THE LS-ANTIGEN OF VACCINIA

I. Inhibition of L- and S-Antibodies by Substances in Treated Vaccine Dermal Filtrate*

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(Received for publication, October 22, 1941)

A relationship between the heat-labile (L) and heat-stable (S) soluble antigens of vaccinia has been shown to exist by several workers. Craigie and Wishart (1) originally regarded the two antigens as occurring in the form of a complex which elicited two separate antibodies in animals. They were led to this conclusion because both antigens regularly are found in equal amounts in filtrates of infected tissue and because absorption with either specific antibody removes both serologically active substances. Parker (2) observed that under certain conditions different results were obtained, *e.g.*, a solution containing both precipitable substances when treated with S-antibody lost its S-antigen but retained some L-antigen. Craigie and Wishart (3), at about this time, made similar observations; they reaffirmed that L-antigen ordinarily occurs in a state of combination with S-antigen but reported that with prolonged storage in the cold "this combination may dissociate into separate L and S fractions prior to ultimate inactivation of the L antigen." The data of Parker and of Craigie and Wishart that suggested dissociation of L- and S-antigens have not been duplicated in our laboratory. This failure we believe indicates that conditions for successful repetition of such experiments occur infrequently. That they do occur occasionally we have no doubt, for, as will be shown in a subsequent paper (4), a solution with L-activity only can be prepared from pure LS-antigen by enzymatic digestion.

In our experience, L-antigen has always been encountered in association with S-antigen except under the special circumstances just mentioned. This was true even though at various times during the past five years we have thought of the L-reacting substance as a protein, a carbohydrate, a fat, or a polypeptide and have attempted to isolate such an hypothetical material from dermal filtrates by appropriate methods. When it became evident that the methods employed were inadequate to procure preparations of L-antigen which were free of S-antigen for comparison with the relatively pure S-protein of Parker

* Presented in New Haven before the Society of American Bacteriologists, December 29, 1939.

and Rivers (5), other means were sought for the study of the relationship of these two antigens of vaccinia.

The inhibition technique used in serological work assisted in establishing the existence of partially degraded forms of the A and B antigens of infectious myxomatosis of rabbits (6). These two protein-like antigens readily lose their specific precipitability on gentle heating or even on storage. Solutions of A-antigen, which are no longer capable of precipitating with A-antibody, still combine with it as demonstrated by the inhibition technique. Solutions of degraded B-antigen behave similarly. Inhibition by the non-precipitable A- and B-antigens is specific, *i.e.*, each antibody combines only with the degraded form of its own antigen. In view of the results obtained with myxoma antigens, inhibition of the L- and S-antibodies of vaccinia was investigated with the hope of demonstrating serologically degraded forms of the L- and S-antigens. It was thought that experimentation with such degraded substances might provide data which would help in interpreting the interrelationship of the two native antigens. The results of these investigations are reported in this paper.

Materials and Methods

Source of Soluble Antigens.—Dermal filtrate containing the soluble antigens of vaccinia was obtained from dermal pulp of rabbits which had been cutaneously infected 3 days previously with the C.L. strain of vaccine virus (1). The pulp from each rabbit was suspended in 30 to 40 cc. of a 1:50 dilution of disodium phosphate-citric acid buffer solution, pH 7.2. The suspension was freed of gross particles and of essentially all of the elementary bodies of vaccinia by differential centrifugation, and, finally, filtered through a Seitz pad to remove all residual virus. These filtrates were stored at 3°C. until used.

Antisera.—L-antiserum was prepared by absorbing S-antibody from hyperimmune antivaccinal serum. Vaccine virus immune rabbits which had been given a course of intravenous injections of active elementary bodies of vaccinia provided the hyperimmune serum; absorption of S-antibody was carried out with proper amounts of heated dermal filtrate. S-antisera were obtained from non-immune rabbits after several courses of injections of heat-inactivated elementary bodies.

Precipitin Tests.—0.25 cc. volumes of serial dilutions of solution of antigen were prepared in physiological saline solution buffered at pH 7.2 and mixed with 0.25 cc. amounts of an appropriate dilution of antiserum. Readings were made after incubation overnight in closed tubes held at 50°C.

EXPERIMENTAL

General Properties of L-Antigen.—Only a few procedures have been found practicable for the concentration of L-antigen from filtrates of dermal pulp obtained from rabbits infected with the virus of vaccinia. These methods are equally efficacious for the concentration of S-antigen. Craigie and Wishart (1) have observed that both serologically active substances can be obtained

from virus-free dermal filtrate by bringing the pH of the solution to 4.5 and then redissolving the insoluble material thus obtained at pH 6.6. In our hands this method has proved the simplest and the most satisfactory for obtaining solutions containing large amounts of L- and S-antigens from which have been eliminated appreciable amounts of serologically inert material. Moderate variations in the pH ranges employed for precipitating and for redissolving the antigens still result in final solutions with equal amounts of the two serologically active substances; in some instances, however, the recovery of antigenic material is less complete than it is with Craigie and Wishart's technique. Bringing the pH to below 4.0 results in a loss of precipitability of L with its antibody, but S-activity is not appreciably reduced even at pH 1.8. On the alkaline side of neutrality both serological substances retain their activity for many days in the cold at pH 9.0. Furthermore, the L-antigen is unaffected by short exposure to borate buffer of pH 11.0, while the S-antigen is inactivated in N/20 NaOH only after minutes or hours depending on whether the procedure is carried out at 56°C. or at 3°C. Treatment with N/20 NaOH rapidly destroys the precipitability of L-antigen.

Under the proper conditions, precipitation of concentrated unheated dermal filtrate with ammonium sulfate yields equal quantities of L- and S-antigen in the globulin fraction. L-activity is generally lost during fractional precipitation with ammonium sulfate unless filtrates which have been concentrated 10 to 20 times by evaporation are used as starting material; preliminary concentrations of dermal filtrate is unnecessary, however, if the objective is purification of S-antigen. Evaporation of dermal filtrate through cellophane sausage casings suspended in an air stream at room temperature provides a ready means of concentrating the soluble antigens, but fails to eliminate any significant amount of serologically inert substances. On the other hand, drying large volumes of dermal filtrate from the frozen state renders some of the inert material insoluble on subsequent resuspension. Although laborious, this procedure can be employed for concentration of the S-antigen, but it is often unsatisfactory for work with L-antigen since temporary thawing during the process may be followed by partial or complete loss of L-precipitability.

The boiling of solutions containing antigens of vaccinia at pH values near neutrality with subsequent removal of coagulated protein has been found by several workers (5, 7, 8) to be of assistance in the partial purification of solutions of the heat-stable antigen, but obviously this procedure has no place in experiments designed for the purification of the heat-labile material. Similarly, treatment of dermal filtrate with large volumes of cold alcohol results in complete disappearance of the substance capable of precipitating with L-antibody even though the stable antigen can be recovered from the alcohol insoluble fraction as has been demonstrated by others (5, 8).

The L-antigen of vaccinia may be regarded, therefore, as having certain

properties in common with S-antigen. Both are insoluble in the neighborhood of pH 4.5 and soluble in the region of pH 6.5, and both are found in the globulin fraction when separated from concentrated dermal filtrate by partial saturation with ammonium sulfate. The heat-labile antigen, however, readily loses its specific precipitability under conditions which do not alter the heat-stable antigen, *viz.*, extremes of pH, treatment with alcohol, and, in certain instances, drying from the frozen state or precipitation with ammonium sulfate. The common solubilities of the two antigens give no clue to their nature, since the data might equally well suggest that the antigens are parts of a single protein molecule, or that they are similar protein substances, or that L is a non-protein material associated with the S-protein molecule.

Inhibition of L-Antibody

Solutions containing both L- and S-precipitable substances in high concentration showed no change in physical appearance when heated at 56°C. for 1 hour. This led us to think that the L-antigen, which was no longer demonstrable by the usual precipitation technique, might still be present in these heated solutions in an altered form which was unable to precipitate with L-antibody. It seemed possible, furthermore, that such an altered antigen might inhibit L-antibody, and, indeed, this was found to be the case as shown by the following experiment.

Experiment 1.—1500 cc. of dermal filtrate were placed in cellophane tubes and concentrated to 100 cc. by evaporation. The material was dialyzed against running water overnight, and while still in the original cellophane tube was again concentrated by evaporation to 50 cc. This solution reacted in a dilution of 1:1024 with optimal amounts of both L- and S-antibodies. Globulin material was obtained from the concentrate by fractional precipitation with ammonium sulfate. 50 cc. of a solution of the reprecipitated globulin material were treated with 5 cc. of standard citric acid-disodium phosphate buffer, pH 4.5. Most of the material insoluble under these conditions redissolved when taken up in 20 cc. of diluted buffer solution, pH 6.6. This solution, containing globulin material insoluble at pH 4.5 and soluble at pH 6.6, had as much L- and S-activity as did the original concentrated dermal filtrate. After heating at 56°C. for 1 hour the solution no longer precipitated with L-antiserum but its titer with S-antiserum was not reduced. One cc. of the heated solution was added to an equal amount of undiluted L-antiserum; the mixture was diluted with 4 cc. of saline solution, incubated at 56°C. for 1 hour, and stored at 3°C. for 24 hours. No precipitate appeared. This treated mixture, which should have contained sufficient L-antibody to react strongly with unheated L-antigen, failed to form a precipitate when added to serial dilutions of unheated dermal filtrate and incubated under the usual conditions. The inhibition experiment was repeated with identical results when portions of the same solution of antigen which had been heated at 60°C., 80°C., and 90°C. for 1 hour were used.

In the experiment just described L-antigen in the concentrated globulin material from dermal filtrate was affected in the usual manner by heating, *i.e.*, it no longer precipitated with L-antibody. A substance was present, however, in the heated solutions which was capable of inhibiting in some way the flocculation of L-antibody with unheated L-antigen. Since the partially purified material employed in the experiment was highly concentrated, it seemed desirable to repeat the inhibition procedure with a more dilute solution. For this purpose crude dermal filtrate prepared according to routine was used.

Experiment 2.—Dermal filtrate, pool 6, which precipitated in a dilution of 1:128 in the presence of optimal amounts of either L- or S-antibody was heated in a closed flask in a water bath at 80°C. for 1 hour. The heated filtrate which no longer precipitated with L-serum was added in varying amounts to 0.5 cc. portions of undiluted L-antiserum. Sufficient saline solution was added to each mixture to bring the final dilution of serum to 1:8 and the mixtures were incubated at 56°C. for 1 hour. Samples of the treated serum, together with a control serum diluted only with saline, were tested for their capacity to precipitate with serial dilutions of unheated dermal filtrate, pool 6. The results, summarized in Table I, show that the addition of an equal volume of heated filtrate to L-antiserum appreciably decreased the power of the serum to react with unheated filtrate. Furthermore, no precipitation occurred when the serum mixtures containing 3 and 5 volumes, respectively, of heat-inactivated filtrate were tested against solutions known to possess active L-antigen.

TABLE I

Inhibition of L-Antibody by Heated Dermal Filtrate

Antigen	Treatment of L-antiserum 6814*	Dilution of antigen					
		1:8	1:16	1:32	1:64	1:128	1:256
Dermal filtrate 6 (untreated)	Control	+++	++++	++++	+++	+	−
	1 volume heated vaccinia dermal filtrate 6	++	++++	+++	±	−	−
	3 volumes heated vaccinia dermal filtrate 6	−	−	±	−	−	−
	5 volumes heated vaccinia dermal filtrate 6	−	−	−	−	−	−
	5 volumes heated myxoma dermal filtrate	++++	++++	++++	+++	±	−

* Dilution of antiserum was 1:8 in each test.

The data obtained in Experiments 1 and 2 indicate that crude or partially purified solutions containing either small or large amounts of L- and S-substances lose their capacity to precipitate with L-antibody after heating, but are, nevertheless, still able to inhibit L-antibody.

Inhibition of anti-soluble substance antibodies of vaccinia has been ob-

served by others. Craigie and Wishart (1) found that certain heated crude dermal filtrates employed for absorption studies with antivaccinal serum contained inhibiting substances which were carried along with the absorbed serum and subsequently interfered with the demonstration of residual antibodies. These authors regarded the inhibitory substances as non-specific in character and found that fractionation of the filtrates resulted in their elimination. Salaman (9) likewise observed reductions in precipitin and agglutinin titers following absorption of antiserum with large amounts of dermal filtrate; he regarded these effects as examples of the familiar inhibition which occurs in the presence of an excess of antigen and thought that they should not be called non-specific. Since the L-antigen had been rendered non-precipitable by heating, the inhibition of L-antibody observed in Experiments 1 and 2 was certainly not dependent upon the presence of an excess of precipitable L-antigen. Moreover, that the inhibition was serologically specific was demonstrated in the following manner.

Experiment 3.—Dermal filtrate was prepared from rabbits cutaneously infected with the virus of myxomatosis by a technique essentially identical with that employed for rabbits infected with vaccinia. The filtrate which was rich in the A and B soluble antigens of myxoma was heated at 56°C. for 1 hour and then mixed with antivaccinal serum. The mixtures were tested for L-precipitins of vaccinia in the usual manner. As indicated by the results summarized in Table I, no inhibition of L-antibody occurred even when 5 volumes of the myxomatous filtrate were added to the L-antiserum. It has already been recorded that vaccine dermal filtrate fails to inhibit the anti-soluble substance antibodies of myxomatosis (6).

Heated dermal filtrate appeared to be capable of specifically inhibiting L-antibody of vaccinia. It will be recalled that L-antisera are regularly prepared by absorbing hyperimmune antivaccinal serum with heated dermal filtrate; this absorption procedure when carried out under the proper conditions removes S-antibody and leaves L-antibody. This apparent contradiction was shown to depend on a quantitative factor; a much smaller amount of heated filtrate was generally needed to absorb S-antibody from immune serum than was necessary to obtain demonstrable inhibition of L-antibody.

Experiment 4.—Unabsorbed hyperimmune serum, number 6814, reacted in a dilution of 1:32 with an optimal amount of unheated filtrate, pool 6, and in a dilution of 1:4 with heated filtrate. Complete removal of S-antibody without a detectable reduction in the quantity of L-antibody resulted when the hyperimmune serum was absorbed with an equal volume of a 1:8 dilution of dermal filtrate, pool 6, which had been heated at 80°C. for 1 hour. L-precipitins, however, were no longer demonstrable in this antiserum after it was treated with an equal volume of four-times concentrated solution of heated filtrate, pool 6. The filtrate had been concentrated by evaporation in a cellophane bag. Results of this experiment are summarized in Table II.

The material having the property of inhibiting L-antibody in the experiments so far presented always occurred in association with S-antigen. It seemed desirable, therefore, to prepare purified S-antigen by the method of Parker and Rivers (5) and to test the power of this substance to block or inhibit L-antibody.

Experiment 5.—625 cc. of dermal filtrate, pool 7, having a titer of 1:128 with optimal dilutions of L- and S-antisera were boiled and subsequently the globulin fraction was salted out with ammonium sulfate. The redissolved globulin fraction was precipitated with alcohol, redissolved in water, precipitated at pH 4.6 with citric acid-disodium phosphate buffer solution, and, finally, redissolved in dilute citric acid-disodium phosphate buffer solution, pH 8.0. This solution was boiled, the pH was then changed to

TABLE II

Preparation of L-Serum by Absorption of Immune Serum with Heated Dermal Filtrate and Inhibition of L-Antibody with the Same Material

Hyperimmune serum 6814	Antigen: Dermal filtrate 6 diluted 1:16	Dilution of serum						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
Untreated	Unheated	++++	++++	++++	++++	++++⁻	?	—
	Heated	++++	++	—	—	—	—	—
Absorbed*	Unheated	++++	++++	++++	++++	++++⁻	?	—
	Heated	—	—	—	—	—	—	—
Inhibited‡	Unheated		—	—	—	—	—	—

* Absorbed serum was prepared by treating 1.5 cc. of serum 6814 with 1.5 cc. of a 1:8 dilution of heated dermal filtrate 6.

‡ Inhibited serum was prepared by treating 6814 with an equal volume of 4 × concentrated solution of heated dermal filtrate 6.

6.0 by the addition of sodium hydroxide, the solution was again boiled, and then dialyzed free of salts. 0.25 cc. of a 1:1024 dilution of this final solution gave a precipitate with optimal amounts of S-antibody. A portion of the 41 cc. of final solution was dried from the frozen state and the residue was weighed; the solution before drying was shown to have contained 5.1 mg. of dry material per cc. Hence, on the basis of precipitin titer and dry weight, it was estimated that 1 part in 800,000 was sufficient to give a specific reaction with S-antiserum. The relative purity of the final preparation appeared to be of the same order as that obtained by Parker and Rivers. Portions of the final preparation of S-antigen as well as samples of different fractions obtained during the process of purification were tested in the usual way for their inhibitory effect on 0.5 cc. volumes of L-antiserum, number 6814. The results are summarized in Table III.

The results summarized in Table III show that the substance in crude vaccine dermal filtrate capable of inhibiting L-antibody was also present in materials

obtained at intermediate stages and in the final stage of the purification of S-antigen. These data indicate that L-inhibitor, like the precipitable form of L-antigen, is closely associated with S-antigen.

Inhibition of S-Antibody

Earlier observations (10) indicated that S-antigen which has been heated with dilute alkali is not precipitated by its antibody. The capacity of such a degraded antigen to combine with S-antibody was investigated by means of the inhibition technique. The following protocol illustrates the results obtained in a typical experiment.

Experiment 6.—40 mg. of the final dried preparation of S-antigen from Experiment 5 were dissolved in 8 cc. of N/20 NaOH. The solution remained clear after heating

TABLE III

Inhibition of L-Antibody by a Preparation of S-Antigen

Antigen	Fraction prepared from heated dermal filtrate	L-antiserum treated with fraction	Dilution of untreated dermal filtrate				
			1:8	1:16	1:32	1:64	1:128
Dermal filtrate		None	+++	++++	++++	+++	+
	Globulin fraction	1 volume	−	−	−	−	−
	Alcohol insoluble fraction of globulin	1 "	−	−	−	−	−
	Final solution of S-antigen	1 "	++	++	+	−	−
	" " "	2 volumes	−	±	−	−	−

Dilution of L-antiserum was 1:6 in all tests. Inhibited sera were obtained by adding 1 or 2 volumes of test material to 0.5 cc. amounts of L-antiserum. S-antigen titer of each test material was 1:1024. Final solution of S contained 5 mg. per cc.

at 56°C. for 90 minutes. A faint opalescence which appeared when the treated solution was brought to pH 7.2 with N/1 HCl was eliminated by ultracentrifugation at 30,000 R.P.M.; the small amount of sediment thus obtained was discarded. Serial dilutions of the clear supernatant fluid which contained practically all of the degraded S-antigen did not form precipitates when incubated with optimal amounts of S-antibody. Varying quantities of the undiluted solution of treated antigen were mixed with 0.4 cc. volumes of S-antiserum, number 274, and with 0.3 cc. volumes of L-antiserum, number 6814; sufficient saline solution was added to each mixture to bring the final concentrations of antisera to their optimal dilutions. The mixtures, all of which remained clear after incubation at 56°C. for 1 hour, were tested for demonstrable precipitins against L- and S-antigens by the usual methods. The results are summarized in Table IV.

The data presented in Table IV indicate that S-antigen of vaccinia which has been degraded by heat and alkali to a stage where it no longer precipitates with

its antibody is still able to combine with this antibody. For example, 0.5 mg. of treated antigen completely inhibited the antibody in 0.4 cc. of undiluted S-antiserum. It is also evident that this preparation of degraded antigen had only a slight capacity to inhibit L-antibody. That the property of the degraded S-antigen to inhibit L-antibody was indeed less than that of the purified precipitable S-antigen from which it was derived may be seen by comparing the data given in the protocols of Experiments 5 and 6; for instance, 2 to 3 times as much of the alkali-treated material was needed to produce the slight inhibitory effect on L-antibody observed in Experiment 5 where undegraded S-antigen was used.

TABLE IV

Inhibition of S-Antibody

Antigen	Antiserum	Treatment of antiserum with degraded S	Dilution of antigen				
			1:8	1:16	1:32	1:64	1:128
		mg.					
		None	+++⁻	++++	++++	++++	++
		0.125	+++	++++	++++	++++	++
	S	0.25	?	+++	++++	+	−
	(diluted 1:4)	0.50	−	−	−	−	−
Dermal filtrate		0.75	−	−	−	−	−
		1.00	−	−	−	−	−
		None	+++	++++	++++	++++	++
	L	1.00	++++	++++	++++	+++	+
	(diluted 1:6)	7.50	+++	++	++	−	−

0.4 cc. volumes of S-antiserum and 0.3 cc. volumes of L-antiserum were treated with the designated amount of S-antigen which had been heated at 56°C. for 90 minutes in the presence of N/20 NaOH.

Inhibition of S-antibody can also be demonstrated by adding the antibody directly to mixtures of native and degraded S-antigen. In this type of experiment, however, the latter material must be present in large amounts in comparison to the former. This is illustrated by the data presented in Table V which summarizes the results obtained when alkali and heat-treated S-antigen from Experiment 6 was added in increasing amounts to 0.4 cc. volumes of unheated dermal filtrate, after which serial dilutions of the mixtures were prepared and incubated with optimal amounts of L- or S-antibody. Results of the titrations made with the mixture containing 0.1 mg. of degraded S-antigen were comparable to the controls. The presence of 1.0 mg. of the non-precipitable or degraded antigen prevented flocculation of the native S-antigen only in the lowest dilutions of the titrations with S-antibody, while 5.0 mg. completely blocked precipitation of native S-antigen with its antibody through-

out the range of dilutions. In none of the mixtures was the reaction with L-antibody appreciably altered.

On several occasions S-inhibitor has been encountered in preparations of vaccine dermal filtrate that were not subjected to alkaline treatment. In these experiments the filtrates were concentrated 10 to 20 times before boiling. The technique for purifying S-antigen was originally applied to crude dermal filtrates (5). In order to avoid working with large volumes, we first concentrated the crude filtrates by evaporation in cellophane tubes. The results obtained when the purification procedure was applied to these concentrates were disappointing in that yield of S-antigen was negligible as determined by pre-

TABLE V

Inhibition of S-Antibody—Continued

Antigen mixture		Antiserum	Dilution of original dermal filtrate			
Dermal filtrate	Degraded S-antigen		1:8	1:16	1:32	1:64
cc.	*mg.*					
0.4	0.1	L	++++	++++	++++	++
		S	++++	++++	++++	+++
0.4	1.0	L	++++	++++	+++	++
		S	−	++++	++++	+++
0.4	2.0	L	++++	++++	+++	++
		S	−	−	−	++
0.4	5.0	L	++++	++++	+++	+
		S	−	−	−	−

Mixtures of dermal filtrate and degraded S-antigen were incubated at 37°C. for ½ hour and then diluted serially. Constant amounts of L-antiserum 6814 diluted 1:6 and of S-antiserum 274 diluted 1:4 were added and the titration results were read after incubation overnight at 50°C.

cipitin tests. The failure to demonstrate the expected amounts of S-antigen in the solutions was found to be dependent upon the presence of an inhibitor which could be removed, leaving behind precipitable S-antigen. The results of this type of work are illustrated by the following experiment.

Experiment 7.—960 cc. of dermal filtrate, pool 8, were dialyzed in cellophane tubes against running water and then concentrated to 90 cc. in the same tubes by evaporation in a stream of air. The clear solution became opalescent after gentle boiling for 5 minutes, and the small amount of precipitate that formed was removed by centrifugation. The globulin fraction obtained by precipitation with ammonium sulfate was redissolved in water, dialyzed free of sulfate ions, and brought back to a volume of 90 cc. The original crude dermal filtrate in a dilution of 1:64 precipitated with L- and S-antisera, but the concentrated solution of heated globulin gave only a slight precipitate in dilutions of 1:8 to 1:32 with S-antiserum. A portion of the solution

of globulin was fractionated by the method of Craigie and Wishart (1) for the concentration of LS-antigen. Only about half of the voluminous white precipitate obtained at pH 4.5 redissolved when it was suspended in buffer solution, pH 6.6. The solution containing the material which dissolved at pH 6.6 had a precipitin titer of 1:128 when tested with S-antiserum. The material which failed to dissolve at pH 6.6 was soluble in dilute buffer solution, pH 9.0, and the solution remained clear when brought back to pH 7.0. While this solution did not precipitate in dilutions of 1:4 to 1:512 in the presence of an optimal amount of S-antibody, it was found to have a marked inhibiting effect on S-antibody. S-antiserum treated with 3 volumes of the solution would not precipitate with native S-antigen. On the other hand, L-antiserum was not appreciably inhibited by 5 volumes of the solution.

The results obtained in Experiment 7 throw some light on the work of Craigie and Wishart (1). For example, it was shown that under certain conditions the heating of dermal filtrate, even in the pH range near neutral reactions, may result in the formation of S-inhibitor. Furthermore, it was found that S-inhibitor can be separated from precipitable S-antigen by means of the different solubilities of the two substances at several pH values.

DISCUSSION

The results of the foregoing experiments may be briefly summarized as follows. Dermal filtrate prepared from the skin of rabbits infected with the virus of vaccinia contains the heat-labile (L) and heat-stable (S) antigens of vaccinia which can be demonstrated by the precipitation technique. Gentle heating destroys the precipitability of L-antigen but leaves in solution a substance capable of inhibiting L-antibody. This L-inhibitor, like precipitable L-antigen, is closely associated with S-antigen and cannot readily be separated from it. Preparations of S-antigen can also be degraded by any of several methods to a stage where precipitation with S-antibody does not occur; however, inhibition of S-antibody with this material is easily demonstrated. Degraded S-antigen, in contrast to precipitable S-antigen, has little power to inhibit L-antibody. The solubilities of S-inhibitor are different from those of S-antigen and L-inhibitor.

The immediate objective of the present experiments, *viz.*, the demonstration of degraded forms of L- and S-antigens of vaccinia which are capable of inhibiting their specific antibodies, was accomplished. The studies were less successful, however, in regard to their ultimate objective which was the elucidation of the relationship between L- and S-antigens. Nevertheless, the observations just reported may serve as the basis for several hypotheses on the relationship of these two serologically active substances.

One of the simplest of the hypotheses is as follows: The native antigen present in infected tissue is L, or the antecedent substance from which it is derived. L is readily degraded during manipulation and storage to a slightly modified

material, S, which is still capable of eliciting a specific antibody in animals and which combines with L-antibody as demonstrated by inhibition tests. S-antigen can be further degraded to a stage where it inhibits but fails to precipitate with its antibody; and, in this form it loses most of its ability to combine with L-antibody. A graphic representation of the various stages of degradation according to this scheme is presented in Text-fig. 1. There are

Hypothesis	Native materials in dermal filtrate	Effect of heat	Effect of heat and alkali
I	L Precipitates with L-antibody	S Inhibits L-antibody Precipitates with S-antibody	S′ No inhibition of L-antibody No precipitation with S-antibody Inhibits S-antibody
II	L Precipitates with L-antibody S Precipitates with S-antibody	L′ Inhibits L-antibody S Precipitates with S-antibody	L″ No inhibition of L-antibody S′ Inhibits S-antibody
III	(L S) Precipitates with L-antibody Precipitates with S-antibody	(L′ S) Inhibits L-antibody Precipitates with S-antibody	(L″ S′) No inhibition of L-antibody No precipitation with S-antibody Inhibition of S-antibody
	Other theoretical combinations		
	(a) (L′ S′) Precipitates with neither antibody Inhibits both antibodies	(b) (L″ S″) Neither precipitates nor inhibits	(c) (L S′) Precipitates with L-antibody No precipitation with S-antibody Inhibits S-antibody

TEXT-FIG. 1

several serious objections to this hypothesis. No reasonable explanation is at hand for the regular occurrence of equal amounts of L- and S-antigen in preparations from infected tissue. Moreover, it should be possible, if the hypothesis were correct, to demonstrate a simultaneous increase in S along with a decrease in L during the various procedures which render L-antigen non-precipitable.

A second hypothesis might begin with the assumption that L- and S-antigens are separate protein molecules which are distinguishable by serological means but not by ordinary physical and chemical procedures. According to this idea each of the antigens could be degraded independently, but, since L is more

easily affected than S, the labile antigen would generally be denatured one step further than the stable one (Text-fig. 1). The similar physicochemical nature of the native substances might also be considered to be characteristic of the mildly degraded antigens; thus, a separation of L-inhibitor and S-antigen would be difficult. This hypothesis, like the first, affords no explanation for the uniform ratio of L- and S-antigens in crude filtrates. Furthermore, it is hard to reconcile it with the almost invariable results obtained in absorption experiments, namely, the removal of both antigens on the addition of either antibody.

A third hypothesis deals with an LS-complex similar to that postulated by Craigie and Wishart (1). No assumptions need be made about the chemical nature of the L-part of this complex or about the type of union between it and the protein-like S-part. As in the second hypothesis, the L-reacting substance would be considered to be degraded more readily than the S under ordinary conditions, and complexes might be formed of L′S and L″S′ on treatment with heat or heat and alkali, respectively (Text-fig. 1). In such a scheme the first degradation product, *e.g.*, L′ would inhibit L-antibody but not be precipitated by it, while L″ would have lost most of its capacity to inhibit L-antibody. Similarly, stages in the degradation of the S-portion of the complex could be postulated. Additional complexes with various combinations of native and degraded stages of the L- and S-parts might be considered; several of these are represented in Text-fig. 1, (*a*), (*b*), (*c*). The objections raised to the first two hypotheses are not valid for the last one. The unusual results of occasional absorption experiments reported by Parker (2) and Craigie and Wishart (3) can be explained by this hypothesis if one assumes that under certain rare conditions a portion of the LS-complex in dermal filtrate is degraded to a form represented as LS′ (Text-fig. 1, (*c*)). A mixture of this complex and native LS when treated with S-antibody could result in the precipitation of LS, leaving in solution LS′ combined with S-antibody. Such a soluble antigen-antibody combination might then be precipitated on the addition of L-antibody.

At the time these experiments were completed we were inclined to accept the third hypothesis as the one which more nearly explained the available date on the relationship of the L- and S-antigens of vaccinia. Work reported in accompanying and subsequent papers (4, 11) has done much to strengthen our belief that this hypothesis is substantially correct.

CONCLUSION

Experimental data are presented which may be interpreted as follows. The heat-labile (L) and heat-stable (S) antigens of vaccinia occur in nature as a complex consisting of a single substance with two serologically active parts, each of which may be degraded independently of the other.

We wish to express our appreciation to Dr. Kenneth Goodner for his advice.

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