THE LS-ANTIGEN OF VACCINIA

II. Isolation of a Single Substance Containing Both L- and S-Activity

By THEODORE SHEDLOVSKY, Ph.D., and JOSEPH E. SMADEL, M.D.

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

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The intimate association of the heat-labile (L) and heat-stable (S) soluble antigens of vaccinia was shown by the work of Craigie and Wishart (1). Moreover, observations on the degraded forms of these antigens, reported in the previous paper (2), further indicated the close relationship of these two antigens. The fact that the L-antigen is readily denatured by a number of procedures which are ordinarily used for purification of immunologically active substances has delayed the acquisition of knowledge concerning its nature. On the other hand, the heat-stable antigen has been studied in considerable detail (3, 4) and has been shown to be a protein substance. Although the results of several experiments (4, 5) have been interpreted as indicating that L-antigen may be obtained free of the S-antigen, these experiments are also subject to a different interpretation (2). The work reported here was undertaken in the hope of obtaining pure L-antigen in sufficient amounts to study its physical and immunochemical properties. As will be shown, the L-antigen is not found free from the S-reacting substance under ordinary conditions. Indeed, the results indicate that the L- and S-immunological activities reside in a single native protein molecule. A method for isolating pure LS-antigen of vaccinia from virus-free filtrates of infected rabbit skin will be given in this communication together with observations on the physical and immunological properties of the native substance and of some of its degraded forms.

Materials and Methods

Vaccine Dermal Filtrate.—Dermal filtrate was available in large quantities as a by-product obtained from the preparation of washed elementary bodies of vaccinia which were used for other studies. The technique of Craigie (6) was employed without significant modification. Dermal pulp from each cutaneously infected rabbit was suspended in 30 cc. of a 1:50 dilution of standard phosphate buffer solution pH 7.2 and the solution which remained after removal of tissue particles and elementary bodies by means of differential centrifugation was filtered immediately through a Seitz pad. Bacteriologically sterile virus-free filtrates were stored at 3°C. until 800 to 1200 cc. had been accumulated, usually a period of a month.

Antiserum.—L-antiserum used throughout this portion of the work was prepared from pooled sera obtained by bleeding vaccine virus immune rabbits which had been hyperimmunized with washed active elementary bodies of vaccinia; the serum was absorbed free of S-antibody with heated crude dermal filtrate. S-antisera were obtained from rabbits which had been immunized with a non-infectious solution of purified S-antigen (4). Precautions were taken to prevent these non-immune animals from becoming accidentally infected with vaccinia during the period of immunization. Our experience, like that of Parker, indicated that solutions of S-antigen prepared in this manner elicit a poor antibody response in rabbits and in order to obtain potent sera several courses of treatment were given. For the final course of immunization, washed and graded collodion particles were added to the solution of S-antigen and the mixture was injected intravenously.

Serological Methods.—The serological techniques employed in this work have been reviewed in a previous paper (2).

Electrophoretic Technique.—Electrophoretic experiments were carried out in the Tiselius apparatus with the schlieren-scanning optical system (7) the details of which have been discussed elsewhere. The temperature of the thermostat containing the cell was regulated at 1°C., but all the values of mobility given in this paper have been corrected to 0°C.

EXPERIMENTAL

Observations on Concentrated Whole Dermal Filtrate.—The results obtained in studies in which the electrophoretic technique was applied to concentrated dermal filtrate, both before and after heating, are illustrated in the following experiments.

Experiment 1.—One liter of dermal filtrate was concentrated to a volume of 100 cc. by means of evaporation through a cellophane sausage casing placed in an air stream at room temperature for 24 hours. The L- and S-titers of this concentrate were 1:1000. A portion of the material was dialyzed against 0.05 M lithium-veronal buffer solution at pH 7.9.[1] Upon electrophoresis this dialyzed material was found to display four boundaries, all of which corresponded to negatively charged substances at this value of pH. A reproduction of the electrophoretic pattern obtained is shown in Fig. 1. The mobilities of the four components, which were designated I to IV, were 6.7×10^{-5}, 5.4×10^{-5}, 4.4×10^{-5}, and 3.4×10^{-5} cm./sec. per volt/cm., respectively. In Fig. 1, as in subsequent figures, the first peak on the right, ϵ, does not correspond to any component, but is due to a gradient of buffer salts.

Experiment 2.—A portion of the same material was heated at 70°C. for ½ hour in a closed vessel (a procedure which inactivates L without destroying S) and an electro-

[1] This solution contained diethylbarbituric acid, its lithium salt, and lithium chloride, each at 0.025 M. The chloride was used to build up the ionic strength, since the diethylbarbituric acid concentration is fixed by its relatively low solubility at 0°C.

phoretic determination was made under conditions identical with those described above. The electrophoretic pattern obtained in this experiment is illustrated in Fig. 2.

It is immediately obvious from Figs. 1 and 2, that the results obtained in the two experiments were strikingly different. As a result of heating, one component (III) had completely disappeared and another component (II) had been

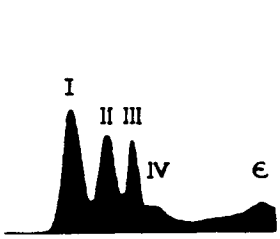

Fig. 1

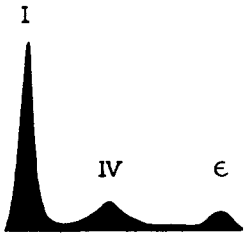

Fig. 3

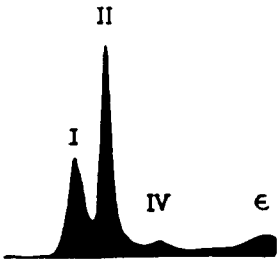

Fig. 2

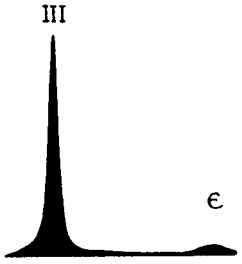

Fig. 4

Fig. 5

increased in amount; on the other hand, components I and IV were unaffected by heating under these conditions. A quantitative estimation of the relative amounts of components II and III in these two preparations was obtained from the measurement of the areas in the electrophoretic patterns. This indicated that the increase in component II which occurred on heating was approximately equal to the loss of component III.

When the observations described above were first made we were not certain whether L- and S-activities were associated with one or with two substances.

Therefore, we were inclined to believe from these data that component III corresponded to L-antigen and that component II corresponded to S-antigen; furthermore, that as a result of heating, L had been transformed to S. Let it be said at once that this hypothesis was subsequently proved wrong. Nevertheless, we immediately attempted to isolate by electrophoretic separation the four components shown in Fig. 1, in order to identify the substances serologically. These results may be briefly summarized as follows: components I, the fastest, and IV, the slowest, were readily isolated and were found to be serologically inert. Sharp electrophoretic separation of components II and III was not possible, due to the fact that their respective mobilities were not sufficiently different for such resolution. Even when electrophoresis was carried out in solutions with pH values from 6.0 to 8.6, such fractions which were obtained invariably showed L- and S-activity in equal amounts. Since the above methods were inadequate for the purpose, fractionation of the L- and S-antigens from crude dermal filtrate was attempted by other means.

Fractionation of Dermal Filtrate by Precipitation.—It has been shown by Craigie and Wishart (1) that the L- and S-antigens can be concentrated from crude dermal filtrate by precipitating at pH 4.5 and redissolving the precipitate at pH 6.5. This procedure enabled these workers to eliminate a considerable amount of serologically inert nitrogenous material, present in the crude filtrate, without appreciably reducing the titer of the antigens in the final preparation. Parker and Rivers (3), in their experiments dealing with the purification of S-antigen from heated dermal filtrate, had also observed that the heat-stable active substance has similar solubility characteristics. Procedures based on these observations were employed in the fractionation of concentrated dermal filtrate and the fractions thus obtained were studied electrophoretically and serologically. The following experiment illustrates the methods employed and the results obtained.

Experiment 3.—1150 cc. of vaccine dermal filtrate, collected over a period of 5 weeks, were concentrated by evaporation from cellophane bags to a volume of 110 cc. The slight amount of insoluble material present in the concentrate was removed by centrifugation and discarded. The clear concentrate was again placed in a cellophane sack and dialyzed overnight against 4 liters of unbuffered physiological saline solution at 3°C. The sack and its contents were then suspended in 450 cc. of 0.05 M acetate buffer solution at pH 4.63, also at 3°C. Precipitation began at once at the surface of the cellophane membrane. The sack was agitated from time to time and by the end of 3 hours no further increase in the precipitate could be noted. The material was then removed from the sack and the precipitate was separated by centrifugation in the cold. The clear, straw-colored supernatant fluid, which had a pH of approximately 4.6, was set aside for further study; this was designated fraction A. The oyster-gray sediment, which occupied a volume of about 2 cc. in the centrifuge tube, was washed in the cold with 45 cc. of the same acetate buffer solution. The resedi-

mented, washed material was then taken up in 50 cc. of 0.05 M cacodylate buffer solution at pH 6.31 and dialyzed overnight against 450 cc. of the same solution at 3°C. Approximately half of the material dissolved under these conditions. The material insoluble at pH 6.31 and the substance dissolved at this pH were separated by centrifugation. The clear, pale straw-colored supernatant solution was labeled fraction B and stored. The sediment was washed with the cacodylate buffer solution and then suspended in 50 cc. 0.05 M veronal buffer solution of pH 8.56. Following dialysis against a large volume of the same buffer solution an appreciable part, but not all, of the material dissolved. The clear liquid was again separated from the precipitate by centrifugation and designated fraction C. The precipitate which was insoluble at pH 8.56 dissolved partially in 0.1 M borate buffer solution pH 11.0 and almost completely in 0.05 M NaOH; since all the material in dermal filtrate was originally soluble in neutral solutions, this portion probably consisted largely of denatured products.

The three fractions which had been saved for further study were: A, the material which remained soluble at pH 4.63; B, the material which was insoluble at pH 4.63 but soluble at pH 6.31; and C, the material which was insoluble at pH 4.63 and 6.31, but soluble at pH 8.56. Portions of fractions A and B were placed in cellophane sacks and dialyzed against 0.05 M lithium-veronal buffer solution at pH 7.91 for 1 or 2 days in the cold, after which they were examined by electrophoresis; similar data were obtained on fraction C in veronal buffer solution pH 8.56. The serological properties of all three fractions were investigated.

Fraction A was found to contain only two components; these had the same electrophoretic mobilities as components I and IV in whole dermal filtrate. Fraction B consisted of a single substance which was electrically homogeneous and which moved at a rate corresponding to that of component III. Fraction C contained a trace of component III, but most of the material appeared to correspond to component II in whole filtrate. The electrophoretic patterns obtained with fractions A, B, and C are shown in Figs. 3, 4, and 5, respectively.

The results of serological studies on the original material and on fractions A, B, and C are summarized in Table I. Briefly it may be stated that fraction B, consisting of the electrically homogeneous component III, contained practically all of the L- and S-reacting material present in the original concentrated dermal filtrate. It gave a precipitate when diluted 1:1600 and incubated in the presence of an optimum amount of L- or of S-antibody. The small amount of L- and S-precipitinogen activity found in the C fraction was completely lost after refractionation; furthermore, all of the serologically active material was now recovered in the solution corresponding to the original B fraction. The A fraction was serologically inert.[2]

This portion of the work may be summarized as follows. Components I and

[2] In several early experiments in which dialysis of crude filtrate against pH 4.6 buffer solution was inadequate appreciable amounts of components II and III were left in fraction A.

IV found in vaccine dermal filtrate may be separated quantitatively from components II and III by precipitation of the latter at pH 4.6. Furthermore, component III can be isolated from this precipitate in uncontaminated form by extracting this precipitate at pH 6.3. Such a solution of component III contains practically all of the L- and S-reactive substance present in the original filtrate.

Observations Indicating that a Single Substance in Its Native State Contains Both L- and S-Activity.—In our laboratory, precipitation titrations with the

TABLE I

Serological Activity of Fractions Derived from Vaccine Dermal Filtrate

Test solution	Anti-serum	Dilution of test solution							
		1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
Concentrated dermal filtrate	L		++++	++++	++++	++++	+++	++	+
	S		±	+++	++++	++++	++++	+++	+
Fraction A	L	−	−	−	−	−			
	S	−	−	−	−	−			
Fraction B	L		++++	++++	++++	++++	+++	++	±
	S		±	++++	++++	++++	++++	++	±
Fraction C	L	++++	++++	+++	+	±			
	S	++++	++++	+++	++	+			
Refractionated C	L	±	−	−	−	−			
	S	+	±	−	−	−			

L-antiserum 1601 was used in a dilution of 1:2 throughout. S-antiserum 493 was used in a dilution of 1:8. 0.01 M lithium-veronal buffer solution, pH 8.6, containing 0.9 per cent of NaCl served as diluent. Test mixtures were incubated at 50°C. overnight.

See text for preparation of test materials.

soluble antigens of vaccinia are ordinarily carried out by incubating the antigen-antibody mixtures at 50°C. for 18 hours in closed tubes. Such an incubation period is sufficient to degrade the heat-labile antigen, L, to a state where it no longer precipitates with its antibody. Consequently, at this stage in the experiments it was necessary to consider the possibility that the material designated as component III was a substance which possessed only L-activity in its native form. According to such an hypothesis, this native material could be degraded by heating to a substance with the electrical mobility of component II and with S-activity.

In order to test this hypothesis a solution containing component III was diluted serially in the usual manner, and portions of each dilution were placed in tubes with

optimal amounts of L- or of S-antibody. Four sets of duplicate titrations were thus made and each set was incubated for 18 hours at a different temperature, namely, at 3°, 20°, 37°, and 50°C.

In each set of titrations the precipitin endpoint in the presence of S-antibody was identical with that obtained in the presence of L-antibody. Flocculation was slower at the lower temperatures, and with incubation at 3°C. periods of 48 to 72 hours were necessary in order to develop maximum precipitation; end-points thus obtained equalled those observed after 18 hours at 50°C.

The presence of an S-reactive substance in solutions of component III which were incubated at temperatures that do not change the precipitability of the heat-labile substance, clearly shows that component III in its native state possesses both L- and S-reacting portions; therefore, S cannot be a degradation product of L-antigen. Observations reported in succeeding sections will clarify the relationship between component II and heated component III.

Evidence based on absorption experiments in which solutions of component III were used with L- and with S-antisera indicates that the two antigenic parts of component III cannot be separated by this method. The following protocol illustrates the results obtained with one of several preparations of antigen absorbed with antisera.

Experiment 4.—40 cc. of an electrophoretically homogeneous solution of component III were obtained from 800 cc. of vaccine dermal filtrate by the method described in the protocol of Experiment 3. The solution had a titer of 1:2048 with L- and with S-antibody. A 0.7 cc. volume of S-antiserum 493 was mixed with 0.1 cc. of the solution of antigen in one instance, and in another with 0.05 cc. of antigen and 0.05 cc. of saline. Each antigen-antibody mixture was diluted with an equal volume of saline solution, and incubated at 37°C. for 4 hours, and then at room temperature for a similar period of time. The voluminous precipitate which formed was removed by centrifugation. Similar absorptions were carried out with 0.8 cc. volumes of L-antiserum 1601 and varying amounts (0.01 to 0.10 cc.) of solution of antigen, sufficient saline solution being added to each mixture to bring the final volume to 1.6 cc. These were tested for residual antigen and for residual antibody. The results of these tests which are summarized in Table II show that absorption with either L- or S-antibody removes both L- and S-antigenic substances completely or reduces both to the same degree.

It is apparent from these observations that the electrically homogeneous[3] component III, which was obtained from the dermal filtrate, contains both L- and S-activity and that the two serologically distinct parts are inseparable in the native substance. Ultracentrifugal and chemical studies on this material, which will be reported in another paper, showed it to be a homogeneous protein

[3] Studies on the material at values of pH between 6.2 and 8.6 consistently revealed but one electrophoretic component. These measurements are discussed in detail in a subsequent paper.

TABLE II

Absorption of Solutions of Component III with L- and S-Antibody

Absorption mixtures		Residual antigen									Residual antibody			
Antigen	Antiserum type	Anti-sera	Dilution of original antigen solution									Dilution of absorbed mixture			
			1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:1	1:2	1:4	1:8
cc.	*cc.*														
	Unabsorbed	L		++++	++++	++++	++++	+++	++	+	−				
		S		−	±	++++	++++	+++	+++	+	−				
0.01	Anti S 0.7	L	−	−	−	−	−								
		S	−	−	−	−	−					−	−	−	−
0.05	Anti S 0.7	L		−	−	−	−	−							
		S		−	−	−	−	−				+++	++++	−	−
0.1	Anti L 0.8	L		++++	++++	+++	+	±				−	−	−	−
		S		++++	++++	+++	++	±							
0.05	Anti L 0.8	L		++	±	−	−	−				−	−	−	−
		S		++	±	−	−	−							
0.025	Anti L 0.8	L			−	−	−	−				++	−	−	−
		S			−	−	−	−							

L-antiserum 1601 was prepared from pooled sera of rabbits hyperimmunized with active elementary bodies of vaccinia by absorption of S-antibody with heated dermal filtrate. S-antiserum 493 was obtained from a rabbit immunized with a non-infectious fraction of heated dermal filtrate rich in the heat-stable soluble antigen of vaccinia. Tests for residual antigen were carried out with optimal dilutions of antisera, *i.e.*, 1:2 for L- and 1:8 for S-antisera. Determinations of residual antibody were made by adding 0.25 cc. of a 1:16 dilution of unheated vaccine dermal filtrate to equal volumes of serial dilutions of the absorbed mixture.

See text for details of absorption technique.

with a molecular weight near that of serum globulins. On the basis of electrophoresis and ultracentrifugal evidence, therefore, we conclude that component III is a single molecular substance containing both L- and S-activity. Henceforth we shall designate it as LS-antigen. This LS-antigen is probably the substance that Craigie and Wishart (1) prepared from the same type of source material and called "LS fraction." Our method of isolation of the antigen is similar to theirs and the serological data obtained following incubation at various temperatures and by absorption with L- and S-antibody agree with their findings. The present experiments supplement the observations of these authors in that they were done with solutions of antigen which were proved to be pure by a number of different criteria and hence these experiments carry additional weight in theoretical discussions of the nature of LS-antigen.

Effect of Heat on LS-Antigen.—Destruction of the precipitability of the labile soluble substance of vaccinia by heating has been well established (1, 5). The combination between heated L- antigen and L-antibody without subsequent flocculation has been discussed in the preceding paper (2). Since pure LS-antigen was now available it seemed desirable to study the effect of heat on its physical and serological characteristics.

Experiment 5.—The observations described in the following experiment are typical of those noted in the study of several different preparations of LS-antigen. A portion of the solution of LS-antigen from Experiment 4 was heated for ½ hour in a water bath at 70°C. in a closed tube. The solution which had been clear now showed a faint opacity. After removing the trace of insoluble material by high speed centrifugation (12,000 R.P.M.) the solution was redialyzed against 0.05 M lithium-veronal buffer solution at pH 7.91 and was examined by electrophoresis.

The electrophoretic mobility of this lot of unheated LS-antigen under these conditions was 4.0×10^{-5} cm./sec. per volt/cm. After heating this solution, a single electrically homogeneous component was still present, but it now moved at a rate of 5.9×10^{-5} cm./sec. per volt/cm. This preparation of heated LS was inhomogeneous when examined in the analytical centrifuge, as will be shown in another paper in this series. The precipitin titer of the solution of unheated LS was 1:2048 with both L- and S-antibody; the heated LS still titered 1:2048 with S-antibody but failed to precipitate over the range of dilutions from 1:8 to 1:4096 in the presence of L-antibody.

This experiment throws considerable light on the early observations which misled us into believing that components II and III represented S- and L-antigens respectively. Heating destroyed the precipitability of the L-part of the LS-antigen and increased the mobility of the degraded molecule to a value almost identical with that of component II in crude dermal filtrate. The augmentation of component II concurrent with the disappearance of component III in heated crude filtrate thus appears to have been a coincidence.

The Occasional Occurrence of Partially Degraded LS-Antigen in Crude Dermal Filtrate.—Although pure LS-antigen had been previously obtained in four successive experiments by means of the fractionation technique which has been described, it was found in one experiment that the fraction which usually contained only LS-antigen was contaminated with a component having the mobility of either component II or of heated LS-antigen.

Experiment 6.—850 cc. of dermal filtrate were concentrated and fractionated in the manner described in Experiment 3. Fraction B was examined by electrophoresis in 0.05 M lithium-veronal buffer pH 7.89 and found to contain two components; one component corresponding to LS-antigen was present in an appreciable amount, and the other, corresponding to either heated LS-antigen or component II, was estimated to be present in about ½ the concentration of LS-antigen. Although the titer of the solution was 1:1600 with L-antibody and 1:3200 with S-antibody this was considered inconsequential at the time. Accordingly, the solution was refractionated to remove the contaminating material. In short, the material was precipitated three times at pH 4.6 and each time the precipitate redissolved almost completely at pH 6.6. The final preparation gave essentially the same electrophoretic pattern and serological titers as did the original fraction B.

Refractionation of the solution which might have been expected to eliminate the contaminating material if it were component II failed to accomplish this. Furthermore, since the contaminant had solubility characteristics similar to those of LS-antigen it appeared likely that it was a degraded form of LS-antigen. Therefore, at this point, it seemed desirable to know whether the technique of fractionation, as regularly employed, was adequate to separate heat-degraded LS-antigen from native LS-antigen.

Experiment 6 (Continued).—14 cc. of the three times fractionated solution B from Experiment 6 were still available. The solution had an S-titer of 1:1600 and L-titer of 1:800. One half of the material was heated at 70°C. for ½ hour. This portion then failed to react with L-antibody but still retained its S-titer of 1:1600. The two solutions were recombined; the precipitin titers were then 1:400 for L-antigen and 1:1600 for S-antigen, as might have been expected.

After dialysis, the mixture was again studied electrophoretically; the pattern revealed the presence of two components with mobilities corresponding to LS-antigen and heated LS-antigen, which were present in concentrations closely corresponding to their respective titers. The material was then subjected to the solubility fractionation process and was again examined electrophoretically. No change in the resulting pattern was noted. The serological titers remained the same.

Essentially similar electrophoretic and serological observations were made on a mixture containing equal amounts of solutions of unheated and heated LS-antigen from Experiment 3. In this case, however, the two electrical components were about equal quantitatively, and the solution had titers of 1:800 to 1:1600 with L- and S-antibody respectively.

The results obtained in Experiment 6 indicate that heat-degraded LS-antigen has essentially the same solubility characteristic as native LS-antigen and hence cannot be separated from it by the procedures employed. This is of importance for it indicates that, in general, dermal filtrate contains no partially degraded LS-substance, *i.e.*, one possessing S-activity but not L-activity. In other words, all of the L- and S-activity in fresh dermal filtrate is carried by component III. In the single experiment in which pure LS-antigen was not isolated by the process of fractionation it appears that the contaminant was a degraded form of antigen similar to heated LS-antigen. The reason for the appearance of this contaminant in a single pool of dermal filtrate is not at hand. It may be pointed out, however, that, unlike the usual preparations, in this case, several of the lots of the angle supernatant fluid from the suspension of crude pulp had not been filtered immediately, but were allowed to stand at ice box temperature for several days before being passed through a Seitz pad. The centrifuged but unfiltered fluid is slightly opalescent and contains some virus, tissue debris and bacteria, and the presence of one or more of these might well have contributed to partial denaturation of LS-antigen by enzymatic digestion or otherwise.

Inhibition of L- and S-Antibodies by Degraded Forms of LS-Antigen.—The interpretation of the experiments described in the previous paper (those dealing with inhibition of anti-soluble-substance antibodies of vaccinia by various preparations of materials from vaccine dermal filtrate) was difficult, due to the lack of critical evidence showing that the degradation products of a single substance were capable of inhibiting the two antibodies. Accordingly, solutions of pure LS-antigen were treated with heat alone, and also with heat and dilute alkali, and the specific inhibitory properties of the resulting preparations were studied (Experiments 7 and 8).

Experiment 7.—A portion of solution obtained from Experiment 3 was heated at 70°C. for ½ hour. The resulting solution still precipitated in a dilution of 1:1600 with optimal amounts of S-antibody but failed to flocculate when dilutions of 1:25 to 1:3200 of the solution were mixed with optimal amounts of L-antibody. Inhibition tests were made in the following manner. Varying amounts of two different L-antisera were treated with varying amounts of heated LS-antigen solution. Sufficient saline solution was added to the test mixtures to make the final dilutions of serum comparable. The mixtures were incubated at 50°C. for ½ hour and then at 37°C. for an additional ½ hour. The solutions remained clear and centrifugation failed to sediment an appreciable amount of material. The test sera, together with controls, prepared by properly diluting antisera with saline and incubating for a similar period, were then set up in constant amounts with serial twofold dilutions of unheated crude dermal filtrate. Illustrative data obtained are summarized in Table III.

It is evident from the results summarized in Table III that a solution of LS-antigen, shown to be pure by electrophoresis and ultracentrifugation, when

degraded by heat to the stage where it fails to give a precipitin reaction with L-antibody, is still capable of combining with L-antibody as demonstrated by the inhibition technique. An additional point of interest may be mentioned regarding the serological behavior of LS-antigen degraded by heat. Mixtures of serial dilutions of such antigen and optimal amounts of L-antibody, which give no precipitate after incubation, were subsequently treated with optimal amounts of S-antibody and incubated in the usual manner. The precipitin titers in these instances were approximately the same as those obtained in control titrations in which no L-antibody was employed. Apparently, the presence of L-antibody, which was presumably combined with the degraded

TABLE III

Inhibition of L- and S-Antibody by Degraded Forms of LS-Antigen

Inhibiting solution	Ratio of inhibiting solution to antiserum	Antiserum	Dilution of dermal filtrate					Saline + test mixture
			1:8	1:16	1:32	1:64	1:128	
None		L	++++	++++	++++	+++	++	
		S	++++	++++	++++	+++	++	
LS heated	1:1	L	+	−	−	−	−	−
	2:1	L	−	−	−	−	−	−
LS heated with alkali	1:1	L	++	++++	+++	++	++	−
	2:1	L	±	+++	+++	++	++	−
	1:1	S	−	−	−	−	−	−
	1/2:1	S	−	−	−	−	−	
	1/4:1	S	±	+++	+	−	−	
	1/8:1	S	++	++++	++++	+++	−	

L-antiserum 1601 and S-antiserum 493 were used in the experiments summarized in this table.

See legend of Table II and text for details of experiment.

L-portion of the antigen, did not interfere with the union of the S-portion with its antibody and subsequent flocculation of the entire aggregate.

Experiment 8.—A portion of the solution of heated LS-antigen used in Experiment 7 was freed of buffer by dialysis and treated with a sufficient amount of sodium hydroxide to bring the final concentration to 0.1 M. The material was heated in a water bath at 50°C. in a closed vessel for 90 minutes with frequent gentle agitation. After neutralization to pH 7 the solution was titered with S-antibody. The treatment failed to effect a complete loss of specific precipitability of the solution, for the S-titer, although much diminished, was still 1:320. Accordingly, the procedure was repeated except that 0.05 M concentration of alkali was used. Although the first treatment did not result in any change in the appearance of the solution, this second treatment was accompanied by the production of a small amount of insoluble material which, incidentally, was arranged in small, elongated ribbon-like fibers which were white in

reflected light and colorless in transmitted light. The insoluble material was removed and the solution tested in dilutions of 1:5 to 1:640 at pH 7 with optimal amounts of S-antibody. No precipitation occurred.

An inhibition experiment of the type described in the previous section was now performed. L- and S-antisera were treated with varying amounts of alkali-heat degraded antigen and subsequently tested for precipitable antibody. The results of the experiment are summarized in Table III.

It is apparent from the data presented in Table III that as little as 0.1 cc. of the solution of LS-antigen which had been degraded by heat and alkali (approximately 0.15 mg. of dried material) was sufficient to inhibit completely the S-antibody in the mixture. Furthermore, even ¼ of this amount of antigen was sufficient to inhibit the antiserum slightly. On the other hand, 1.0 cc. (1.5 mg.) was incapable of inhibiting the amount of L-antibody employed. Thus, degradation of LS-antigen by means of heat and alkali completely destroys the serological activity of the L-portion of the molecule and changes the S-portion of the molecule to a form which no longer precipitates with S-antibody but is capable of inhibiting it. It will be noted that the inhibitory action of the degraded S-portion of the molecule on S-antibody is of a greater order of magnitude than that of the degraded L-portion for its corresponding antibody.

Electrophoretic studies on the solution of LS-antigen in Experiment 8 which had been degraded by heat and alkali showed that it contained one electrically homogeneous component with a mobility of 6.4×10^{-5} cm./sec. per volt/cm. at pH 7.9 and ionic strength 0.05. The mobility of this substance is close to that of component I in dermal filtrate. The two are not identical, however, for the latter failed to inhibit S-antibody. Furthermore, the two substances have different solubilities; the S-inhibitor is precipitated at pH 4.5 and fails to dissolve appreciably at pH 6.3, but it can be brought into solution between pH 8 and 9, and will then remain in solution when the pH is lowered to nearly 7.0.

DISCUSSION

The experiments presented in this paper indicate that all of the serological activity associated with the heat-stable and heat-labile soluble antigens of vaccinia are present in a single protein molecule. This differs somewhat from the concept of Craigie and Wishart (5), who considered the two antigens to occur, ordinarily, in the form of a complex which could be dissociated into two separate antigens under certain circumstances.

It is possible to state with assurance that various levels of degradation of LS-antigen can be accomplished, leaving either the L- or S-part of the molecule in a stage where it can still combine with the corresponding antibody without precipitation. The observations on inhibition of L- and S-antibody reported in the previous paper were not sufficiently conclusive to enable us to decide as to the nature and origin of the inhibiting substances. On the basis of the

present studies it is clear that they arise directly from the LS-antigen through partial degradation of the molecule.

Here it has been shown that the L-portion of the LS-molecule can be degraded by heat without serological alteration of the S-portion. In a subsequent paper in this series, it will be demonstrated that by means of enzymatic digestion the S-portion of the molecule can be degraded without altering the serological activity of the L-portion. On the basis of this latter observation it is possible to explain directly (2) the results occasionally obtained by other workers (4, 5), which had been interpreted (5) to indicate a dissociation of their LS-complex antigen into two separate components.

SUMMARY

Virus-free filtrate, obtained from suspensions of vaccine virus-infected dermal pulp of rabbits and rich in the soluble substances of vaccinia, was shown to contain four distinct components in electrophoresis experiments. Electrophoretic and serological observations served as a guide in developing a method for separating these components from one another. This method depended upon changes in the solubilities of the components with alterations of pH.

Three of the four components appeared to be serologically inert when tested with anti-vaccinia sera. All of the L- and S-activity was found to be associated with a single component which was electrically homogeneous at several values of pH and which was homogeneous in the ultracentrifuge.

This single substance, designated as LS-antigen, precipitates in equal titers with optimal amounts of L- and of S-antibody and is completely removed from solution by absorption with either antibody.

The LS-antigen of vaccinia appears to be a protein molecule with two antigenically distinct parts, L and S. Heating modifies the L-portion in such a manner that the substance no longer precipitates with L-antibody; this degraded antigen still combines with L-antibody, as is shown by inhibition tests, and still precipitates with S-antibody. Similarly, treatment with heat and dilute alkali modifies the S-portion of LS-antigen so that it combines but does not precipitate with S-antibody; and at the same time all recognizable immunological properties of the L-portion are destroyed.

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