STUDIES ON VACCINIA HAEMAGGLUTININ

II. SOME IMMUNOLOGICAL PROPERTIES

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(With 3 Figures in the Text)

The antigens of vaccinia virus have been the subject of both intensive and extensive studies by many workers. The early conflicting situation was much clarified by the work of Craigie and his co-workers (Craigie, 1932; Craigie & Wishart, 1934, 1936*a*, *b*; Wishart & Craigie, 1936) who reported the separation of the soluble L-S antigen from the virus and described the relationship between the two. Their results have been amply confirmed and extended by Salaman (1934, 1937, 1938) and Rivers, Parker, Smadel, Hoagland, etc. (Review by Smadel & Hoagland, 1942). The latter authors showed that besides the L-S antigen which appears to coat the surface of the virus, the vaccinia elementary bodies contain at least two other antigens: the nucleoprotein antigen and an antigen of obscure nature responsible for eliciting the production of neutralizing antibodies. The L-S antigen which is chiefly responsible for agglutination, precipitation and complement fixation phenomena with immune serum does not combine with the neutralizing antibody nor is it capable of eliciting the production of neutralizing antibody or any appreciable amount of immunity to infection when inoculated into animals.

The more recent observations of Nagler (1942, 1944), Burnet & Stone (1946) bring into light a new serological reaction for the study of vaccinia immunity—the haemagglutinin inhibition test. The evidences upon which Burnet based his suggestion that vaccinia haemagglutinin (v.h.) probably represents a soluble antigen of vaccinia responsible for the production of anti-haemagglutinin in a vaccinated subject have already been briefly reviewed in a previous paper (p. 42). It is not clear what relationship exists between the v.h. and the other, better known, antigens, nor was there much experimental evidence for the antibody nature of serum anti-haemagglutinin developed after vaccinia infection. It appeared that a more detailed examination of these points might be desirable on both theoretical and practical grounds. Experiments have been performed to investigate: (1) the nature of anti-vaccinia haemagglutinin (hereafter abbreviated as anti-v.h.); (2) the haemagglutinin anti-haemagglutinin reaction; (3) the immunological relationship of the haemagglutinin anti-haemagglutinin system to that of other antigens. The results of such experiments are recorded in this paper.

The preparation of v.h., e.b.s., L-S soluble antigens, and the method of haemagglutinin titration have already been described in the previous paper (p. 42). Salaman's rabbit adapted strain of vaccinia virus was used throughout this investigation. Both convalescent and hyperimmune rabbit sera have been used. All titres are expressed in terms of the initial dilution of the material tested. In all immunological studies, a normal rabbit serum and a standard anti-vaccinia serum were included as controls.

Haemagglutinin inhibition test. The serum under test was diluted from 1/10 upwards in 2-fold dilutions of 0·25 c.c. amounts. To the serum dilutions, an equal amount of 0·5 % susceptible fowl red cells was added. The v.h., also in 0·25 c.c. quantity, was added last and contained four complete (+ +) agglutinating doses. Previous experiments have shown that the agglutination end-point is essentially the same irrespective of whether the total volume of the reaction mixture is 0·5 or 0·75 c.c. provided that the time of reading is fixed at approximately 1 hr. after set up. The test was incubated at room temperature for an hour. The anti-haemagglutinin titre was represented by the dilution which showed either negative (−) or trace agglutination (±). This end-point is clearcut and regularly reproducible results have been obtained. The titre thus obtained is called the 'Standard Titre'. As it will be noted from later studies on the v.h. anti-v.h. reaction the titre obtained depends on the dose of v.h. used and up to a certain point also on the time of incubation after the haemagglutinin and anti-haemagglutinin are mixed. By varying either or both of these factors, more sensitive tests can be devised at will.

Elementary body suspension agglutination test. This test was performed in small tubes of 0·6 × 5·0 cm. size. The e.b.s. had been preserved in approximately 2 % ether and was diluted in m/250 McIlvaine's buffer pH 7·0 to match No. 9 of Brown's opacity tubes. The serum was also diluted in saline buffered to pH 7·0. To 0·1 c.c. of each of a series of

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2-fold dilutions of serum, 0·1 c.c. of E.B.S. was added. The test was incubated in a 50° C. water-bath for 18 hr. The tube showing partial but definite agglutination was taken as the end-point.

Precipitin test. The soluble antigen consists of a Seitz E.K. filtrate of the supernatant fluid obtained from the first angle centrifugation in the preparation of E.B.S. This antigen had not been heated and therefore contained both the L and S components. Filtration through a Seitz E.K. pad usually removed the bulk of V.H. In some cases when an antigen completely free from V.H. was desired, the residual haemagglutinin was removed by adsorption on to susceptible fowl cells. The antigen was standardized against a 1/5 dilution of a standard anti-vaccinia serum. The dilution of antigen which gave maximum precipitation was used in the titration of unknown sera. In this way, the titre of unknown sera can always be compared with reference to each other and to the standard. The test was performed in small tubes of 0·6 × 5·0 cm. size. A series of dilutions of either the serum or the antigen was prepared and 0·1 c.c. of each of the reagents were mixed and incubated in a 50° C. water-bath for 18 hr. The tube showing maximum precipitation was taken as the titre.

Complement fixation test. This test was performed with 0·1 c.c. each of the following reagents: anti-vaccinia serum; soluble antigen, the same preparation used for precipitin test; guinea-pig complement, 2½ complete haemolytic doses; rabbit anti-sheep haemolysin, 5 complete haemolytic doses and 2% sheep red cells. The antigen was standardized against a 1/5 dilution of the standard anti-vaccinia serum. In preliminary 'chess-board' tests with different dilutions of antigen titrated against different dilutions of antibody, it was found that the titre of one reagent increased proportionally as the concentration of the other was increased and no zone phenomenon occurred. It was therefore decided to choose the highest antigen dilution showing approximately 50% haemolysis as the titre. A quantity of antigen eight times this titre was used for the titration of unknown sera. The antigen-antibody-complement system was allowed a period of 18 hr. at 4° C. for fixation. After this, haemolysin and sheep cells were separately added and given a further incubation of ½ hr. at 37° C. The tube showing approximately 50% haemolysis was chosen as the serum titre. All appropriate controls were included.

Neutralization test. Estimation of the content of neutralizing antibody in anti-vaccinia serum was performed according to the technique of Ledingham, Morgan & Petrie (1931). To a series of 10-fold dilutions of virus, a constant amount of serum was added. Crude rabbit-skin virus was used in most experiments because of the tendency of purified E.B.S. to aggregate in saline. The mixtures were incubated at room temperature for 1 hr. 0·2 c.c. amounts were inoculated intradermally into the shaved back of rabbits. Comparative titrations were always performed on the same rabbit. Both the end-point and the size of the reaction were read on the third and again on the fifth day after inoculation.

THE SPECIFICITY OF ANTI-VACCINIA HAEMAGGLUTININ

The possible presence of haemagglutinin-inhibiting substances in normal sera of man and several species of animals has been investigated. In order to detect traces of anti-haemagglutinin, a supersensitive titration procedure employing one complete agglutinating dose of V.H. and 1 hr. incubation at room temperature of the serum-V.H. mixtures was used. With an immune serum, the titre thus obtained is sixteen times the standard titre. Serum dilutions were tested from 1/10 upward. Over ten normal rabbit sera were tested with consistently negative results. Out of five normal adult human sera, only one had a titre of 1/10. These sera were collected for other purposes and had been kept at 4° C. for 15 months. The other animal sera tested included ox, sheep, fowl and horse. Only horse sera showed any appreciable amount of inhibiting activity. The respective titre of three horse sera were 1/10, 1/20 and 1/160. Under the same conditions, the inhibiting titre of a convalescent rabbit serum was 1/2560.

These results indicate that with the exception of one horse, haemagglutinin-inhibiting substances were not found to be present in normal sera to any appreciable titre.

LONG TERMED VARIATIONS OF AGGLUTININ, PRECIPITIN, COMPLEMENT-FIXING ANTIBODY AND ANTI-VACCINIA HAEMAGGLUTININ IN IMMUNE RABBITS

An area of about 3 × 5 cm. on the shaved back of two rabbits was gently scarified with the edge of a piece of wire gauze. 1 c.c. of dilute purified E.B.S. containing no detectable V.H. was rubbed in. The rabbits developed good confluent lesions after 3 days. Blood specimens were drawn before, 3 days after and 2 weeks after, inoculation. The animals were then hyperimmunized by three weekly injections of 1 c.c. of purified E.B.S. containing no detectable V.H. Blood specimens were again collected 2 weeks, 3 months and 5 months after the last injection. The agglutinin, precipitin, complement-fixing antibody and anti-V.H. were titrated. The sera were kept in frozen state at −20° C. after collection and before titration. All comparative tests were performed on the same day using the same batch of antigens. The sera were inactivated at 56° C. for half an hour just before testing. The results of such titrations are shown in Fig. 1.

The curves clearly show that within the limits of technical accuracy, the agglutinin, precipitin and complement-fixing antibody ran parallel to each other, whereas the anti-v.h. titre varied independently and in general remained fairly steady throughout the experiment. In fact, in rabbit 1307, following hyperimmunization, there was a sharp rise in agglutinin, precipitin and complement-fixing antibody with a concurrent drop in anti-v.h. titre. These results indicate that the anti-v.h. titre of any serum is independent of its content of antibodies to L-S antigen. Incidentally, it may be noted that the injection of purified e.b.s. did not serve as an effective antigenic stimulus to the production of more anti-v.h.

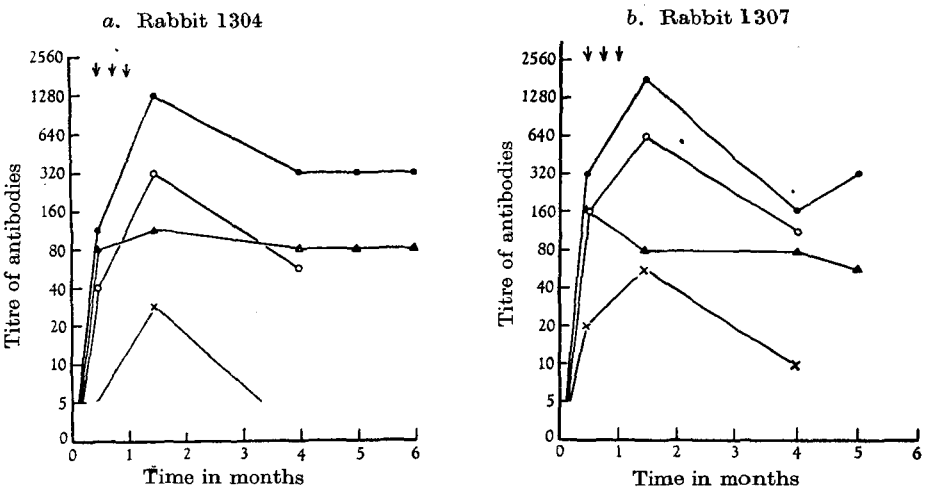

Fig. 1. Graphs showing variations of agglutinin, precipitin, complement-fixing antibody and anti-v.h. in two rabbits during six months.

Arrows = Hyperimmunization by intravenous injections of e.b.s. 1 c.c.
● Agglutinin. × Precipitin. ○ Complement fixing. ▲ Anti-v.h.

THE NATURE OF ANTI-VACCINIA HAEMAGGLUTININ

Experiments have been performed to see whether the anti-v.h. in anti-vaccinia serum possessed the usual properties ascribed to antibodies.

A portion of vaccinia convalescent serum was diluted 1 : 10 in saline and heated to different temperatures. The heated sera were titrated for anti-v.h. activity together with an unheated serum control. The results were: unheated, 1/160; 56° C. 30 min. 1/160; 70° C. 30 min. 1/160; 80° C. 30 min. < 1/10; boiling water-bath 5 min. < 1/10. Sera heated to 80° C. or higher developed marked turbidity and precipitation indicating protein denaturation.

Another portion of the same serum diluted 1 : 5 in saline was fractionally precipitated with ammonium sulphate. A saturated solution of the salt was added first to 50 % saturation, then solid crystals were added to full saturation. The two precipitates obtained and the final supernatant fluid were dialysed against distilled water at 4° C. for 48 hr. Some insoluble precipitate appeared in the 50 % saturation fraction after dialysis. All fractions were titrated for anti-v.h. activity with the following results:

Original serum	1/160
50 % saturation precipitate soluble in distilled water (pseudoglobulin)	1/80
50 % saturation precipitate insoluble in distilled water (euglobulin)	1/10
100 % saturation precipitate (albumin)	< 1/10
Soluble fraction	< 1/10

These experiments show that the anti-v.h. was stable at 70° C. but destroyed at 80° C. The bulk of anti-v.h. activity occurred in the pseudoglobulin fraction, but some was also present in the euglobulin fraction.

THE VACCINIA HAEMAGGLUTININ ANTI-VACCINIA HAEMAGGLUTININ REACTION

Proportional combination under given conditions

In a 'chess-board' titration in which both the v.h. and antiserum were tested in serial 2-fold dilutions, it could be readily demonstrated that under the given experimental conditions, the v.h. and anti-v.h. appeared to combine in simple multiple proportions. The result of such an experiment is given in Fig. 2. These results do not purport to show the kinetics of the reaction and they are entirely compatible with the possibility that under other experimental conditions, v.h. and anti-v.h. can combine in different proportions as do other well-known antigen-antibody mixtures.

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Effect of time of incubation on the anti-vaccinia haemagglutinin titre

When to serial 2-fold dilutions of an anti-vaccinia serum there was added a fixed dose of V.H. (4+ + doses) and the mixtures were incubated at room temperature from 0 to 6 hr. before the addition of the indicator red cells, a change of anti-V.H. titre was obtained. Such results are graphically represented in Fig. 3. It will be seen that within the first hour after mixture, the anti-V.H. titre increased proportionally as the time of incubation was prolonged so that the curve ran as a straight line. The apparent increase in titre then proceeded more slowly up to 3 hr. There was a slight but definite reversing at 6 hr. This may be due to the true dissociation of the

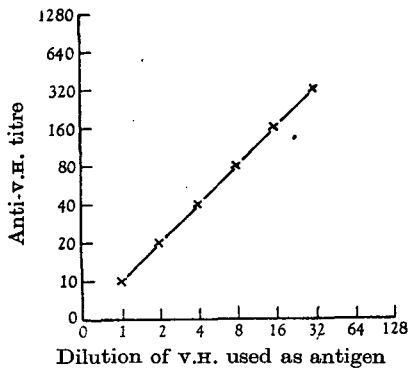

Fig. 2. Graph showing the anti-V.H. titres obtained with different dilutions of V.H. as antigen.

combination or to the slow denaturation of anti-V.H. at high dilutions. The fact that the V.H. anti-V.H. combination is a reversible one appeared to be substantiated by later experiments on the heating of V.H. anti-V.H. mixtures.

Based on these findings, it has been possible to devise methods of differing sensitivity for titrating anti-V.H. For example, with a suspected weak serum, the anti-V.H. titre would be four times higher than the standard titre when one complete agglutinating dose of V.H. was used instead of the routine four doses. Since incubation of V.H. anti-V.H. mixtures for 1 hr. before the addition of red cells further increases the apparent titre of anti-V.H. 4 folds, the combined use of one agglutinating dose of V.H. together with 1 hr. of incubation produces a 16-fold increase in the sensitivity of the test.

Recovery of vaccinia haemagglutinin from neutralized vaccinia haemagglutinin anti-vaccinia haemagglutinin mixture

By exploiting the different heat sensitivity of V.H. and anti-V.H., it has been possible to recover haemagglutinin activity from an artificial inactive mixture of the two. This is illustrated by the following experiment.

To 1 c.c. of V.H. from chorioallantoic membrane, was added 1 c.c. 1 : 10 dilution of anti-vaccinia serum. The mixture was incubated at room temperature for 1 hr. A portion of the mixture was then heated in the boiling water-bath for 5 min. Portions of V.H. preparation and anti-vaccinia serum 1 : 10 were also heated separately as controls. The heated and unheated mixtures and the controls were then titrated for haemagglutinin activity against susceptible fowl red cells. The result of this experiment is shown in Table 1.

The result shows that free V.H. could be recovered from the inactivated mixture after the anti-V.H. was destroyed by heat. The reactivated V.H. can further combine with fresh anti-V.H. This proves that the V.H. anti-V.H. combination is a reversible process.

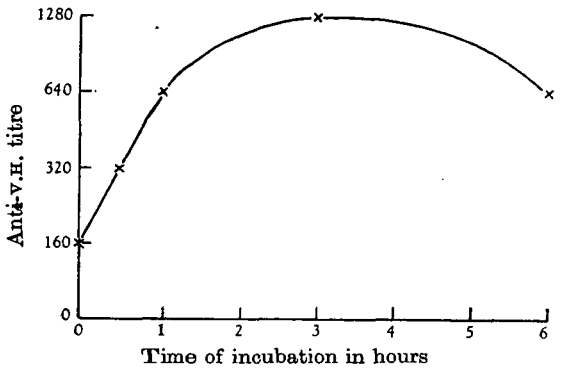

Fig. 3. Graph showing the anti-V.H. titres obtained after different periods of incubation.

Dissociation of vaccinia haemagglutinin from red cells

Red cells agglutinated by V.H. can be rendered completely stable again by anti-vaccinia serum but not by normal serum (Burnet & Stone, 1946). This is the more interesting because the combination of V.H. with susceptible red cells is a very firm one and could not be reversed by variation of temperature, pH and salt concentrations within the limits the cells can stand. That V.H. was actually eluted from the cell surface was shown by the following experiment.

To 4 c.c. of V.H. from chorioallantoic membrane with a titre of 1/256 was added 0·4 c.c. of washed packed cells from a susceptible fowl. After 4 hr. at 37° C., all the haemagglutinin was adsorbed. The agglutinated red cells were deposited and washed twice in saline. No haemagglutinin appeared in the washings. 1 c.c. of anti-vaccinia serum at 1 : 10 dilution was then added to the packed cells which had adsorbed all the V.H. After 1 hr. at room temperature, the cells were deposited and the supernatant serum separated. The original serum had an anti-V.H. titre of 1/640 by a supersensitive titration method which magnified the standard titre sixteen times. After absorption, the serum titre dropped to

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1/40. The serum was then heated in boiling water-bath for 5 min. The heated serum agglutinated red cells to a titre of 1/64. Thus approximately 1/16 of the original V.H. was recovered. Neither normal serum treated in the same way nor the same anti-vaccinia serum treated with normal red cells yielded any haemagglutinin. Cells previously treated with anti-vaccinia serum and washed were agglutinated by V.H. as usual.

The result of this experiment proves that V.H. and anti-V.H. actually combine with each other and that the anti-V.H. appears to possess a greater affinity for its antigen than do the susceptible red cells.

Table 1. *Effect of heating on a vaccinia haemagglutinin anti-vaccinia haemagglutinin mixture*

Materials	Heating	Titre of haemagglutinin in presence of: Saline	Normal rabbit serum 1/10	Anti-vaccinia serum 1/10
V.H., chorioallantoic membrane preparation	Unheated	256	—	—
	Boiling bath 5 min.	64	—	—
V.H. anti-V.H. mixture	Unheated	<4	—	—
	Boiling bath 5 min.	128	128	<4
Anti-vaccinia serum 1/10	Boiling bath 5 min.	<4	—	—

< used here and in subsequent tables denotes negative result at the lowest dilution tested.
— not tested.

Table 2. *Dissociation of haemagglutinin titre of a vaccinia antigen preparation from its precipitinogen and complement-fixing titres*

Material	Haemagglutinin titre	Precipitinogen titre	Complement-fixing antigen titre
Crude rabbit skin virus	256	12	1280
Supernatant after centrifugation	256	12	1280
Seitz filtrate	32	8	1280
Seitz filtrate absorbed with R.B.C.	<1	8	1280
160 mμ filtrate	<1	2	320

RELATIONSHIP OF VACCINIA HAEMAGGLUTININ TO L-S ANTIGEN

A preparation of rabbit skin virus was centrifuged at 5000 r.p.m. on the angle centrifuge to remove the bulk of virus. The supernatant fluid possessing high V.H. activity was subjected to differential filtration through a Seitz E.K. disk and collodion membranes of 160 mμ A.P.D. All preparations, the crude virus, the centrifuged supernatant fluid and the filtrates were tested for precipitin, complement-fixing and haemagglutinin activities. The Seitz filtrate possessing a V.H. titre of 1/32 was absorbed with fowl red cells added to a final concentration of 5%. After 3 hr. at 37° C., all detectable V.H. activity was removed. Both the original and absorbed filtrates were then titrated for precipitin and complement-fixing activities. The result of this experiment is shown in Table 2.

The data indicate that a vaccinia preparation could be freed of its haemagglutinin content either by differential filtration or by red cell absorption without appreciable impairment of its precipitin and complement-fixing activity. The result also suggests that V.H. possesses relatively little if any precipitating or complement-fixing capacity. The bulk of L-S antigen therefore is clearly distinct from V.H. This experiment does not exclude, however, the possibility that V.H. may represent a complex incorporating or associated with a small fraction of L-S antigen. That such association is unlikely was indicated by the absorption test and supplemented by the apparent failure of V.H. to fix complement in presence of its immune antibody.

FAILURE OF VACCINIA HAEMAGGLUTININ TO FIX COMPLEMENT IN PRESENCE OF ANTI-VACCINIA HAEMAGGLUTININ

The complement fixation technique was essentially the same as that used for work with L-S antigen. The anti-vaccinia serum used had a standard anti-V.H. titre of 1/80. A normal rabbit serum was used as a control. Both sera had previously been absorbed with fowl red cells to eliminate any normal antibody against fowl cells. Serum dilutions from 1/10 to 1/320 were tested. As antigen, V.H. coated fowl red cells were prepared as follows:

10 c.c. of chorioallantoic membrane suspension with a V.H. titre of 1/256 and most of the virus removed by centrifugation was absorbed with 1 c.c. of packed washed fowl red cells at 37° C. for 4 hr. All detectable haemagglutinin was adsorbed. The red cells after twice washing in saline were resuspended to 10%. Volume by volume, this antigen should theoretically have the same V.H. content as the

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original crude suspension. Normal red cells from the same fowl were used as a negative control. After serum-antigen-complement mixture, the test was incubated at 37° C. for 1 hr. The fowl cells were then spun down and removed. Sensitized sheep cells were then added to the supernatant fluid and readings were taken after reincubation for ½ hr. All tubes were haemolysed indicating no fixation of complement. A similar test was repeated with a more potent anti-V.H. serum with a standard titre of 1/160 with the same negative result. The V.H. anti-V.H. system under the present experimental conditions therefore failed to fix complement.

SERUM ABSORPTION TEST

In order to define more precisely the relationship between V.H., L-S and antigens which engender the production of neutralizing antibodies, absorption of anti-vaccinia serum with the respective antigen was carried out.

Absorption Exp. I

Antiserum. A preparation of convalescent rabbit serum was used. Pre-vaccination serum from the same rabbit was used as control. The antigens under test were added to 0·25 c.c. of the serum in two instalments. Absorption was carried out at 37° C. for 2 hr. and then 4° C. overnight. The final volume was made up to 5 c.c. representing a serum dilution of 1/20.

Antigens. All antigens used were derived from the same batch of rabbit skin virus:

E.B.S. Washed five times to reduce the V.H. titre to a minimum. The infectivity was titrated on the chorioallantoic membrane of chick embryos and presented as the average of two countable dilutions of two eggs each.

V.H. The supernatant fluid from the preparation of E.B.S. was used. Its infectivity was similarly titrated.

L-S. A Seitz filtrate of the supernatant, absorbed free of V.H. with fowl red cells. It contained no infective virus.

Only the L-S antigen was completely free from other antigens. The other antigens used, both the E.B.S. and V.H. were slightly contaminated by each other and the V.H. also contained a large amount of L-S. It was thus necessary to control the quantitative relations of the materials very carefully. For the sake of clarity, their relative proportions as determined by titration methods and the actual amounts of each added during absorption are presented in Table 3*a* and the result of the test in Table 3*b*. The absorbed sera were tested for agglutinin for E.B.S., for anti-V.H. and for neutralizing antibodies. The agglutination test was chosen as a rough estimation of the L-S antibodies because the absorbed sera were too dilute for precipitin test and often anti-complementary, presumably due to the presence of fine antigen-antibody aggregates. This is justified since it has been demonstrated that agglutination of E.B.S. is mainly due to L-S antibodies (Smadel & Hoagland, 1942).

Absorption Exp. II

This experiment was carried out in the same manner except that:

(1) A different batch of E.B.S. and L-S antigens was used.

(2) The V.H. antigen used for absorption consisted of susceptible fowl red cells coated with V.H. The antigen was derived from chorioallantoic membranes infected with the same strain of virus (Salaman's strain). The egg virus contained relatively more V.H. and less L-S antigen. 40 c.c. of the suspension with the bulk of virus removed by centrifugation was absorbed with 4 c.c. of packed washed red cells. After two washings, the red cells were used for absorption of serum. To serve as a control, red cells from the same fowl treated in a similar way with 20% normal chorioallantoic membrane suspension were used to absorb another portion of the serum. The protocol and result of this experiment are shown in Tables 4*a* and 4*b*.

Great difficulties were experienced when attempting to remove anti-V.H. from the serum by absorption. Excessively large amounts of V.H. preparation were required and even then failed to remove the last trace of anti-V.H. The absorption in Exp. I probably represents more inhibition than genuine absorption. Absorption in Exp. II is more reliable since the absorbed serum had been filtered through 200 mμ Gradocol membrane which should remove any inhibiting V.H. Moreover, no V.H. could be reactivated from the serum by heating.

An analysis of the result of the two experiments shows clearly that the anti-vaccinia serum contained a neutralizing antibody which, besides reducing the size of lesions with low virus dilutions, lowered the actual virus titre by approximately 100-fold. Absorption of anti-serum with E.B.S. removed both the agglutinin and the neutralizing antibody but left anti-V.H. intact. Absorption with L-S removed the agglutinin but neither the neutralizing antibody nor the anti-V.H. was affected. In Exp. I, absorption with V.H. removed all the agglutinin. This was to be expected since the V.H. used for absorption contained a large amount of L-S as well. In Exp. II, when V.H. from chorioallantoic membranes coated on to red cells was used as the absorbing antigen, the resulting serum, while showing an 8-fold reduction of anti-V.H. titre, retained its agglutinin titre intact. These findings, besides confirming the result of previous workers that L-S antigen does not combine with neutralizing antibody, clearly indicate that L-S and

v.h. are distinct and unrelated antigens. The effect of absorption with v.h. on the neutralizing antibody is a little more intriguing. Here in both experiments, the size of lesions with lower virus dilutions (10^{-3}, 10^{-4}, 10^{-5}) was very significantly reduced and approached that of unabsorbed serum, indicating a variably contaminated with some virus which may have absorbed a certain amount of antibody. Secondly, as Keogh (1933) has demonstrated, the neutralization of vaccinia virus conforms to the percentage law of Andrewes & Elford (1933), so that it is not altogether surprising that absorption pro-

Table 3*a*. *Absorption Exp. I: details of absorption of anti-vaccinia serum*

	Antigens for absorption				Relative amounts of antigens added		
Final sera*	Antigen	Infectivity titre/0·1 c.c.	v.h. titre	Precipitinogen titre	Infective particles	v.h.† units	Precipitinogen‡ units
Absorbed with e.b.s.	e.b.s. 2 c.c.	$4{\cdot}8\times10^{9}$	1/4	?	$9{\cdot}6\times10^{10}$	32	?
Absorbed with v.h.	Supernatant 4·75 c.c.	$3{\cdot}0\times10^{5}$	1/128	1/8	$1{\cdot}4\times10^{7}$	2432	380
Absorbed with L-S	Seitz filtrate 0·5 c.c.	—	—	1/8	—	—	40
Unabsorbed control	0	—	—	—	—	—	—
Normal rabbit	0	—	—	—	—	—	—

* Final volume all made up to 5 c.c. at the end of absorption, final serum dilution 1/20.
† v.h. unit = amount of v.h. giving + agglutination with 0·25 c.c. 0·5 % susceptible r.b.c.
‡ Precipitinogen unit = amount of L-S antigen giving maximum precipitation with 0·1 c.c. 1/5 of a standard anti-vaccinia serum.
? Quantity of precipitinogen unknown.
0 No antigen added.
— Negative.
Conditions of absorption: Antigens divided in two equal doses added in two steps. Absorption carried out at 37° C. 2 hr. and 4° C. overnight. Final sera spun clear and filtered through Seitz E.K. previously treated with dilute normal rabbit serum.

Table 3*b*. *Absorption Exp. I, continued: titration of absorbed sera*

			Neutralizing antibody titration Virus dilutions						
Sera	Agglutinin titre	Anti-v.h. titre	Serum control no virus	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Absorbed with e.b.s.	< 20	120	—	17 × 18	14 × 12	10 × 10	7 × 7	6 × 5	—
			—	16 × 16	15 × 15	10 × 12	9 × 9	5 × 5	—
Absorbed with v.h.	< 20	20	—	15 × 15	12 × 10	8 × 8	*p*	*p*	—
			—	12 × 10	10 × 10	9 × 8	*p*	*p*	—
Absorbed with L-S	< 20	120	—	12 × 10	10 × 10	4 × 6	*p*	—	—
			—	10 × 10	7 × 8	5 × 5	—	—	—
Unabsorbed control	160	160	—	8 × 9	8 × 8	*p*	—	—	—
			—	9 × 9	10 × 9	5 × 5	—	—	—
Normal rabbit	< 20	< 20	—	18 × 18	14 × 12	12 × 12	7 × 7	6 × 6	—
			—	17 × 17	13 × 12	10 × 10	8 × 8	*p*	—

Figures under neutralizing antibody titration give actual measurement of diameters of lesions in mm. Each horizontal subcolumn gives the result on one rabbit.
p Just visible papule.
— No visible reaction.

definite neutralizing action. However, higher virus dilutions (10^{-6}, 10^{-7} in Exp. I, 10^{-7}, 10^{-8} in Exp. II), which were completely neutralized by the unabsorbed serum, produced a definite persisting papular reaction when mixed with the absorbed serum. Two factors may contribute to explain this result. First, the v.h. preparation used for absorption was induced a slight lowering of neutralizing antibody content, which manifested itself only with the high virus dilutions. Taking all the evidences together, especially the fact that complete removal of neutralizing antibodies by e.b.s. did not affect anti-v.h. at all, it is probable that v.h. is also distinct and unrelated to the neutralizing antigen of the virus.

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DISCUSSION

The duality of V.H. and infective particles, as reported by Burnet & Stone (1946), has been confirmed. The constant occurrence of specific haemagglutinin in vaccinia materials derived from different sources, however, strongly indicates a close association between the two. Calf lymph was not available in the present series of experiments but the studies of Stone & Burnet (1946) suggest that, even in this case, the apparent inactivity of calf lymph was probably due to the simultaneous presence of an inhibitor (anti-haemagglutinin). In view of the thermo-resistance of V.H. and its reactivation from mixture with immune anti-V.H. by heating, it would be interesting to see whether heating would restore the haemagglutinin activity of calf lymph. Curnen & Horsfall (1946) have demonstrated such a case of 'masked haemagglutinin' with the pneumonia virus of mouse. This virus, as prepared by the usual

Table 4*a*. *Absorption Exp. II: details of absorption of anti-vaccinia serum*

	Antigens for absorption				Relative amounts of antigens added		
Final sera	Antigen	Infectivity titre/0·1 c.c.	V.H. titre	Precipitinogen titre	Infective particles	V.H. units	Precipitinogen units
Absorbed with E.B.S.	E.B.S. 1 c.c.	$7{\cdot}5\times10^{9}$	1/16	?	$7{\cdot}5\times10^{10}$	64	?
Absorbed with* V.H.	Packed R.B.C. 4 c.c. (40 c.c. supernatant)	3×10^{6}	1/2560	—	$1{\cdot}2\times10^{7}$	40,900	—
Absorbed with L-S	Seitz filtrate 1 c.c.	—	—	1/8	—	—	80
Absorbed with† R.B.C.	Packed R.B.C. 4 c.c. (40 c.c. normal CA suspension)	—	—	—	—	—	—
Unabsorbed control	0	—	—	—	—	—	—
Normal rabbit	0	—	—	—	—	—	—

* Figures given in this horizontal column were calculated from the amount lost from the crude V.H. suspension, therefore representing the maximal amount absorbed by the red cells.

† The serum was also absorbed with R.B.C. which had been treated with centrifuged supernatant of normal chorioallantoic suspension as a control to V.H. absorption.

?, 0 and — as in Table 3*a*.

Conditions of absorption: Antigen added in two doses. Absorption carried out at 37° C. 2 hr. and 4° C. overnight. Final sera spun clear and filtered through 200 mμ Gradocol previously treated with broth.

Table 4*b*. *Absorption Exp. II, continued: titration of absorbed sera*

				Neutralizing antibody titration Virus dilutions					
Sera	Agglutinin titre	Anti-V.H. titre	Serum control no virus	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Absorbed with E.B.S.	< 20	160	—	14 × 14	16 × 16	12 × 16	11 × 11	7 × 8	*p*
			—	23 × 23	21 × 22	20 × 18	15 × 15	8 × 8	4 × 4
Absorbed with V.H.	120	20	—	11 × 11	9 × 9	7 × 8	4 × 4	*p*	*p*
			—	16 × 16	16 × 16	14 × 12	5 × 5	*p*	*p*
Absorbed with L-S	20	120	—	9 × 9	6 × 10	4 × 6	*p*	—	—
			—	16 × 16	11 × 10	9 × 9	4 × 4	*p*	—
Absorbed with normal CA, treated R.B.C.	120	120	—	10 × 10	5 × 8	5 × 5	*p*	—	—
			—	18 × 21	15 × 16	11 × 11	8 × 8	—	—
Unabsorbed control	160	160	—	14 × 14	6 × 6	6 × 6	*p*	—	—
			—	18 × 18	15 × 18	9 × 9	7 × 7	*p*	—
Normal rabbit	< 20	< 20	—	12 × 11	14 × 14	10 × 10	6 × 6	4 × 4	*p*
			—	20 × 20	20 × 20	15 × 16	13 × 13	8 × 6	5 × 5

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method of tissue grinding, was present in a bound form and as such was devoid of haemagglutinating and complement-fixing activities both of which were restored after heating to 80° C. In this case, however, the virus itself appears to be the haemagglutinating agent and heating liberates the free form of the virus.

The antigenic nature of V.H. appears to be reasonably supported by experimental evidence. At least two host species, the man and rabbit, have been shown to develop specific anti-V.H. after vaccination. The above studies on the course of development and nature of anti-V.H. and the behaviour of V.H. anti-V.H. combination, appear to justify the conclusion that anti-V.H. is an antibody and that the combination is of the nature of antigen antibody reaction. The apparent failure of V.H. to fix complement in the presence of its specific antibody may be due to the small quantity of antigen used or to some peculiar nature of the antibody, or to the presence of the antigen in a masked form. An instance of such masked antigen has already been referred to in the case of mouse pneumonia virus. Incidentally, it may be mentioned that V.H. does not appear to be an antigen of a heterophile nature, since immune sera possessing high anti-V.H. titre did not show any higher sheep agglutinin or haemolysin than pre-vaccination sera.

The early experimental difficulties in completely clearing purified E.B.S. from haemagglutinin are probably due to ignorance of the size of V.H. particles. In view of the result of later centrifugation studies, it is not surprising to find particles of the order of V.H. contaminating the elementary bodies which are after all selected solely by their behaviour in centrifugal fields. The presence of aggregates of V.H. will naturally further increase the difficulty of complete separation. Repeated fractionation however did succeed in getting rid of the last detectable trace of V.H. The facts that inoculation of such V.H.-free E.B.S. into convalescent rabbits did not enhance their serum anti-V.H. content, and that absorption of an immune serum with E.B.S. did not lower its anti-V.H. titre, suggest that V.H. is not only separable from the elementary bodies but does not form a constituent part of them. The same conclusion may be drawn as to the relationship of V.H. with L-S antigen, both the antigens and their antibodies having been shown to be distinct. It must be noted however that no attempt has been made to study the L and S components separately and the anti L-S immune serum used probably contained more L than S antibodies. Judging from the result of absorption tests alone, the relationship of anti-V.H. to neutralizing antibody appears to be a little more complex. However, taking all the evidences together it is believed that V.H. and neutralizing antibody are also distinct, because absorption with E.B.S. removed all the neutralizing antibodies while leaving anti-V.H. intact. A titration of anti-V.H. content therefore does not measure the neutralizing activity of a serum. Nagler (1944) noted that anti-V.H. developed regularly and abundantly in man following primary vaccination and in those cases of secondary vaccination with a primary type of reaction, while cases with an accelerated or immune reaction showed rather poor anti-V.H. response. In the rabbit we have gained the impression that the production of both V.H. and subsequently anti-V.H. parallels the extent of lesion and of virus multiplication. Nagler's results in man, and the author's observation in rabbit, together with the evidence presented in this paper that rabbits hyperimmunized with E.B.S. failed to develop more anti-V.H., indicate that this antibody probably only develops when there has been active virus multiplication and tissue damage.

Vaccinia haemagglutinin particles are fairly readily extracted from tissues. Gentle grinding of chorioallantoic membranes without abrasives is adequate to liberate most of them, for subsequent vigorous and prolonged grinding of the detritus with pyrex powder fails to yield much more activity. Burnet has suggested the possible lipo-protein nature of V.H., the present evidence regarding size, density, thermo-stability, ready aggregation and adsorption, all seem to be compatible with such a suggestion. The occurrence in vaccinia-infected tissues of non-infective particles of a size and density entirely different from those of elementary bodies, and possessing distinct but specific antigenic properties cannot fail to stimulate speculation. It may be recalled that sedimentable particles mainly consisting of ribonucleoprotein and phospholipids have been isolated from normal tissues (Claude, 1941). Non-infective particulate components possessing distinct immunological properties and capable of conferring protection against homologous tumour transplantation have been reported in Brown Pearce and V2 carcinoma (Kidd, 1946). It is not known how far V.H. may be analogous to these components. There has already been much speculation about a possible life cycle and a non-infectious phase of vaccinia virus. In the absence of any concrete knowledge concerning the mode of virus reproduction, the experiments here reported do not allow anything profitable to be added. Likewise with arguments as to whether V.H. has a tissue or a virus origin. In so far as the virus itself is ultimately derived from the tissues, it does not appear that a sharp line of demarcation can be easily drawn. On the evidence at present available however, the author is rather inclined to believe that V.H. is not a product of virus disintegration but more likely a virus-host interaction product. The presence of such antigenic products in infected hosts has been

suggested in yellow fever (Davis, 1931; Hughes, 1933) and perhaps more concretely in anthrax (Cromartie, Watson, Bloom & Heckley, 1946; Watson, Cromartie, Bloom, Kegeles & Heckley, 1946). In the latter case, the antigen is present in the edema fluid of anthrax lesions and apparently absent from *B. anthracis* itself whether in vegetative or in spore form. This antigen confers effective immunity on rabbits against virulent *B. anthracis*, but the protection appears to have nothing to do with the ordinary protective antibody found in the serum of animals immunized with attenuated strains. The antigen fails to absorb any of the protective antibody. Recently Gladstone (1946) reported a similar substance in cell-free culture filtrate of *B. anthracis* which, however, was formed only in presence of animal plasma and then only found extracellularly. These findings serve to caution us against drawing the conclusion that since V.H. is distinct from the virus, and anti-V.H. from the neutralizing antibody, the antigen in question cannot play any serious role in the pathogenesis and immunity in vaccinia. The final proof regarding this point can only be settled by immunizing animals with purified V.H. separated from the virus and subsequently challenging the immunized animals with active vaccinia virus.

Haemagglutinin of the vaccinia type has so far been found to occur only in a group of three antigenically related viruses, i.e. vaccinia, variola and ectromelia. The haemagglutinin activity of each appears to be at least partially neutralizable by the anti serum of the others. Burnet & Boake (1946) have been able to show that actual antigenic relationship by cross-immunity test exists between vaccinia and ectromelia viruses. It is possible that haemagglutinin of this type may yet be discovered in other virus diseases, particularly of the pox group. Since specific haemagglutinin neutralizable by anti-vaccinia serum has been described in variola, this test coupled with specific inhibition may be of diagnostic aid in doubtful cases of smallpox.

SUMMARY

1. Vaccinated rabbits develop serum anti-haemagglutinin after infection. The anti-haemagglutinin is not found to any appreciable titre in normal human and animal sera tested. It is present in the globulin fraction of rabbit serum and is stable at 70° C. for 30 min. but destroyed at 80° C. for 30 min.

2. Observations over a period of 6 months of variations in serum antibodies in vaccinated rabbits demonstrate that whereas the agglutinin, precipitin and complement-fixing antibody varied in parallel with each other, the anti-haemagglutinin varied independently and in general remained more or less steady throughout the period.

3. Some experimental observations of the haemagglutinin anti-haemagglutinin reaction are reported. The haemagglutinin inactivated by immune serum can be reactivated by boiling. Haemagglutinin adsorbed on to red cells can be eluted by subsequent addition of immune serum.

4. Failure to obtain complement fixation with the haemagglutinin anti-haemagglutinin system is recorded.

5. Vaccinia haemagglutinin is distinct from the L-S antigen.

6. By absorption test, it has been shown that the anti-haemagglutinin is a distinct antibody related neither to L or S antibodies nor to neutralizing antibody.

7. It is concluded that vaccinia haemagglutinin represents a new and distinct antigen occurring in a relatively large particulate state in vaccinia infected tissues. The possible theoretical implications of such findings are discussed.

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