STUDIES ON THE SOLUBLE PRECIPITABLE SUBSTANCES OF VACCINIA

II. The Soluble Precipitable Substances of Dermal Vaccine

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In addition to the agglutinable elementary bodies, suspensions of dermal vaccine contain soluble precipitable substances which may be obtained free of virus (Craigie, 1932; Parker and Rivers, 1935). Craigie, by means of cross-absorption tests, showed that the same antibodies which were involved in the agglutination of washed elementary bodies also precipitated these soluble substances. More recently Craigie and Wishart (1934 a) have reported that two distinct agglutinins occur in antivaccinia sera, the corresponding antigens being distinguished by a marked difference in thermostability. One of these antigens, the L agglutinogen, is inactivated at 56°C. while the S agglutinogen is stable at 95°C. It has also been found that the soluble substances which dissociate *in vitro* from washed elementary bodies contain thermolabile and thermostable components corresponding to the L and S agglutinogens (Craigie and Wishart, 1936). The amount of LS antigen which dissociates *in vitro* from washed elementary bodies is small compared with the amount of soluble precipitable substances (p.p. substances) encountered in fresh vaccine suspensions. It is not known whether the latter substances exist *in vivo* in the state of solution in which they are obtained or whether they are liberated by the virus when vaccine pulp is subjected to manipulation *in vitro*. At present the question of the relationship of these substances to the vegetative form of the virus can be approached only indirectly. One method of approach is by the serological analysis of these substances with reference to the agglutinogens of the elementary bodies. Craigie (1932) found that the elementary bodies

and the p.p. substances (Seitz filterable flocculable substance) showed a serological relationship. This relationship has been reinvestigated since the elementary bodies have been found to possess both thermolabile and thermostable antigens.

EXPERIMENTAL

The preparations of the soluble precipitable substances of vaccinia used in this investigation were derived from dermal lapine processed to obtain suspensions of elementary bodies. The method used has been described in a previous paper (Craigie and Wishart, 1934 a) which should be consulted for details. When the elementary bodies are deposited by angle centrifugation from the lapine extract the supernatant fluid is passed through a Seitz EK filter. These filtrates contain the soluble precipitable substances in solution, generally in titres ranging from 1 in 400 to 1 in 1600. These crude filtrates have a protein content corresponding to 1 to 2 mg. of nitrogen per cc. or more. Although they give satisfactory precipitation reactions with antivaccinia serum, they are usually unsuited for more elaborate serological tests. If heated for the purpose of inactivating thermolabile substances, crude filtrates tend to develop an undesirable degree of opacity or instability. Even more important, however, is the presence of substances which inhibit precipitation. When a serum is absorbed with untreated filtrate, these inhibiting substances are carried over into the absorbed serum and interfere with its subsequent examination for residual antibodies. In a number of instances, however, satisfactory cross-absorption tests have been carried out with selected filtrates. These tests clearly indicated that substances serologically identical with the L and S components of the LS agglutinogen and the dissociated LS antigen were present in these filtrates. In order that these observations on selected filtrates might be confirmed and extended, it was found necessary to devise a method of eliminating the properties of crude filtrates which generally render them unsuitable for absorption tests.

The LS Fraction of Vaccine Filtrates

Wilson Smith (1932) described the preparation of a heat-stable precipitable extract from testicular vaccinia which contained protein

and carbohydrate radicles, while Ch'en (1934), using an elaboration of this method, has obtained a precipitable substance apparently of the nature of a carbohydrate haptene. In repeating and modifying these procedures results were obtained which suggest that the non-antigenic precipitable substances of Smith and Ch'en are components of the antigenic S agglutinogen of elementary bodies. Moreover, evidence has been obtained which suggests that the S antigen occurs in combination with the highly labile L antigen (Craigie and Wishart, 1936). It was apparent, therefore, that any method used to purify the precipitable substances prior to serological investigation must avoid disruption of the LS antigen or inactivation of the L component. A preliminary investigation of the precipitation of the precipitable substances by carbon dioxide, chilled alcohol, and by alum yielded unsatisfactory results, but precipitation by an acid buffer solution seemed more promising. A number of buffer systems were tested and the most consistent results were obtained with the HCl-citrate-NaOH system described below. Accordingly, the pH at which maximum precipitation of the LS antigen occurred was determined for various concentrations of electrolyte and subsequently the conditions of pH, electrolyte concentration, and time for maximum solution of the precipitated antigen with minimum solution of precipitated rabbit protein were investigated. A method which has given consistently satisfactory results was thus arrived at. It should be borne in mind that the pH at which the antigen is precipitated from crude filtrates may be influenced by the nature and concentration of the accompanying proteins. The hydrogen ion concentrations stated in the following description of the method are those found to be requisite for 3rd day dermal lapine produced by the C.L. strain of elementary bodies. Before this method is applied to dermal vaccine filtrates from other strains of vaccine virus, particularly strains such as the Armstrong testicular strain, or to extracts of other organs and tissues infected with vaccinia, preliminary tests should be undertaken to determine whether modification of the pH of the acid buffer is necessary.

Preparation of the LS Fraction.—The vaccine filtrate is first subjected to dialysis in cellophane bags. A 1/10 volume of Sørensen's HCl-citrate buffer (Clark, 1928), pH 4.45, is added to the dialysed filtrate and the mixture centrifuged for 30 minutes. The deposit is drained and fluid adhering to the walls of the tube dried

with filter paper. The deposit is then suspended in Sørensen's citrate-NaOH buffer, pH 6.65, the volume used being 1/10 of the final volume of fraction desired. Four times this volume of distilled water is added and after thorough dispersion of the deposit the suspension is centrifuged and the supernatant set aside. The deposit is extracted a second time with the same volumes of buffer (pH 6.65) and distilled water as previously used. The two supernatant fluids thus obtained are pooled and emulsified with ether and after 24 to 48 hours in the cold room are again centrifuged to remove the precipitate which appears. The supernatant fluid thus obtained will be referred to as the LS fraction. Saturation of the fraction with ether provides a convenient method of preventing subsequent bacterial contamination. It should be noted that this method also provides a means of

TABLE I

Nitrogen Content of Lapine Filtrates and Derived LS Fractions*

No. of filtrate and LS fraction	N per cc.	Precipitating titre (L)	N per 100 units of L antigen (approximate)
	mg.		*mg.*
Filtrate 71	2.39	1 in 400	0.6
LS fraction 71	0.51	1 in 800	0.064
Filtrate 139	1.26	1 in 200	0.63
LS fraction 139	0.12	1 in 400	0.03
Filtrate 175	1.16	1 in 200	0.58
LS fraction 175	0.21	1 in 400	0.053
Filtrate 283	0.882	1 in 400	0.22
LS fraction 283	0.276	1 in 800	0.035

1 unit of LS antigen = amount just yielding perceptible precipitation with optimum amount of L serum in a volume of 0.5 cc.

* Estimated by micro Kjeldahl method.

concentrating the LS antigen by limitation of the volume of buffer used to dissolve the precipitated antigen. The LS fraction thus obtained is, of course, still impure but is much more suitable for serological tests than the original crude filtrate. A considerable amount of protein, however, is soluble at pH 4.45, while further amounts fail to dissolve at pH 6.65 or precipitate when the LS fraction is treated with ether. The proportion of protein to LS antigen is thus reduced and nitrogen estimations indicate that the reduction is an appreciable one.

In Table I the nitrogen content of several crude filtrates and the LS fraction derived from them are shown (*a*) per cubic centimeter of the material and (*b*) corrected for the amount of LS antigen present. In

processing, the material was concentrated 2.5 times and the titres of the LS fractions were approximately twice those of the original filtrates.

There is evidence that reprocessing of the LS fraction results in greater purification, but since the inhibitory substances are reduced to a negligible amount by a single treatment, further manipulation has not been found necessary.

Properties of the LS Fraction

Antigenicity.—The LS fraction prepared by the method which has just been described stimulates the production of agglutinins, precipitins, and complement-fixing antibodies when inoculated into rabbits. It is proposed to deal with these antigenic responses in detail in a subsequent paper. For the present, it may be pointed out that the L and S sera referred to in this and the previous paper (Craigie and Wishart, 1936) were produced by inoculation with LS fraction. The S sera were prepared by the inoculation of LS fraction heated in order to inactivate the L component, while the L sera were derived from sera prepared by inoculation of untreated LS fraction by absorbing the S antibody from them with heated LS fraction.

Thermolability and Thermostability.—The demonstration of thermolabile and thermostable precipitable substances in vaccinia filtrates, or the LS fraction derived therefrom, is dependent on the use of sera which contain only one or other of two distinct precipitins. Both precipitins are present in the sera of rabbits during the 2nd week after vaccination but subsequently diminish or disappear. Hyperimmunization of the vaccinated animal at a later date with (*a*) elementary bodies or (*b*) LS fraction, provides, in most cases, sera of considerably higher titre than does vaccination alone. Both precipitins are developed in response to inoculation with either of these antigens. This may be shown by absorption of the serum with LS fraction heated at 70°C. for an hour, when the absorbed serum will be found to retain its capacity to precipitate unheated LS fraction although the precipitins for the heated fraction have been removed. Table IV provides an example of this phenomenon.

At this point the method of precipitin absorption used should be described. Prior to the absorption tests, Dean and Webb tests are set up to determine the

equivalent amounts of the serum and the antigen preparation. The serum and antigen are then mixed in the ratio indicated by this test, are held for 1 hour at room temperature, and then, after incubation for 1 hour at 50°C., the precipitate is removed by centrifugation. The controls required in tests on the absorbed serum include (*a*) a test for excess antigen by the addition of further precipitin and (*b*) a test for nonspecific inhibitory substances derived from the antigen preparation. In the latter test minimal amounts of antigen and serum which will give evident precipitation are added to a series of dilutions of the absorbed serum. This test is particularly necessary when crude filtrates are used for absorption, since otherwise inhibition of precipitation may be mistaken for evidence of absorption.

Sera, before being used as pure L or pure S sera, must be checked by means of absorption tests. Absorption is, of course, necessary in the case of L sera from which the S antibody must be removed. S sera, although prepared by injection of heated antigen, require to be checked by absorption in case L antibody, formed in response to earlier vaccination, has persisted in the serum. By means of pure L and pure S sera the difference in thermostability of the corresponding antigens may be readily demonstrated (Table II).

As Table II shows, the precipitable substance which reacts with one antibody is thermolabile at 56°C., while that which reacts with the other is stable at 99°C. These substances therefore correspond in their thermostability with the previously described L and S agglutinogens, and the L and S components of the LS antigen dissociated *in vitro* from elementary bodies. It has been the practice to carry out vaccinia precipitin reactions at 56°C. In view of the thermolability of the L substance the question arises as to whether 50°C. is not too high a temperature of incubation when precipitation of this substance is involved. Tests carried out at various temperatures have indicated that 50°C. promotes a greater extent of precipitation than 56°C. or 37°C. Table III shows the results obtained when a constant amount of antigen was mixed with various dilutions of serum and incubated at various temperatures.

As this table shows, L precipitation is weakened at 56°C., compared with that at 50°C., and is also decreased at temperatures lower than 50°C. The comparatively feeble precipitation which is obtained at cold room temperature is worthy of note since maximum complement fixation occurs at this temperature, presumably on account of the

greater antigen-antibody surface retained with delayed aggregation (Craigie and Wishart, 1934 b). Other tests carried out at 50°C. in which serum and antigen were preheated to 50°C. and also mixed and allowed to stand at room temperature for periods up to 1 hour showed no difference in the degree of precipitation. Apparently, therefore,

TABLE II

Thermostability of L and S Precipitinogens

Treatment of LS fraction 238	L serum 105, 1 in 30, and LS fraction diluted					S serum 335, 1 in 40, and LS fraction diluted				
	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1600	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1600
Untreated	+++	+++	++	+	−	+++	+++	++	+	−
1 hr. at 56°C.	−	−	−	−	−	+++	+++	++	+	−
1 hr. at 70°C.	−	−	−	−	−	+++	+++	++	+	−
15 min. at 99°C.	−	−	−	−	−	+++	+++	++	±	−

Plus signs indicate amount of precipitation.

− = no precipitation or opacity.

Tests read after incubation at 50°C. for 1 hour.

TABLE III

Influence of Temperature of Incubation on Precipitation

Temperature of incubation for 18 hrs.	LS fraction 234, 1 in 300, and LS serum 329 diluted				LS fraction 234, 1 in 300, and L serum E6 diluted				Heated LS fraction 225, and S serum 243 diluted		
	1 in 20	1 in 40	1 in 80	1 in 160	1 in 20	1 in 40	1 in 80	1 in 160	1 in 20	1 in 40	1 in 80
°C.											
56	++++	++++	++	+	+++	±	−	−	+++	+	−
50	++++	++++	+++	++	+++	++	±	−	+++	±	−
37	++++	+++	+++	++	+++	+	−	−	+++	±	−
18	+++	++	++	+	+	±	−	−	++	−	−
5	+	+	−	−	±	−	−	−	±	−	−

50°C. is a suitable temperature for the incubation both of L and S precipitin tests and the preliminary period of contact at room temperature, up to 1 hour, has no significant effect. For complete precipitation with small amounts of antigen or antibody a period of at least 8 hours is necessary and it has been found convenient to incubate the tests overnight in open tubes in a closed water bath at 50°C.

Relationship of L and S Antigens.—By heating the LS fraction, the L substance may be eliminated, thus yielding preparations which apparently contain only the S substance. In the hope that corresponding preparations containing only the L substance might be obtained, attempts were made to absorb the S substance from the LS fraction by means of pure S serum. It was found, however, that as in the case of the dissociated LS antigen (Craigie and Wishart, 1936) both substances were removed from solution when either pure S or pure L sera were added. As suggested in the previous paper, this probably indicates that the L and S antigens are different antigenic

TABLE IV

Treatment of serum 366*	Serum titre for					
	Elementary bodies		Dissociated antigen		LS fraction	
	N	H	N	H	N	H
Unabsorbed	1 in 640	1 in 320	1 in 80	1 in 40	1 in 40	1 in 40
Absorbed with heated elementary bodies	1 in 640	0	1 in 40	0	n.t.	n.t.
Absorbed with LS fraction	0	0	0	0	0	0
Absorbed with heated LS fraction	1 in 640	0	1 in 80	0	1 in 40	0

N = untreated.
H = heated for 1 hour at 70°C.
0 = negative 1 in 5.
n.t. = not tested.
* Obtained by hyperimmunization with LS fraction.

components of a complex LS antigen rather than two independent antigens. The corresponding antibodies, however, are independent and sera containing either may be readily obtained. The relative amounts of L and S antibody formed in response to inoculation with LS antigen vary in different rabbits. In contrast to this, the titres of L and S substances in filtrates, or dissociated from elementary bodies, show an almost constant ratio to each other irrespective of the actual amounts present.

Serological Relationships to the Elementary Body Agglutinogens.—Evidence that the L and S antibodies which precipitate the L and S

soluble substances are also involved in the agglutination of washed elementary bodies has been obtained by absorption tests. A number of such tests have yielded results similar to those shown in Table IV, which indicate that the LS antigen found in solution in fresh vaccine pulp is serologically similar to antigens retained by washed elementary bodies.

As illustrated in Table IV, complete removal of agglutinins results on the addition of the equivalent amount of LS fraction to many sera prepared by hyperimmunizing rabbits with LS fraction. In contrast with this, it has been found that LS fraction may not remove all agglutinins from sera prepared by hyperimmunization with infective elementary bodies (Table V).

TABLE V

Serum No.	Treatment of serum	Serum titre for		
		Elementary bodies	LS fraction	Heated LS fraction
204*	Unabsorbed	1 in 1280	1 in 80	1 in 40
	Absorbed with LS fraction	1 in 320	0	0
331*	Unabsorbed	1 in 1280	1 in 160	1 in 80
	Absorbed with LS fraction	1 in 320	1 in 5	1 in 5

0 = negative 1 in 5.

*Obtained by hyperimmunization with infective elementary bodies.

These residual agglutinins presumably indicate additional antigens in the elementary bodies and it is suggestive that sera prepared with LS fraction are usually deficient in respect of these agglutinins. It has also been found that different elementary body suspensions vary considerably in their sensitivity to agglutination by these residual agglutinins. Further investigation of these additional antigens is being undertaken.

DISCUSSION

The observations reported in this and in two previous papers (Craigie and Wishart, 1934 a, 1936) indicate that the agglutination and precipitin reactions of vaccinia are for the most part referable to

the LS antigen. This antigen produces two distinct kinds of antibody, one of which reacts with its thermolabile L component, the other with its thermostable S component. Each of these antibodies may produce agglutination or precipitation according to whether the antigen is present on elementary bodies or is in a state of solution. Agglutinin and precipitin absorption tests provide evidence that the LS antigen occurs (*a*) on the elementary bodies of vaccinia, (*b*) in solution in fresh suspensions of dermal vaccine. *In vitro*, the LS antigen may be observed to dissociate from the elementary bodies and it would seem reasonable to assume that the LS antigen found in solution in fresh dermal vaccine represents antigen dissociated from the virus. However, it is not known whether the LS antigen of dermal vaccine exists *in vivo* in the state of solution in which it is recovered or whether its liberation from the virus is brought about by manipulation of the vaccine pulp. The question of the virus origin of this antigen has been previously discussed (Craigie and Wishart, 1936) when it was pointed out that the highly resistant elementary bodies which may be isolated from vaccinia-infected tissue do not, from a functional point of view, represent the only form in which vaccine virus may exist. It was suggested that the LS antigen was a specific product of vaccine virus, not in the resting, resistant state shown by elementary bodies *in vitro*, but in the active phase associated with its proliferation in a suitable cellular environment.

The LS antigen is specifically related to the viruses of vaccinia and variola. It has been found in dermal vaccine extracts of the guinea pig and calf as well as the rabbit (Craigie and Wishart, 1935). In the rabbit the C.L. strain of vaccine virus has been principally used in these investigations but unpublished observations show that the same LS antigen is formed by other strains, such as the Armstrong testicular strain, the Mulford Z encephalitogenic strain, and variola strains adapted to the rabbit. In all circumstances in which this antigen is found, L and S components are present in an approximately constant ratio unless the material has been exposed to conditions which inactivate the L component.

The thermolability of the L antigen at 56°C. is of interest. In this respect it resembles the Vi agglutinogen of *B. typhosus* but it differs in regard to its stability to ether. The L antigen of vaccinia is stable

to ether but the Vi agglutinogen of *B. typhosus* is inactivated by this reagent. Craigie and Tulloch (1931) found that the flocculable substance separated by CO_2 precipitation from vaccinia extracts was heat-labile at 56°C. It is now known that both L and S components are precipitated by CO_2 and the previous failure to detect the latter is to be attributed to the use of sera in concentrations in which only L precipitin was present in effective concentrations. When the LS antigen is heated at 70°C. the L component is destroyed but the S component remains antigenic. A few tests indicate that the antigenicity of the S component is considerably reduced by steaming it and the heat-stable precipitable substances described by Wilson Smith (1932) and by Ch'en (1934) were obtained by boiling vaccinia extracts in acid and these were found by them to be nonantigenic. It seems probable that the carbohydrate obtained by Ch'en represents a polysaccharide haptene of the S component of the LS antigen, but further enquiry in this direction is desirable. Hughes, Parker, and Rivers (1935) applied the Molisch test and precipitin reaction to each of seven wash waters obtained during the purification of a batch of elementary bodies, since previous analyses of washed elementary bodies showed comparatively small amounts of carbohydrate. Examination of the wash waters from the elementary bodies showed that the Molisch and precipitin tests roughly paralleled each other and that the amounts of carbohydrate and precipitable substance progressively decreased in successive wash waters. Further study of the carbohydrate fraction in relation to the LS antigen dissociated from elementary bodies should be of interest.

The LS antigen is probably not the only antigen of vaccinia virus which reacts *in vitro*. In addition to a neutralizing antibody which inactivates the elementary bodies *in vitro*, agglutinins distinct from the L and S agglutinins are found in some antivaccinia sera. While the terms L and S were first used in reference to the agglutinogens of elementary bodies it is no longer desirable to define them in this way, at least until further investigation of the additional agglutinogens has been carried out. LS fraction prepared and preserved by the method described in this paper appears to be deficient in these additional antigens and the L and S antigens are preferably to be defined as the thermolabile and thermostable components of the LS antigen obtained in this way.

We are indebted to Mr. M. D. Orr, Connaught Laboratories, for his kindness in undertaking the nitrogen estimations recorded in Table I.

CONCLUSIONS

1. Thermolabile (L) and thermostable (S) antigens occur in solution in suspensions of vaccine pulp.

2. These antigens are also present on the elementary bodies of vaccinia and participate in the agglutination of these bodies by anti-vaccinia serum.

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