THE LS-ANTIGEN OF VACCINIA

III. PHYSICAL-CHEMICAL PROPERTIES OF LS-ANTIGEN AND SOME OF ITS DEGRADATION PRODUCTS

BY THEODORE SHEDLOVSKY, Ph.D., ALEXANDRE ROTHEN, Ph.D., AND JOSEPH E. SMADEL, M.D.

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

(Received for publication, November 13, 1942)

The isolation of a substance, LS, with the serological properties of both the heat-table (L) and heat-stable (S) antigens of vaccinia in a single protein molecule has been described in an earlier paper of this series (1). In the present communication we shall present the results of additional physical-chemical studies on this substance.

LS is a globulin-like protein which is relatively insoluble at values of pH below 5.5. It contains no phosphorus, nucleic acid, lipid, or glucosamine (2). Preparations of the purified native material, which are homogeneous by electrophoresis and in the analytical ultracentrifuge, can be degraded by heat, heat and alkali, or chymotrypsin with interesting results.

Gentle heating of LS-solutions destroys the specific precipitability of the L-antigen with L-antibody, but not its inhibiting power, nor the serological properties of the S-antigen (1). The altered substance (L'S) remains homogeneous electrophoretically, although the mobility is changed. Examination in the ultracentrifuge reveals, however, some polydisperse material. This is not surprising since the process of degradation may produce alterations in the molecule other than those affecting the L-portion specifically. But it appears from electrophoresis that the altered molecules all have the same average charge distribution.

Heating in the presence of 0.1 N alkali destroys all serological properties of the L-antigen with L-antibody, but not its inhibiting power, nor the serological properties of the S-antigen (1). The altered substance (L'S) remains homogeneous electrophoretically, although the mobility is changed. Examination in the ultracentrifuge reveals, however, some polydisperse material. This is not surprising since the process of degradation may produce alterations in the molecule other than those affecting the L-portion specifically. But it appears from electrophoresis that the altered molecules all have the same average charge distribution.

Carefully controlled enzymatic digestion with chymotrypsin results in a substance (LS*) which no longer possesses any S-properties but retains the original L-activity intact (2). This material comes out of solution in the form of needle-like crystalloids which are relatively insoluble at pH values below 9. It has not been examined by electrophoresis nor in the ultracentrifuge.

We shall submit at this time the results of electrophoretic and ultracentrifugal measurements on the parent substance, LS, and the related substances, L'S and L'S'. Determinations of the diffusion coefficient and specific volume of LS will also be reported. From these data it is possible to estimate the isoelectric point, molecular weight, and shape of this protein.
Materials and Methods

Solutions of purified LS-antigen were prepared in the manner previously described from the dermal filtrate of vaccinia-infected rabbits (1).

The methods employed in our serological and electrophoretic studies have been mentioned in the earlier papers of this series (1, 3, 4). In order to determine the isoelectric point of LS it was necessary to carry out some of the electrophoretic mobility determinations at pH values at which the substance is scarcely soluble. This was accomplished in the Tiselius apparatus after removing the larger precipitated particles with low speed centrifugation. Measurements were made of the electrical migration of the boundaries between the slightly turbid preparations thus obtained and the clear buffer solutions which served in the preparative dialysis.

In obtaining the ultracentrifugal data we employed an air turbine-driven analytical centrifuge of the Bauer and Pickels type (5) with the optical system of Philpot and Svensson (6, 7).

Density measurements, which served to determine the partial specific volume of LS, were carried out in the usual manner with the aid of a precision pycnometer and a constant temperature bath.

The Longsworth schlieren-scanning method (8) in conjunction with the Tiselius apparatus was used for the diffusion measurements as well as for electrophoresis.

RESULTS AND DISCUSSION

Electrophoresis.—The electrophoretic homogeneity of LS is demonstrated by the presence of only one significant boundary in experiments carried out with solutions at different values of pH. A typical pattern, obtained at pH 7.9 and an ionic strength, 0.05, is shown in Fig. 1. The small, relatively immobile
boundaries marked ε and δ in the descending and rising sides of the cell, respectively, are not due to any impurities in the preparation, but to salt and total protein gradients the nature of which is well known (9). A summary of the

<table>
<thead>
<tr>
<th>Preparation</th>
<th>pH</th>
<th>Mobility (cm./volt·sec./cm.) × 10^-5</th>
<th>Buffer composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>E19</td>
<td>4.47</td>
<td>2.0</td>
<td>Lithium acetate—acetic acid</td>
</tr>
<tr>
<td>E19</td>
<td>5.41</td>
<td>-2.6</td>
<td>&quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>E19</td>
<td>6.58</td>
<td>-3.98</td>
<td>Lithium cacodylate—cacodylic acid</td>
</tr>
<tr>
<td>E20</td>
<td>6.64</td>
<td>-4.02</td>
<td>&quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>E18</td>
<td>7.86</td>
<td>-4.61</td>
<td>Lithium chloride = lithium veronal—veronal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(acid)</td>
</tr>
<tr>
<td>E18</td>
<td>7.90</td>
<td>-4.73</td>
<td>&quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>E19</td>
<td>7.90</td>
<td>-4.81</td>
<td>&quot; &quot; &quot; &quot;</td>
</tr>
</tbody>
</table>

Fig. 2. Plot of mobility, U, vs. pH for LS at 0°C, in 0.05 M monovalent buffers.

results of mobility measurements at 0°C., 0.05 ionic strength, and different values of pH is given in Table I and plotted in Fig. 2. As we have already mentioned, LS comes out of solution below pH 5.5. Accordingly, the patterns for the experiments at pH 4.47 and 5.41 are of "turbidity" boundaries, one of which is shown in Fig. 3. The broken portion of the curve in Fig. 2 corresponds to this turbid region of pH. It crosses the zero axis of mobility at pH 4.8,
Fig. 3. Electrophoretic pattern of LS "turbidity" boundaries in 0.05 M acetate buffer at pH 5.41. The descending boundary is at the left, the rising boundary at the right.

Fig. 4. Electrophoretic patterns of (a) L'S and (b) L'S' in 0.05 M lithium veronal buffer at pH 7.85. The descending boundaries are at the left, the rising boundaries at the right.
thus locating the isoelectric point of LS at this value. The mobility of proteins is not independent of salt concentration, however (10). Experiments carried out in lithium-veronal buffer at pH 8.45 gave mobility values for LS of $-4.1 \times 10^{-5}$ and $-8.0 \times 10^{-6}$ cm./sec. per volt/cm. at ionic strengths of 0.10 and 0.01 respectively.

Patterns on L'S (LS heated at 56°C. for 30 minutes) and on L"S' (LS heated at 56°C. for 90 minutes with 0.10 M sodium hydroxide) are shown in Fig. 4a and b. Here the ionic strength was 0.05, pH 7.85. The material remains electrophoretically homogeneous although the mobility, which is $-4.6 \times 10^{-5}$ for LS under similar conditions, is $-6.0 \times 10^{-5}$ for L'S and $-6.4 \times 10^{-5}$ for L"S'. Thus it appears that destruction of the precipitability of the L-antigen with L-antibody is paralleled by a considerable reduction in the net electric charge of LS, but that further degradation, which completely inactivates the L-antigen and alters the S-antigen, has relatively little effect on the net charge as L'S changes to L"S'.

**TABLE II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>LS concentration per cent</th>
<th>Salt concentration</th>
<th>pH</th>
<th>Temperature °C.</th>
<th>Sedimentation constant in Svedberg units* (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E19</td>
<td>0.3</td>
<td>0.05 lithium chloride veronal</td>
<td>7.9</td>
<td>4.1</td>
<td>4.3 6.6</td>
</tr>
<tr>
<td>E20</td>
<td>0.4</td>
<td>0.05 lithium cacodylate</td>
<td>6.4</td>
<td>20.1</td>
<td>6.3 6.3</td>
</tr>
<tr>
<td>E22</td>
<td>0.3</td>
<td>0.125 lithium chloride veronal</td>
<td>7.8</td>
<td>18.1</td>
<td>5.9 6.2</td>
</tr>
<tr>
<td>E22</td>
<td>0.3</td>
<td>&quot;&quot;</td>
<td>7.8</td>
<td>21.0</td>
<td>6.5 6.2</td>
</tr>
<tr>
<td>E22</td>
<td>0.15</td>
<td>0.125 &quot;&quot;</td>
<td>7.8</td>
<td>17.4</td>
<td>5.9 6.3</td>
</tr>
</tbody>
</table>

*1 S = 10^{-13} seconds.

Ultracentrifugation.—Three different preparations of LS were studied in the ultracentrifuge. A summary of the results is presented in Table II. Two samples (E19 and E20) were homogeneous with respect to size, but another (E22) contained about 14 per cent of material which sedimented 0.65 times slower than the main component. This can be seen in Fig. 5a. In Fig. 5b exposures at different time intervals are shown. It can be seen from the table that a change in concentration from 0.3 per cent to 0.15 per cent does not pro-

This preparation was also found by electrophoresis to contain a second component. Attempts to remove it by several reprecipitations failed. It was subsequently shown that the minor component was L'S, which cannot be removed by the precipitation procedures we employed once it appears with LS as a contaminant. We have no exact knowledge as to how the L'S was formed in this instance. However, we have noted previously that this degradation product occasionally occurs.
duce any appreciable change in the sedimentation constant, $s_{20\text{w}}$. These values, corrected to 20°C, listed in the last column of the table, have been computed from the measured sedimentation constants, $s_t$, in column 6, at the corresponding temperatures in column 5. The apparent discrepancy between $s_{20\text{w}} = 6.6$ S (11) and $s_{20\text{w}} = 6.3$ S for the other four determinations (E20) and E22 near 20°C.) is probably due to the much larger and therefore less certain temperature correction required in the first case than in the others (Table II). We therefore assign the value of 6.3 S for the sedimentation constant of LS at 20°C.

The results obtained with L'S (heated LS), in the ultracentrifuge were not consistent, as we see in Table III. The first preparation (Experiment 1),
Fig. 6 a and b, showed two fairly homogeneous components with sedimentation constants of 5.6 S and 4.1 S at 20°C. Another preparation (Experiment 2) showed one homogeneous component ($s_{20w} = 6.3 \text{ S}$) and some very heterogeneous material. The third preparation (Experiment 3), Fig. 6c showed very heterogeneous material with an average value of $s_{20w} = 3.0 \text{ S}$. It seems possible that endproducts of different size may be obtained as a result of small variations in the conditions during the heating procedure. Experiment 3 of Table III was carried out in a “separation” cell which permitted the isolation of most of the heterogeneous matter from the more homogeneous component. Serological examination of these two fractions showed them to be equally active.

A sample of L"S' obtained by heating LS in 0.10 n alkali for an hour and a half was also examined in the centrifuge. It was found to be completely heterogeneous, no boundary being apparent at any time with speeds as high as 57,000 R.P.M. Yet L"S' was found to be quite homogeneous in electrophoresis (Fig. 4b), indicating similar charge distribution on the surface of the various fragments.

**Diffusion of LS**.—The coefficient of diffusion, $D$, which serves to estimate molecular weight and molecular shape, can be obtained from schlieren-scanning patterns of an initially sharp boundary, taken at intervals over sufficient time, depending on the rate of diffusion. The following equation was used in the computation (12):

$$D = \frac{1}{4\pi t} \left( \Delta \frac{n}{X_m} \right)^2 = \frac{\Delta n^2}{4\pi t X_m^2}$$  \hspace{1cm} (1)

in which $\Delta n$ is the refractive index increment of the boundary, which remains constant in a given experiment and is proportional to the area under the “peak,” $t$ is the time, and $X_m$ is the height of the peak. If one prepares a plot of $X_m$ values against $1/\sqrt{t}$ from the data a straight line should result passing through
Fig. 6. Ultracentrifuge patterns of L/S at 57,000 r.p.m. The material is sedimenting from right to left.

(a) Pattern 68 minutes after reaching maximum speed.

(b) Three superposed patterns of the same experiment. The three peaks correspond to the position of the main component at different times. The time intervals between the first and second and the first and third exposure are 70 and 119 minutes respectively.

(c) Pattern obtained with a separation cell. The discontinuity observed is due to the presence of a perforated partition of the cell. Two exposures are superposed taken 16 minutes apart. The heterogeneity is apparent from the large decrease in the height of the peak and also from the upward displacement of the baseline.

The origin (12). The slope, $m$, of this line gives us the diffusion coefficient $D$ by equation 1.

Such a plot, for L/S at 4°C. at pH 6.47 and ionic strength 0.15, is shown in Fig. 7. In this experiment $\Delta n$ was 0.99 and from the plot $m$ was found to be
720 cm. sec. The resulting value for $D$ is $1.43 \times 10^{-7}$ cm.$^2$/sec. Another determination with a different preparation, at the same ionic strength but at pH 7.8, gave a value of $1.52 \times 10^{-7}$ cm.$^2$/sec. for $D$. Accordingly, we have used $D = 1.50 \times 10^{-7}$ cm.$^2$/sec. for computing the molecular weight and shape factor for LS.

Density and Partial Specific Volume of LS.—LS-protein contains 15.8 per cent nitrogen (2). The density of a solution containing 0.255 mg./cc. of nitrogen, which corresponds to 1.61 mg./cc. protein, was measured at 4°C. in a pyconometer with a volume of about 10 cc. The value found was $d_4^0 = 1.39$ gm./cc., or $v = 0.72$ cc./gm. for the partial specific volume of this substance.

Molecular Weight and Shape of LS.—To compute the molecular weight of LS we make use of the familiar equation (5):

$$M = \frac{RTs}{(1 - \bar{\rho})D}$$

in which $M$ is the molecular weight, $s$ is the sedimentation constant at the absolute temperature $T$, $\rho$ is the density of the solvent, $\bar{\rho}$ is the partial specific volume of the substance whose diffusion coefficient is $D$, and $R$ is the gas constant. Thus we find from the data reported in this paper a value of 214,000 for the molecular weight of LS protein.
The asymmetry factor, $f/f_0$, from which the shape of the molecule may be estimated, is given by the equation (5):

$$f/f_0 = \frac{RT}{6\pi\eta D} \left(\frac{4\pi N}{3M}\right)^{1/3}$$

Here $\eta$ is the viscosity of the solution, $N$ is Avogadro's number ($6.06 \times 10^{23}$), and the other quantities have already been defined. The factor, $f/f_0$, which is unity for spheres, turns out to be 2.05 for LS. This value indicates a very elongated molecule, with an axis ratio of 1:20 if one assumes an oblong ellipsoidal shape.

**SUMMARY**

Studies on LS-protein, the soluble double antigen of vaccinia, and on the degradation products L'S and L'S' have been made with electrophoresis and in the analytical ultracentrifuge.

LS, which is homogeneous electrically and in the ultracentrifuge, has an isoelectric point at pH 4.8. At 4°C, its partial specific volume is 0.72 cc./gm., and its diffusion constant is $1.50 \times 10^{-7}$ cm$^2$/sec. The sedimentation constant is 6.35 at 20°C, the molecular weight is 214,000, and the molecule appears to have an elongated ellipsoidal shape with an axis ratio of 1:20.

L'S and L'S' are homogeneous electrically but not in the ultracentrifuge, L'S' being extremely polydisperse.

**BIBLIOGRAPHY**