiments.

To explain the slight pathogenicity of many of the "neutral" mixtures of papilloma virus and its antibody one might suppose that not all of the virus particles are fully coated with antibody in the "neutral" mixture, those only partially coated remaining infectious. A finding in favor of this view has been several times noted, namely, that the infectiousness of a "neutral" mixture is considerably greater if the whole mixture is rubbed into scarified skin than if the supernatant fluid is tested after centrifugation at 4400 R.P.M. for 20 minutes,—a procedure that throws down the virus-antibody aggregate, though not the free virus. This finding gives reason to suppose that the small amount of virus that remains infectious in "neutral" mixtures may have been acted upon at least partially by antibody and perhaps incorporated into virus-antibody complexes that can be thrown down by the centrifugation. Another possible explanation is that a few virus particles, and perhaps a few antibody molecules as well, may remain uncombined throughout most of the whole broad "equivalence zone." The literature on antigen-antibody reactions yields several instances presumably of the sort. Heidelberger and Kendall found, for example, using

the pneumococcus SSS III as antigen, that both the antigen and its antibody might, in some cases, be present in the supernatant fluid at the neutral point (13), and Taylor, Adair, and Adair noted similar results when optimal proportions of serum albumin and its antiserum were mixed together (14), while Chester had results of the same sort in experiments with tobacco mosaic virus and its antiserum (10). It seems probable, as pointed out by Marrack (15), that some of the results cited may have been due to a lack of uniformity of a small proportion of the antigen and antibody molecules, and this explanation may perhaps account also for the findings with the papilloma virus and its antibody.

However this may be, it should be pointed out that the amount of virus escaping neutralization in "neutral" papilloma virus-antibody mixtures is very small in proportion to the total amount present. In the tests summarized in Table IX, for example, a very few discrete papillomas (one to four) resulted from the broadcast inoculation of a mixture (virus filtrate 1:10) that was known to contain at least 10,000 neutralized virus entities per unit volume. It is of interest to note further that the amount of virus remaining infectious was about the same in all four "neutral" mixtures of Table IX. There was no evidence in any of the experiments that a constant percentage of the infectious virus entities survived neutralization, as happens when similar experiments are set up with bacteriophages and some other viruses (11, 12); nor was infectious virus detected in mixtures containing antibody in excess, as was the case in the bacteriophage experiments. The papilloma virus that remains infectious in "neutral" mixtures is not "antibody-resistant" in any very strict sense, for it is quickly neutralized in mixtures containing antibody in excess. From the findings as a whole, it seems most likely that the portion remaining infectious in "neutral" mixtures consists of virus particles, or aggregates of these, which are only partially coated by antibody.

SUMMARY AND CONCLUSIONS

Experiments are described which show that the rabbit papilloma virus elicits an antibody of one type only, this being capable both of neutralizing the virus and of fixing complement in mixture with it. The virus and its antibody have a powerful, specific affinity for one another, each being capable of absorbing the other in great excess when they are brought together in the test tube. The union formed by them *in vitro* cannot be dissociated in any demonstrable degree by dilution or centrifugation.

In many respects the findings differ from those with most viruses previously studied.

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