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Soluble Antigens of Vaccinia-infected Mammalian Cells

I. Separation of Virus-induced Soluble Antigens into Two Classes on the Basis of Physical Characteristics

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ABSTRACT

COHEN, GARY H. (University of Pennsylvania, Philadelphia), AND WESLEY C. WILCOX. Soluble antigens of vaccinia-infected mammalian cells. I. Separation of virus-induced soluble antigens into two classes on the basis of physical characteristics. J. Bacteriol. 92:676-686. 1966—Infection of mammalian cells with members of the poxvirus group elicits production of a number of virus-induced, soluble antigens. Immunoelectrophoresis and immunodiffusion techniques employing soluble antigen preparations obtained from vaccinia virus-infected KB cells revealed at least seven well-defined immunoprecipitin bands. On the basis of fractionation and subsequent characterization of the soluble antigen mixture by gel filtration, calcium phosphate chromatography, isoelectric precipitation, disc electrophoresis, and ultracentrifugation studies, two distinct classes of virus-induced antigens differing markedly in molecular weight were recognized. A high molecular weight class (200,000 and greater) contained at least three virus-induced antigens; a low molecular weight class (50,000 to 100,000 range) contained at least four immunoprecipitins. Further separation of the antigens within the two groups was accomplished. The two classes were distinguished also by their ability to stimulate synthesis of virus-neutralizing antibody. Antisera prepared against the high molecular weight class proved effective in neutralizing vaccinia virus. In contrast, the low molecular weight antigens showed little, if any, ability to induce formation of neutralizing antibody.

Infection of mammalian cells with members of the poxvirus group elicits the production of a number of virus-induced, soluble antigens. There is, at present, no unanimity of opinion regarding the exact number of such antigenic proteins. Recent estimates range from 8 to 20 individual immunogenic proteins (1, 12). Specific information regarding the role of the virus-induced, soluble antigens in the infectious process is lacking, although one may surmise from available evidence that some of these antigens represent virus-induced enzymes (8, 10, 11), and that others are virus structural proteins produced in excess (2). One must also entertain the possibility, however unlikely, that other classes of virus-induced proteins concerned neither with enzyme activity nor virus structure exist.

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Studies have been undertaken in this laboratory to isolate and identify individual virus-induced soluble antigen proteins, to relate the appearance of these proteins to the time course of virus biosynthesis, and, wherever possible, to ascertain the role of each of these materials in the infectious process. It is the purpose of this communication to present data which show that the vaccinia-induced soluble antigens fall into two distinct physical classes. In addition, data are presented which define some of the immunological characteristics of the soluble antigens and which relate these classes to the LS fractions originally described by Cragie and Wishart (3).

MATERIALS AND METHODS

Cell culture. KB or L cells were grown as monolayers in bottles containing Eagle's minimal essential medium supplemented with 10% calf serum. For certain experiments, KB cells were grown in suspension cultures also supplemented with 10% calf serum.

Virus. A substrain of the vaccinia IHD strain was employed throughout. This substrain, designated as IHD^-, does not elicit production of hemagglutinin in infected mammalian cells.

Virus purification. Virus obtained from homogenates of infected KB cells was purified as described by Joklik (6).

Virus titration. The plaque technique was employed with the use of primary chick embryo monolayers and agar overlays in essentially the same manner described by Salzman et al. (14).

Soluble antigen preparations. Virus-infected cells, washed and concentrated 50- to 100-fold in phosphate-buffered saline (PBS), were disrupted by sonic treatment for three intervals of 1 min each at 10 kc with a Raytheon sonic oscillator. The preparations thus obtained were centrifuged for 30 min at 100,000 $\times$ *g*, after which the supernatant fluids were collected and dialyzed versus 0.01 M phosphate buffer (*p*H 7.2). This crude material containing less than 0.01% of the infectivity of the uncentrifuged concentrate and exhibiting very high titers of virus-induced complement-fixing antigens was termed the soluble antigen mixture (SAM).

The LS antigen fraction was prepared from SAM by a modification of the procedure of Cragie and Wishart (3). Briefly, after membrane filtration (GS, 220 mμ; Millipore Filter Corp., Bedford, Mass.), SAM was dialyzed versus acetate buffer (*p*H 4.6) to yield a heavy precipitate and a clear supernatant fraction. The precipitate, when collected by centrifugation and dialyzed overnight against cacodylate buffer (*p*H 6.3), is partially soluble. This soluble fraction, by convention, is termed the LS antigen. The insoluble fraction was dissolved in barbital buffer and 0.1 N NaOH. The original supernatant fractions, containing proteins and other macromolecules not precipitated at *p*H 4.6, is referred to hereafter as the acid-soluble (AS) fraction. The LS and AS fractions were adjusted to neutrality, and the procedure, just described, was repeated to effect a further purification. The final fractions, which contained essentially all of the original soluble-complement-fixing activity, were then dialyzed versus 0.01 M phosphate buffer (*p*H 7.2) and centrifuged at 100,000 $\times$ *g* for 1 hr.

Preparation of antisera. Antisera directed against virus and presumably against the entire spectrum of virus-induced proteins were produced by infecting rabbits with purified virus via the intradermal route. After recovery, such animals were hyperimmunized by four intravenous (iv) injections of 1 ml each of the same virus preparation. In contrast, antisera directed solely against virus-induced soluble antigens were prepared by immunizing rabbits with a series of three intramuscular injections of the appropriate antigen suspended in Freund's complete adjuvant. Such antigen preparations were rendered free from virus by three cycles of centrifugation (23,000 $\times$ *g*, 30 min), followed by filtration (GS, 220 mμ; Millipore Filter Corp.). The efficacy of this procedure for the total removal of infectious virus was monitored by appropriate virus titrations. Sera produced in this manner were adsorbed with concentrated sonic-treated preparations of KB or L cells, as required, to remove antibodies directed against normal cell and serum components. Such adsorbed sera did not react with sonic-treated preparations of normal cells or with calf serum components when tested by complement fixation, immunoelectrophoresis, and immunodiffusion.

Virus-neutralization tests. Inasmuch as only a rough comparison of the ability of various antigen fractions to induce production of neutralizing antibody was needed, a simple plaque-reduction test was employed. Serial twofold dilutions of the various antisera were added to an equal volume of virus diluted in PBS to yield a final concentration of 80 to 150 plaque-forming units per tube. The serum-virus mixture was incubated at 37 C for 1 hr, and 0.25 ml of each mixture was placed on each of four chick embryo monolayers. After a 1.5-hr adsorption period, agar overlays were added, and the titration was carried out as described above. The greatest dilution of serum causing a 70% or greater reduction of the plaque count as compared with virus-preimmune serum mixtures (serum dilution, 1:20) was arbitrarily selected as the neutralizing titer. The comparative neutralizing capacity is expressed as the reciprocal of this dilution.

Complement fixation. A microtechnique, described by Sever (15), was employed. Titers are expressed as the highest dilution of antigen yielding complete fixation. The test system employed 4 units of antibody.

Gel filtration. The technique utilized for gel filtration studies was similar to that described by Flodin (Ph.D. Thesis, Pharmacia, Uppsala, Sweden, 1962). Sephadex G 50, G 75, and G 200 (Pharmacia, Uppsala, Sweden), as well as Biogel 300 (Bio-Rad Laboratories, Richmond, Calif.), were utilized. The buffer system was 0.1 M NaCl in 0.05 M phosphate buffer (*p*H 7.2).

Disc electrophoresis. Electrophoresis in 5.5% polyacrylamide gel was performed by a modification of the method of Davis (5). Samples were applied in 20% sucrose rather than polymerized in gel prior to electrophoresis. The R_F values were calculated for each stained protein band by dividing the migration distance of each band by the migration distance of the front moving bromophenol blue dye band.

Immunodiffusion and immunoelectrophoresis. Immunodiffusion was performed by the method of Ouchterlony (13) as modified by Crowle (4), with the use of 1% Ionagar No. 2 (Colab Laboratories, Inc., Chicago Heights, Ill.) in saline. Immunoelectrophoresis was carried out by the method of Scheidegger (17) on glass slides (3 by 2 inches; 7.6 by 5 cm). Runs of 100 min at 10 ma per slide were generally employed.

Ultracentrifugation. Ultracentrifugal studies were carried out in linear density gradients of 5 to 20% (w/v) sucrose made up in 0.001 M phosphate buffer (*p*H 7.2). Gradients were formed after the addition of a cushion of 0.1 ml of 40% sucrose to each tube. Such gradients were centrifuged at 115,000 $\times$ *g* for 22 hr in the SW 39 rotor of a Spinco model L ultracentrifuge; the tubes were punctured and drop fractions were collected from the bottom. The method of Lowry et al. (9) was employed to determine protein concentration.

Calcium phosphate. Calcium phosphate was prepared according to the method of Swingle and Tiselius (18). Samples were dialyzed against 0.01 M PO_4 (*p*H

7.2) and added to the columns. The columns were washed with 0.01 M phosphate buffer and eluted with linear gradients of NaCl in the same buffer.

Radioactivity determinations. Uniformly labeled C^{14}-valine with a specific activity of 205 mc/mmole (Schwarz BioResearch Inc., Orangeburg, N.Y.) was added to cultures at time of infection to label protein. Duplicate samples were counted in a Nuclear-Chicago gas-flow windowless automatic counter. The number of disintegrations counted per sample was adjusted so that the counting error was less than 5%.

RESULTS

Crude fractionation of sonic-treated vaccinia-infected KB cells. One can, by means of centrifugation, separate sonic-treated vaccinia-infected mammalian cells into crude fractions as shown in Table 1. The first fraction, sedimented by centrifugal forces of 15,000 × *g* or over, contained cellular debris and most of the newly synthesized virus. This material, however, exhibited only a relatively small amount of virus-specific complement-fixing activity. The supernatant fluids, in contrast, contained little virus and the bulk of the virus-induced complement-fixing antigens. These are referred to by convention as soluble antigens.

One may, in fact, submit sonic-treated material to centrifugal forces as great as 100,000 × *g* for 30 min or more and still retain 50% or more of the original complement-fixing activity in the supernatant fluids. There is no evidence that centrifugal forces in excess of 15,000 × *g* selectively remove antigens of a particular species from suspension.

Examination of soluble antigen preparations by immunological and physical methods. A number of independent studies indicated that vaccinia-infected cells synthesize a complex mixture of soluble antigens (1, 12). In this laboratory the following results were obtained under the conditions employed.

(*i*) *Immunodiffusion.* The complex pattern shown diagrammatically in Fig. 1a was generally obtained with concentrated SAM. One may conservatively estimate from these data that

TABLE 1. *Fractionation of vaccinia-infected sonic-treated cells by centrifugation*

Fraction	Infectivity (pfu/ml)	CF activity (per 0.025 ml)
Original sonic preparation..	7.6×10^8	16,384
Sediment, 15,000 × *g*.....	5.5×10^8	2,048
Supernatant fluid, 15,000 × *g*	9.0×10^5	16,384
Supernatant fluid, 100,000 × *g*.....	3.6×10^3	8,192

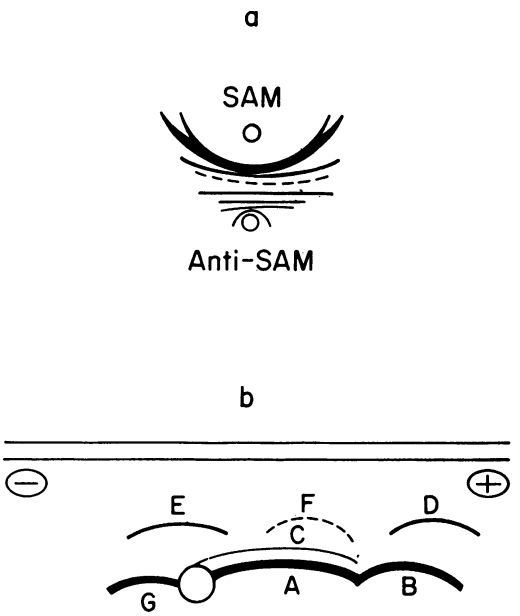


FIG. 1. *Immunoprecipitin patterns obtained from soluble antigen mixture employing (a) agar gel immunodiffusion and (b) immunoelectrophoresis. In each case antisera directed against a virus-free soluble antigen mixture (anti-SAM) were employed.*

there are at least seven, perhaps more, distinct immunoprecipitin bands.

(*ii*) *Immunoelectrophoresis.* This technique effected a better physical separation of the various virus-induced antigens and revealed once again the presence of at least seven antigens (Fig. 1b). One must, however, entertain the possibility that some of the more prominent arcs represent more than one antigen and, therefore, that seven represents only a minimal number. To facilitate study of the soluble antigen fractions, the well-defined precipitin arcs have been assigned letters (A through G), as designated in Fig 1b.

(*iii*) *Gel electrophoresis.* Disc acrylamide electrophoresis of concentrated SAM, when compared with normal cell extracts, revealed the presence of two prominent protein bands unique to infected cells (Fig. 2). These bands migrated with characteristic and highly reproducible rates, permitting the calculation of the following R_F values for each: upper band, R_F 0.25; lower band, R_F 0.45. Although antigens obtained from the elution of the R_F 0.25 and 0.45 bands appeared identical by immunodiffusion, immunoelectrophoresis revealed certain physical differences. The R_F 0.25 protein or proteins appeared identical to the A precipitin arc and the R_F 0.45 antigen, to the B precipitin arc, as seen after

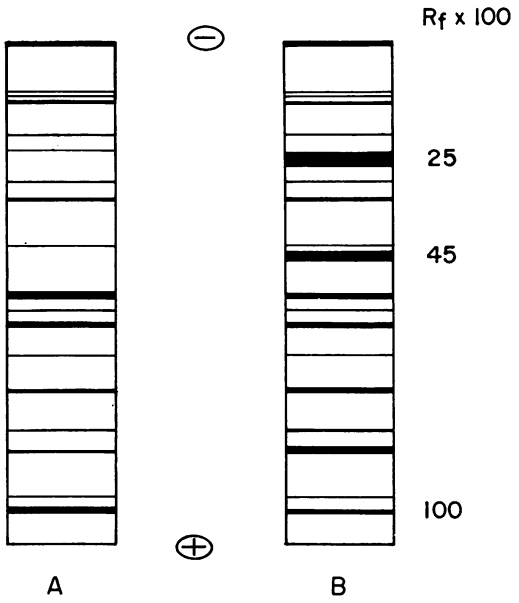


FIG. 2. *Major protein bands found after disc electrophoresis in 5.5% acrylamide gel of (A) normal KB cell extracts and (B) vaccinia-infected KB cell extracts. Total running time was 1 hr at 4 ma per gel.*

immunoelectrophoresis of SAM (Fig. 1b). This rather paradoxical behavior was resolved, in part at least, by the observation that electrophoresis of eluted R_F 0.25 band material in acrylamide invariably resulted in the formation of two bands, R_F 0.25 and 0.45. In contrast, electrophoresis of the eluted R_F 0.45 band resulted in only a single band identical to that of the starting material (R_F 0.45). These data are interpreted as indicating the possibility that the strongly staining R_F 0.25 band represents an aggregation or polymerization of virus-specific proteins normally migrating with an R_F of 0.45. The usefulness of disc electrophoresis for examination of complex SAM material is limited inasmuch as the numerous host-cell proteins of R_F greater than 0.50 prevent the identification of less prominent virus-induced protein bands. However, the presence of virus-specific proteins in this area can be demonstrated by complement-fixation tests upon extracts from serially sectioned gels.

Further characterization of virus-induced soluble antigens by gel filtration. In view of the complexity of the soluble antigen fraction, it seemed probable that a preliminary grouping of these antigens could be made on the basis of molecular weight. Accordingly, sonic-treated IHD$^-$-infected KB cells were added to Sephadex G 200 columns after dialysis of the sample against elution buffer and centrifugation at 100,000 $\times$ *g* for 30 min to remove virus and large aggregates. Similarly prepared sonic-treated, normal host cells labeled with C^{14}-valine were mixed with SAM for a comparison of the elution profiles of normal-host proteins with that of virus-induced protein. The elution profile of virus-induced protein was established by titering each eluate for specific CF activity by use of antibody directed against SAM. The asymmetry of the elution profile seen in a typical experiment (Fig. 3a) suggested that, although many of the virus-induced soluble antigens were of a molecular weight in the vicinity of 200,000 or greater, an appreciable fraction of the soluble proteins was retarded in elution, indicating a fraction of lower molecular weight.

The eluates under bars I and II in Fig. 3a were pooled separately, concentrated and passed, in turn, through Sephadex G 200 once again. The results of these runs are superimposed for purposes of comparison in Fig. 3b. Antisera specific for pooled eluates Ia and IIa of this second run (Fig. 3b) were made and are referred to as anti-HMW (high molecular weight) and anti-LMW (low molecular weight). Pooled fractions Ia and IIa, representing high and low molecular weight classes, were subjected to further gel filtration to gain a somewhat closer approximation of mean molecular weight. In addition, these fractions were examined by immunoelectrophoresis and acrylamide gel electrophoresis with the following results.

HMW (fraction Ia). This fraction contained about 85% of the total CF activity of the starting material. When this material was placed on a column of Biogel 300, two peaks of specific complement-fixing activity were obtained. The first, eluting with the front, appeared to represent a single antigen with a molecular weight greater than 300,000. On the basis of immunoelectrophoresis at *p*H 8.5, this antigen appears to be a basic protein. The second peak, whose elution was significantly retarded, contained most of the complement-fixing activity. It is apparent, therefore, that the crude HMW material designated as fraction Ia contains virus-specific antigenic material (soluble antigens) ranging from about 200,000 to over 300,000 in molecular weight.

Disc electrophoresis of the HMW material showed the R_F 0.25 and 0.45 bands and comparatively few bands attributable to normal-host-proteins. It is notable, however, that the procedure, as applied, would not be expected to reveal the high molecular weight basic protein. Immunoelectrophoresis of the HMW material (fraction Ia) revealed only the A, B, and G arcs (Fig. 1b). In contrast, when this same technique was em-

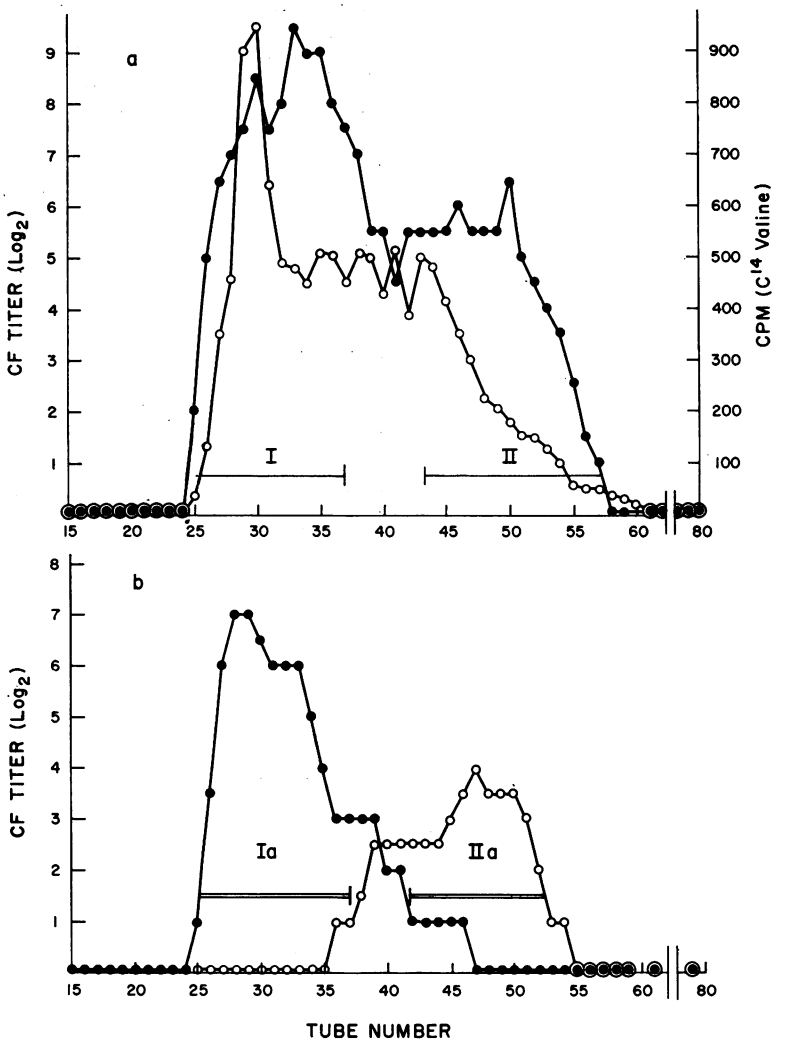


FIG. 3.(*a*) *Gel filtration of a crude soluble antigen mixture (SAM) in Sephadex G 200. Symbols:* ○, *radioactivity (C¹⁴-valine) profile of normal host-cell protein;* ●, *elution profile of virus-induced complement-fixing antigens. Flow rate, 15 ml/hr; fraction volume, 2.8 ml.* (*b*) *Refiltration on Sephadex of pooled fractions I and II. Symbols:* ●, *elution profile of complement-fixing activity of pooled fraction I;* ○, *profile of complement-fixing activity of pooled fraction II. Antiserum to virus-free soluble antigen mixture (anti-SAM) was employed in each case.*

ployed to examine the HMW material which had been further fractionated on Biogel 300, the very high molecular weight subfraction (molecular weight of 300,000 and greater) exhibited only the G arc. The remaining HMW material with an apparent molecular weight of roughly 200,000 formed the A and B arcs.

LMW (fraction IIa). The antigens contained in this pool exhibited less than 15% of the total CF activity of the starting material. Upon gel filtration of this fraction through Sephadex G 75, it was noted that the antigens, as detected by specific antisera, were not found in the front eluates, but were retarded somewhat. To better illustrate this retention of LMW antigens, SAM was passed through Sephadex G 75, and successive eluates were tested for complement-fixation activity with anti-HMW, anti-LMW, and anti-SAM. The results of this experiment (Fig. 4) showed some retardation in the elution of the LMW antigens. In contrast, the LMW antigens eluted with the front from G 50 columns. One can, therefore, assign an approximate molecular weight range of 50,000 to 100,000 for the soluble antigens in this fraction.

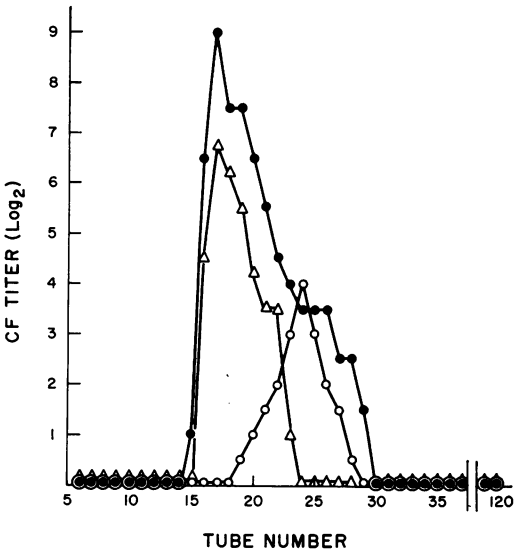


FIG. 4. *Gel filtration of a crude soluble antigen mixture (SAM) through Sephadex G 75. Elution profile of complement-fixing activity with the use of antisera to: (●) soluble antigen mixture; (△) high molecular weight antigens; (○) low molecular weight antigens. Flow rate, 20 ml/hr; fraction volume, 2.8 ml.*

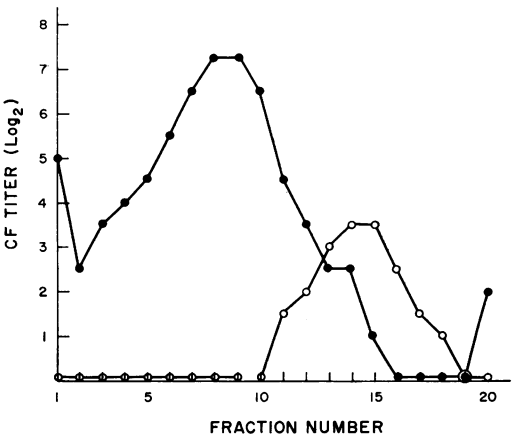


FIG. 5. *Comparison of sedimentation of high and low molecular weight antigen classes on sucrose density gradients (5–20% sucrose). Centrifugation carried out for 22 hr at 115,000 × g. Symbols: ●, profile of complement-fixing activity after centrifugation of high molecular weight antigens; ○, distribution of specific complement-fixing activity of low molecular weight antigens. The antiserum employed was directed against the soluble antigen mixture (anti-SAM).*

Disc electrophoresis of this material showed a total absence of the R_F 0.25 and 0.45 bands characteristic of crude infected material and subsequently identified as high molecular weight antigens. It was not possible to identify with certainty any unique viral protein bands for this fraction because of the reasons set forth previously. Immunoelectrophoresis indicates, however, that the low molecular weight antigens from this fraction are identical with the C, D, and E arcs seen after immunoelectrophoresis of the unfractionated infected material (Fig. 1b).

Ultracentrifugation through sucrose density gradients (5 to 20%) was employed to provide further evidence for the fractionation of virus-induced antigens on the basis of molecular weight. The results of such an experiment comparing the specific soluble-antigen fraction Ia (HMW) and fraction IIa (LMW) are shown superimposed for purposes of comparison in Fig. 5. Antiserum specific for SAM was employed to test successive samples from each tube. These data, combined with that from gel filtration, are consistent with the separation of virus-induced soluble proteins into two complex classes, based on what appears to be molecular weight.

Fractionation of soluble antigens by other procedures. The indication that vaccinia-induced soluble antigens were comprised of two classes led to the re-examination of the fractions obtained after chromatography of SAM on $CaPO_4$ columns (Cohen and Wilcox, Bacteriol. Proc., p. 113, 1965), as well as those obtained during the preparation of LS antigen by the classical method of Cragie and Wishart (3).

Chromatography of SAM on $CaPO_4$ columns. Homogenates of IHD$^-$-infected L cells were chromatographed on calcium-phosphate columns with the use of linear phosphate gradients. The elution pattern of virus-induced soluble antigen was traced by testing successive eluates for antigen-specific CF activity, whereas elution of normal-host protein was traced by addition of C^{14}-valine-labeled normal L-cell homogenates. Results of a typical experiment employing different antisera to outline elution profiles are shown in Fig. 6. The pooled eluates comprising peak 4 in Fig. 6a, eluting at molarities between 0.15 and 0.17 M PO_4, contained roughly 80% of the CF activity of the original preparation. Disc electrophoresis indicated that the material comprising this peak gave the distinctive R_F 0.25 as well as a trace of the 0.45 band. Immunoelectrophoresis revealed the A and a trace of the B antigen arcs, as expected from preceding data. Material designated as peak 3, eluting at molarities between 0.10 and 0.12 contained the R_F 0.45 band which has been shown by immunoelectrophoresis to represent antigen arc B. Peak 2 (0.05 to 0.07 M phosphate) contained none of the high molecular weight (R_F 0.25 to 0.45) antigens. Immunoelectro-

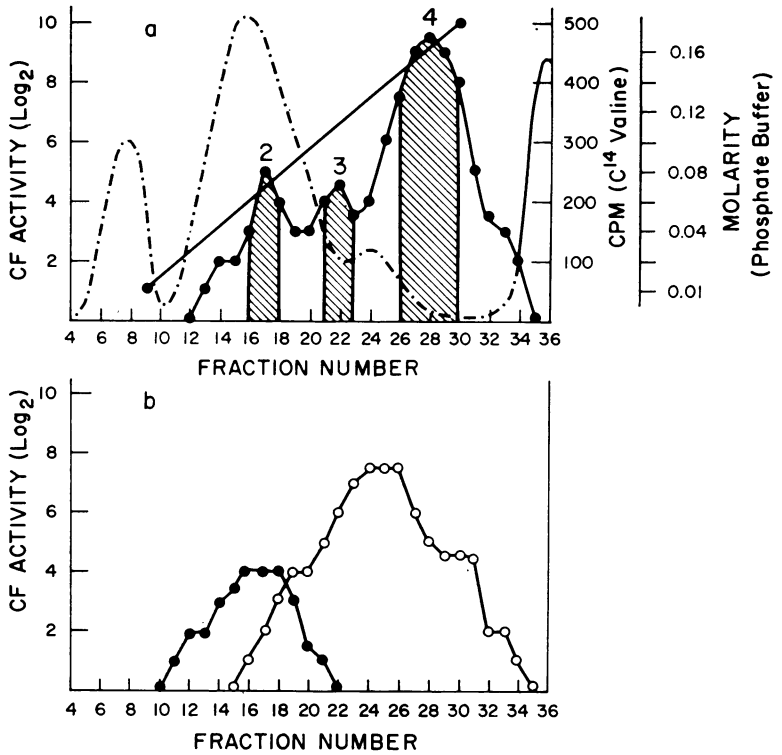


FIG. 6. *Chromatography of soluble antigens on calcium phosphate. (a) Elution pattern of normal L-cell protein (radioactivity) and virus-induced soluble antigens (complement-fixing activity). Symbols: – – – –, radioactivity (C^{14}-valine-labeled normal L-cell extract); ●, complement-fixing titer of vaccinia-infected L-cell extract (anti-serum against soluble antigen mixture, anti-SAM was employed). (b) Elution profile of low and high molecular weight antigens obtained by testing the same eluates with specific antisera. Symbols: ●, versus anti-low molecular weight serum; ○, versus anti-high molecular weight serum.*

phoresis of this material revealed the D, E, and F precipitin arcs characteristic of the low molecular weight species. It is notable, however, that antigen C was consistently missing from $CaPO_4$ eluates. It is apparent, from these data and from testing each sample for CF activity with the use of antisera specific for pooled high and low molecular weight antigens (Fig. 6b), that chromatography on calcium phosphate separates the soluble virus-induced antigens into essentially the same two major groups as revealed by gel filtration. Indeed, antigens of the high and low molecular weight species obtained from two cycles of gel filtration elute from calcium-phosphate columns at the molarities one would predict from Fig. 6b (i.e., 0.16 and 0.06 M).

Properties of the classical LS antigen and associated fractions. LS antigen was prepared from SAM by the procedure outlined in Materials and Methods. One obtains, as a by-product of this technique, an acid-soluble (AS) fraction and a relatively insoluble precipitate. Table 2 summarizes the distribution of CF activity and the relative concentration of protein in these and two other fractions obtained during the preparation of LS antigen. Roughly 90% of the original CF activity was recovered by this procedure. Of the recovered CF activity, 84% was in the LS fraction, with essentially all of the remaining activity (13%) residing in the AS material. This latter observation is at variance with early reports (16), which contended that the AS material contains no detectable virus - specific antigens. Immunodiffusion of the LS material showed it to be a complex material comprised of no fewer than three antigens, as defined by precipitin lines. Immunoelectrophoresis of this material revealed strong A and B precipitin arcs (Fig. 1b), and disc electrophoresis, as expected, showed the corresponding R_F 0.25 and 0.45 bands and only a minimal amount of host-cell protein contamination of this fraction. In contrast, the AS fraction, containing roughly 10% of the total recovered CF activity, exhibited the C, D, and E antigen arcs when examined by immunoelectrophoresis (Fig. 1b). These arcs have been shown, in the previous section, to represent the

TABLE 2. *Distribution of protein and CF activity in fractions derived from isoelectric precipitation of soluble antigen (SAM) into acid-soluble and LS fractions*

Sample	Protein recovered	CF recovered
	%	%
Acid-soluble	45.0	13
LS	8.5	84
Insoluble residue (NaOH extract)	47.5	3
Total	100.0	100.0

[a] Of the original protein 65 to 75% was recovered.

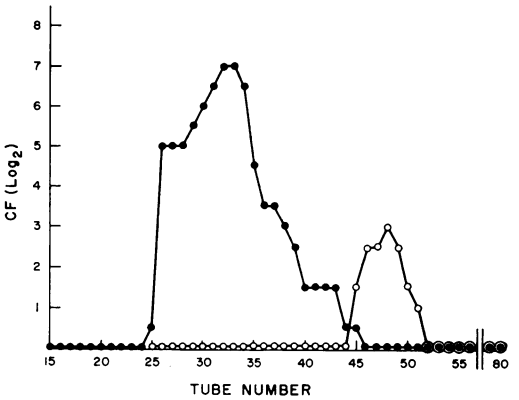


FIG. 7. *Gel filtration of the LS and AS fractions with Sephadex G 200. Symbols: ●, elution profile of LS complement-fixing activity; ○, elution profile of AS complement-fixing activity. Antiserum to soluble antigen mixture (anti SAM) was employed.*

LMW antigen species. Disc electrophoresis revealed no trace of the HMV antigens (R_F 0.25, 0.45) seen in the LS fraction, but indicated, as do the data in Table 2, that a major part of the normal - host protein resided in this fraction.

It would appear from these data that the procedures employed for the preparation of LS antigens serve also to segregate the crude antigen mixture (SAM) into essentially the same high and low molecular weight species revealed by gel filtration. To test this more directly, gel filtration of LS and AS material was carried out on Sephadex G 200 columns. The results of this experiment (Fig. 7) showed the LS antigen eluting in the HMW region, indicative of a molecular weight of around 200,000 or greater, and the AS antigen being significantly retarded, as expected of proteins of the lower molecular weight fraction. Similarly, when the migration of the LS and AS antigens in sucrose density gradients (5 to 20%) with the use of anti-SAM to test successive samples for specific CF activity was examined, almost precisely the same sedimentation patterns seen in Fig. 5 for the HMW and LMW antigens were obtained. On the basis of these data, as well as that presented in the preceding section, it appears that the LS and AS antigen complexes are very similar in composition to those obtained by gel filtration; the whole of these results are consistent with the idea that vaccinia-induced soluble antigens can be rather sharply separated into two classes.

Additional immunological evidence for the separation of the LS and AS antigens through use of homologous antiserum and sera prepared against the calcium phosphate fraction and against high and low molecular weight fractions from Sephadex, is shown in Table 3. These data serve also to relate the major calcium phosphate peaks (Fig. 6) and Sephadex fractions to the LS and AS materials.

TABLE 3. *Relationship of the LS and AS antigens to the high and low molecular weight antigen classes as revealed by complement fixation with specific antisera*

Class	Antiserum to	Complement-fixation titer	
		LS antigens	AS antigens
High molecular weight	LS	768	8
	HMW	384	24
	$CaPO_4$ peak 4	1,024	12
Low molecular weight	AS	4	96
	LMW	6	96
	$CaPO_4$ peak 2	24	256
Total	SAM	1,024	128
	Virus	384	96

Virus neutralization by antisera specific for soluble-antigen fractions. To characterize further the various soluble-antigen fractions, the ability of each to produce specific virus-neutralizing antibody was ascertained. It was necessary, of course, to insure the absence of infectious virus in these antigen mixtures inasmuch as infection of the animal would result in production of antibody to the entire spectrum of virus-induced antigens. Antigen fractions to be employed for immunization were first added to monolayers of normal KB cells. After a 4-day incubation period, these cells were harvested, concentrated 10-fold, and then sonic-treated. The resulting undiluted material was added, in turn, to normal KB monolayers, and the procedure was repeated. The final preparation was tested for the presence of virus

on chick embryo monolayers. Only virus-free soluble-antigen preparations were employed for immunization. The resulting antisera were tested for their ability to inhibit vaccinia virus plaque formation. The results of such tests are expressed simply as the greatest dilution of serum resulting in a 70% or greater reduction of plaque numbers as compared with appropriate virus-normal serum controls. The results of such an experiment are shown in Table 4. These data are interpreted as follows:

(*i*) Antiserum prepared against the entire soluble-antigen mixture (anti-SAM) proved very efficient in neutralizing virus. We found that such sera consistently neutralized virus to at least as great an extent as several batches of sera prepared by infecting rabbits with the infectious agent (anti-IHD).

(*ii*) Antisera prepared against the high molecular weight antigen species obtained by gel filtration (anti-HMW) similarly exhibited a powerful neutralizing capacity. In contrast, low molecular weight antigens showed little, if any, ability to induce formation of neutralizing antibody.

(*iii*) Sera specific for the LS and AS fractions (anti-LS, anti-AS) exhibited little ability to neutralize virus. In view of the relationship of the AS antigens to the low molecular weight antigens, this result was not unanticipated. On the other hand, the failure of the LS complex to induce production of neutralizing antibody, a result which has been reported previously (19), has been interpreted to indicate that the antigenic composition of this material is not entirely identical to that of the high molecular weight fraction obtained by gel filtration. One may postulate either that a critical antigen is missing from the LS fraction or that an antigenic grouping is altered or destroyed at acid *p*H. In this regard, it is notable that the high molecular weight antigen which behaves like a basic protein on immunoelectrophoresis (the G arc, Fig. 1b) has not been found in any of the LS preparations.

TABLE 4. *Virus neutralizing capacity of various antisera specific for soluble-antigen fractions*

Antiserum prepn vs.	Relative neutralizing titer[a]
Soluble antigen mixture	1:1,024
High molecular weight antigens	1:4,096
Low molecular weight antigens	<1:16
LS antigens	<1:16
Acid supernatant fraction	<1:16

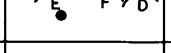
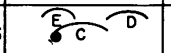
[a] Arbitrarily chosen as the greatest dilutions of serum resulting in a 70% or greater reduction in plaque titer (approximately 150 plaque-forming units used per test).

DISCUSSION

The complexity of the soluble-antigen fraction obtained from vaccinia-infected KB cells can best be demonstrated by immunodiffusion. Because of the complicated nature of the Ouchterlony patterns and the consequent problems of interpretation, no effort was made in this study to enumerate the total number of virus-induced antigens on this basis. When the same soluble antigen preparations are examined by immunoelectrophoresis, a technique of somewhat less sensitivity but greater resolving power, seven easily differentiated and well-defined precipitin arcs characteristically appear. It is probable that two of these arcs (A and B, Fig. 1b) represent a complex of closely related antigens rather than single antigens and, consequently, the number seven represents only a lower limit for the total number of antigens produced in this system. Nevertheless, the ease of identification of these arcs and the reproducibility of this technique provides a tool by which the effectiveness of procedures designed to separate individual antigens or groups of antigens may be measured.

The virus-induced soluble antigens of the system described can be rather sharply divided into two classes: those with an apparent molecular weight of 200,000 and greater and those antigens with a molecular weight range from 50,000 to 100,000. The characteristics of these antigen classes are summarized in Fig. 8.

It had been noted formerly in this and other laboratories (2) that virus-free, soluble-antigen preparations can elicit production of virus-neutralizing antibody. Examination of the soluble antigen fractions in this regard reveals two things: first, that injection of rabbits with the HMW soluble-antigen fraction results in the production of neutralizing antibody, and second, that the LMW soluble-antigen fraction had little, if any, capacity to elicit production of neutralizing antibody. With regard to the latter, it has been noted by a number of investigators that infection of mammalian cells by poxviruses is followed by "early" synthesis of a number of enzymes (10, 11), of which at least one appears to be unique to the virus-infected system (7). These early enzymes would be expected to fall roughly within the range of molecular weights encompassed by the LMW fraction. It is possible, therefore, that the LMW soluble antigen fraction contains no

Procedure / Sample		Immuno-electrophoresis	R_f* x 100	Calcium Phosphate elution Molarity †	Sephadex G-200
Unfractionated Soluble Antigens (SAM)			—	—	HMW+LMW
Sephadex G-200	HMW		25 45	0.11, 0.16	HMW
	LMW		>50	0.06	LMW
Calcium Phosphate Peaks	2		>50	0.06	LMW
	3		45	0.11	HMW
	4		25,45	0.16	HMW
Acid Precipitation Procedure	LS		25,45	0.16	HMW
	AS		>50	0.06	LMW
Biogel 300 (Void peak)			—	—	Front HMW

* *Acrylamide gel electrophoresis*

† *pH 7.4 phosphate buffer*

FIG. 8. *Summary of certain characteristics of the soluble antigen fractions obtained by various methods.*

virus structural proteins but rather represents virus-induced enzymes. An alternative hypothesis would be, of course, that the LMW fraction contains both early enzymes and virus structural proteins but that the viral components are internal proteins which would not be expected to elicit production of neutralizing antibody. This latter interpretation is supported by studies upon the time course of synthesis of soluble antigens and the composition of purified virus, which will be reported separately.

There seems to be a consensus among current investigators that the so-called LS antigen is indeed an antigen complex comprised of three or more antigenic moieties. It is notable that injection of the material into rabbits does not result in the production of neutralizing antibody (19) despite the fact that this fraction resembles closely the HMW antigen class obtained by gel filtration. One may observe a difference between the HMW fraction and the LS fraction by immunoelectrophoresis: the LS material does not exhibit the G (Fig. 1b) arc which represents a single antigenic moiety of 300,000 or greater molecular weight behaving like a basic protein under the conditions employed for immunoelectrophoresis. There is no evidence, however, to indicate that this antigen is responsible for the production of neutralizing antibody.

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