normal level of DNA synthesis is not necessary for that process. Two possibilities suggest themselves: (1) that the mechanism of recombination is a conservative one, with little or no concomitant DNA synthesis, or (2) there occurs a special kind of synthesis, restricted primarily to DNA molecules which have penetrated the cells and a portion of the DNA inside the cells.

The results indicate that transforming factors provide a most fruitful approach to the study of genetic recombination on the molecular level.

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*PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF TYPES 4 AND 5 ADENOVIRUS-SOLUBLE ANTIGENS**

BY WESLEY C. WILCOX AND HAROLD S. GINSBERG

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA

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It has become increasingly apparent in many systems that the interaction of animal virus and host cell results in the production of antigenic materials readily separable from virus.[1–5] Although in most instances little is known about the nature or origin of these antigens, evidence has been presented in at least two instances which may be interpreted to indicate that certain of these materials are related to infectious virus, either as virus precursor materials,[6] or alternatively, as materials released by the degradation of newly synthesized virus.[7]

A primary obstacle to a more thorough study of such antigens as regards their relationship to the virus synthetic process has been the problem of separating these materials, first from normal cell components, and secondly, one from the other, so that each could be characterized independently of the others.

Adenovirus-infected HeLa cells offer a particularly good model for the study of virus-induced antigens for two reasons: (1) infected cells synthesize several easily identifiable products of infection (soluble complement-fixing antigens and a toxin-like material) in addition to infectious virus,[8-11] and (2) separation of these antigens by a simple procedure can be accomplished.[12-15]

The objectives of the study to be described were threefold: (1) to devise a technique of separating and isolating virus-induced antigens of type 5–infected HeLa cells in order to obtain them in a highly purified state, (2) to identify and characterize these antigens by immunological means, and (3) to compare the antigens associated with type 5–induced infections with those produced in type 4–infected cells. Type 4 virus was chosen for purposes of comparison because in terms of cytopathology, kinetics of synthesis, and neutralization by specific antiserum, this agent is markedly different from type 5 virus.[16] During the course of this study, Klemperer and Pereira[13] employed similar methods to effect the partial separation of antigens from types 2 and 5 adenovirus–infected HeLa cells.

Materials and Methods.—Tissue culture: Epithelial cells of the HeLa (Gey) strain and, on one occasion, of the Hep #2 strain[17] were employed throughout. These cells were propagated either in 32-ounce prescription bottles with Eagle's basal medium containing 10 per cent human serum or in screw-capped 14 × 150 mm test tubes with 40 per cent human serum in Hanks' balanced salt solution (BSS) by methods described elsewhere.[18]

Viruses and virus infections: Prototype strains of adenovirus types 4 and 5 were employed. Virus pools were prepared by infecting bottles as previously described.[18,19] Infected cells were incubated at 36°C until cytopathic effects were complete (5 to 6 days), at which time the cells were recovered from maintenance mixture by centrifugation at 1,000 rpm for 20 min and resuspended in phosphate buffered saline (PBS) at one-tenth the original volume. Resuspended cells were frozen and thawed 6 times and cell debris removed by centrifugation at 10,000 rpm (6,600 × *g*) for 15 min in a Spinco Model L preparative ultracentrifuge. The resultant supernatant material, hereafter referred to as crude virus, was stored at −28°C until used.

Virus infectivity and toxin titrations: Infectivity titrations were carried out in tube cultures of HeLa cells using $10^{-0.5}$ (1:3.2) dilution increments as previously described.[18] Toxic activity was assayed in normal HeLa cells prepared in the same manner as those used for infectivity titrations.[18] Serial 2-fold dilutions of the material to be tested were made in Hanks' balanced salt solution. Two-tenths ml of each dilution was added to duplicate tubes of HeLa cells which were incubated at 37°C. Six hours later, these cultures were examined in comparison with normal control tubes for evidence of cytopathic alterations. Changes which ranged from clumping and separation of the entire cell sheet from the glass surface to a rounding up of the cells at the periphery of the cell sheet only were arbitrarily scored from 5^+ to 1^+; the latter degree of cytopathology was designated as the titration endpoint. The reaction increased very little in severity from the 6th to the 20th hour, at which time changes attributable to virus multiplication often became evident.

Complement-fixation titrations: Various eluates obtained from DEAE columns were tested as complement-fixing antigens by means of a standard test employing approximately 1.5 exact units of complement.[20] Heat-inactivated (56°C for 30 min) type-specific rabbit antisera or convalescent human sera were employed according to the aims of the experiment. An antibody excess was used in all antigen titrations. Veronal buffer containing 0.005 *M* $MgCl_2$ and 0.0015 *M* $CaCl_2$ was employed as diluent throughout.[21] Titers were expressed as the highest initial dilution of antigen giving complete fixation.

Neutralization titrations: Neutralization titrations were carried out as described elsewhere.[22] The end-point was expressed as the highest final dilution of serum in the serum-virus mixture which prevented cytopathic changes in one-half or more of the HeLa cells in a culture tube. With two exceptions, toxin neutralization tests were carried out in the same manner as were the virus neutralization tests:[22] first, rather than virus, an amount of toxin sufficient to cause extensive cytopathic alterations was added to each tube of HeLa cells; and secondly, the serum-toxin mix-

ture was incubated at 36°C for 6 hr rather than for 6 days as in the virus neutralization test. Titers were expressed as the highest final serum dilution which completely suppressed cytopathic changes in the cultured cells.

Preparation of antisera: Rabbits were given 3 intramuscular injections of antigen in Freund's adjuvant at weekly intervals followed a week later by one intraperitoneal injection of antigen without adjuvant. Animals were bled 12 days after the last injection. Sera were separated by centrifugation, inactivated at 56°C for 30 min, and stored at −28°C.

Agar diffusion tests: The agar gel techniques employed were modifications of the double-diffusion plate technique of Ouchterlony.[23] Large plates, 9 cm in diameter, were used for the double-diffusion tests. Twenty-five ml of Ionagar[24] at a concentration of 0.6 per cent in water was used for each plate. All wells were cut 6 mm in diameter and the spacing between cups was 8 mm. Prior to use, a single drop of agar was placed in each well to prevent leakage. Specific precipitates were visible within 24 to 48 hr following incubation at room temperature.

Chromatographic procedures: Thoroughly washed DEAE cellulose[25] was used to make columns 1.1 cm in diameter and from 3 to 7 cm in height, depending upon the amount of protein to be added. DEAE columns were packed under pressure and equilibrated with 0.01 *M* phosphate or acetate buffer of the desired pH prior to the addition of the sample. Following this, a program of step-wise elution employing solutions of constant pH and increasing molarity was carried out. Such solutions were prepared by adding solid NaCl to 0.04 *M* buffer of the desired pH. Elution was carried out under 5 psi of air pressure at 2 to 4 ml of effluent per min. Following the collection of a suitable number of samples at each molarity, the column was washed with buffer of the same molarity in an amount equivalent to 10 times the volume of the original sample. In this manner, loss of resolution due to "smearing" of the eluting fractions was minimized. Eluates so obtained were tested for infectivity, toxicity, and the ability to fix complement.

Determinations for DNA, RNA, and protein: The Burton[26] modification of the diphenylamine reaction for deoxyribose was employed for the determination of DNA. Thymus DNA was used as a standard. RNA was determined by the orcinol method,[27] which measures ribose. D-Ribose was utilized as the standard. The method of Lowry *et al.*[28] was employed to determine protein concentration with crystalline bovine albumin employed as a standard.

Results.—Separation of the virus-induced antigens of type 5 virus on DEAE cellulose at pH 7.2: Homogenates of type 5 adenovirus-infected cells were adsorbed to DEAE as described under *Methods* and eluted with increasing concentrations of NaCl in 0.04 *M* phosphate buffer at pH 7.2. Infectivity, complement-fixation, and toxin titrations were undertaken with each eluate. Both human convalescent and type-specific rabbit antisera were employed in the complement-fixation tests. Figure 1 illustrates the characteristic and highly reproducible elution patterns exhibited by the antigens from type 5 adenovirus–infected cells. It is notable that in contrast to homotypic rabbit antiserum (Fig. 1*a*), human convalescent serum failed to reveal the presence of the complement-fixing (CF) antigen which eluted maximally with 0.08 *M* NaCl (Fig. 1*b*). This antigen will be termed the "early-eluting or E antigen," whereas the material which eluted maximally at 0.26 *M* NaCl will be called the "late-eluting or L antigen." The data graphically summarized in Figure 1*a* indicate that the toxin and the E antigen, collectively termed the E-T complex, may be separate and distinct entities. This will be considered in more detail in a later section. The elution characteristics of type 5 virus, toxin, and CF antigens as revealed by rabbit immune serum are similar to those recently observed by Klemperer and Pereira with types 2 and 5 adenoviruses.[13] These investigators, however, presented no data on the reaction of the soluble CF antigens with human convalescent serum.

When the eluates comprising the L antigen and virus peaks were pooled separately, redialyzed and chromatographed one or more times under the original

conditions, the elution characteristics of these moieties remained unchanged. This procedure led to a considerable improvement in the homogeneity of the antigens comprising each fraction. In this manner, it was possible to obtain antigenic materials suitable for preparation of immune sera. It should be emphasized, however, that under the conditions described repeated chromatography of the eluates comprising the E-T complex failed to effect a further separation of these antigens.

Immunological characterization of the antigens of type 5–infected HeLa cells: Specific antisera to the L antigen and the E antigen-toxin (E-T) complex were prepared in rabbits with Freund's adjuvant. Prior to use, each antigen preparation was rechromatographed three times and centrifuged four times at 105,000 × *g* for 120 min to remove contaminating antigens and residual virus. No infectious virus was detected in the final preparations of purified antigen. Antiserum to the E antigen-toxin complex will be referred to as "anti-E-T," whereas that to the L antigen will be termed "anti-L." Anti-5, anti-4, etc. refer to antiserum produced in response to homogenates of infected cells of the virus type designated by the number and may be assumed to contain antibodies to the "soluble" antigens in addition to neutralizing antibody for the virus type designated. Antiserum to homogenates of normal HeLa cells was also prepared and is designated anti-normal cell extract (anti-NCE).

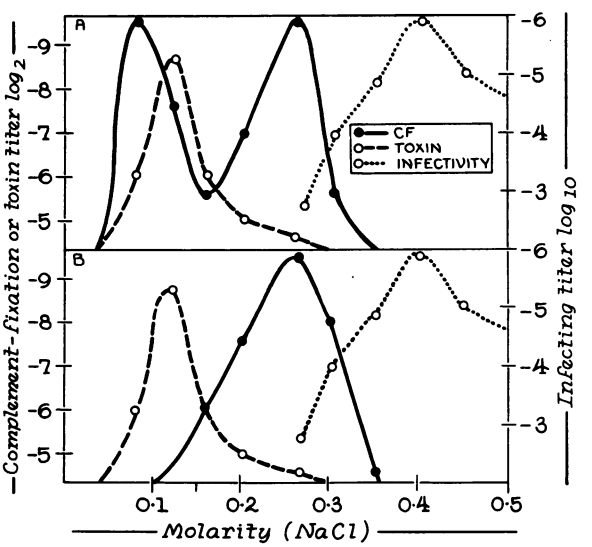

FIG. 1.—Fractionation of crude homogenates of type 5–infected HeLa cells on DEAE cellulose by gradient (stepwise) elution. Figure 1*a* illustrates the elution pattern obtained when homotypic rabbit antiserum was employed in the complement-fixation test; Figure 1*b* when convalescent human serum was used to test the same eluates for complement-fixing antigens.

Eluates obtained from chromatography of type 5 virus were examined for their ability to fix complement with various antisera. Figure 2*a* illustrates the characteristic type 5 elution pattern that was obtained when type-specific rabbit antiserum was employed for the complement-fixation tests. For purposes of orientation, the elution curve of toxin is included. The L antigen of type 5 adenovirus reacted strongly with antibodies specific for heterologous virus types; the use of antisera specific for types 2 and 4 virus are shown as examples (Figs. 2*b* and 2*c*). These results imply that the L antigen is the adenovirus group antigen, an interpretation which is further supported by the observation that antibodies in human serum reacted only with the L antigen (Fig. 2*d*). In contrast, type-specific rabbit antiserum reacted strongly with the E-T complex as well as with the L antigen (Fig. 2*a*). These data suggest that the E-T complex contains a type-specific antigen, as has been previously suggested by Klemperer and Pereira.[13]

These results provide a basis for the observation that type-specific rabbit antisera, unlike human convalescent serum, may be employed to type-specific adenoviruses by means of the complement-fixation technique.[8, 29, 30] Rabbit antisera react strongly with the type-specific antigens of the various adenoviruses, whereas human serum reacts only with the group-specific adenovirus antigens.

Since sera to heterologous virus types reacted, to a limited extent at least, with the eluates comprising the E-T complex (Figs. 2*a* and 2*c*), it may be assumed that this material contained a cross-reacting component in addition to the type-specific antigen previously mentioned. It seemed possible that the toxic moiety may represent this cross-reacting antigen; indeed, neutralization titrations employing various type-specific antisera and toxin preparations derived from different adenovirus types indicated that toxin behaved in a manner expected of a common antigen (unpublished data).

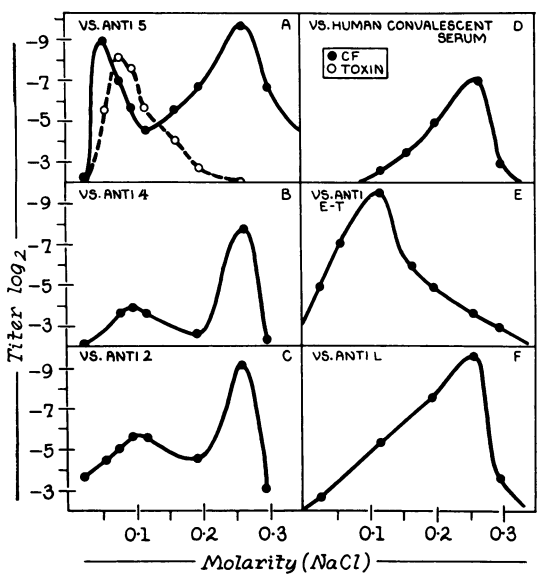

FIG. 2.—Complement-fixation patterns revealed by the reaction of various antisera with eluates obtained from the stepwise chromatography on DEAE of a crude homogenate of type 5 adenovirus-infected HeLa cells.

The reaction of antigen-specific antisera with the antigens of type 5–infected HeLa cells is shown in Figures 2*e* and 2*f*. There is relatively little cross-reactivity between these serum preparations, indicating that the chromatographic separation of E-T complex and L antigen was relatively complete.

It was possible to identify certain of the adenovirus-induced antigens by their precipitation reactions in agar with antisera from immunized animals. Eluates containing the E-T complex and the L antigen were tested in comparison with crude types 4 and 5 viruses against antiserum to unfractionated type 5 virus (anti-5). The results of this experiment are shown diagrammatically in Figure 3. Crude type 5 virus characteristically exhibited three well-defined lines of precipitate when reacted with homotypic rabbit antiserum. In contrast, crude type 4 virus preparations showed only a single sharp line when tested with the same antiserum. The type 4 precipitin band was continuous with that of the L antigen as well as one of the type 5 lines and may be considered to represent the group-specific or common adenovirus antigen. This observation is in agreement with the complement-fixation data regarding the immunologic characteristics of the L antigen. The weak diffuse zone of precipitation immediately adjacent to the center-well can be seen to represent normal cell antigen and was often encountered when crude antigen preparations were employed—in this case types 4 and 5 virus antigens. The two lines exhibited by the E-T complex were continuous with the first and second of the type 5 virus

lines but, unlike the L antigen, had no counterpart in the reaction of type 4 virus with type 5 antiserum. It would appear from this that one or both of these antigens, which presumably represent the toxin and the E antigens are type-specific. In the case of the E antigen this observation is compatible with complement-fixation data which would appear to indicate that this antigen is type-specific (Fig. 2). The toxin, however, seems to be a group rather than a type-specific antigen on the basis of cross-neutralization tests (unpublished data). It is pertinent to note at this point that infection with type 4 virus results in the production of only small quantities of toxin. The apparent type-specificity of this antigen as revealed by agar diffusion may therefore be a quantitative phenomenon.

When antisera prepared against the E-T complex and the L antigen of type 5-infected HeLa cells were tested against crude types 4 and 5 viruses, the results (Fig. 4) were compatible with the hypothesis that the L antigen was a group antigen common to type 4 as well as type 5 virus and that the E-T complex was apparently type-specific (also illustrated in Fig. 3). The result is, of course, subject to the same interpretation as discussed in the preceding paragraph.

To determine which of the two precipitin lines of the E-T complex was associated with the toxin, crude type 5 virus preparations were reacted with an amount of trypsin sufficient to destroy all traces of toxin activity. When trypsin-treated type 5 virus was tested against type 5 antiserum in parallel with untreated virus, the first of the E-T lines (Fig. 4) was not present. The results of this experiment are illustrated in Figure 5. This evidence made it possible to identify with reasonable surety the various precipitin bands associated with crude type 5 virus preparations (Fig. 5).

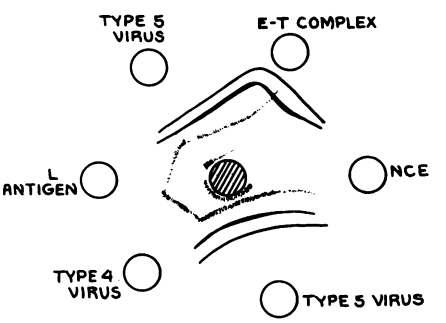

FIG. 3.—Use of the agar double-diffusion technique to reveal the relationships of various antigens. The center well contains antiserum to crude, unfractionated type 5 virus. NCE designates normal HeLa cell homogenate.

Chromatography of type 5 virus–infected cell lysates at pH 5.2: The preceding experiments suggested that the E-T complex consisted of two separate antigens. Therefore, a more satisfactory separation of this fraction was required to obtain a clear picture of the immunologic nature of the antigens. Assuming that the differences in the absorptive capacity of these antigens might be increased at pH values closer to their isoelectric points,[31] experiments designed to explore this possibility were undertaken.

Infected cell lysates were dialysed against 0.01 *M* acetate buffer at a pH of 5.2. The heavy precipitate which formed during this procedure did not contain detectable complement-fixing antigens and was discarded. The supernatant fluid was placed on DEAE columns previously equilibrated with an identical buffer. Eluates obtained with increasing NaCl concentrations in 0.04 *M* acetate buffer at pH 5.2 were titrated for toxin, infectious virus, and complement-fixing antigens. Various antisera were used in the latter tests. Figure 6 illustrates the results of a representative experiment. Under acidic conditions, the bulk of the E antigen passed through the column unadsorbed and was recovered in the original effluent and, to a

lesser extent, the 0.01 *M* acetate wash. The L or common CF antigen also exhibited a shift to the left in its elution characteristics and was recovered maximally in the 0.16 *M* NaCl eluate. In contrast, toxin was more strongly adsorbed to the exchange resin under acidic conditions than it had been at pH 7.2. Because of this, toxin was eluted with the L antigen rather than with the E antigen as had been the case at pH 7.2 (Fig. 1*a*). The identities of the CF antigens were verified by testing each of the eluates both with convalescent human serum (Fig. 6*b*), which has been shown to react only with the L or common antigen, and with specific rabbit antiserum to the L antigen (Fig. 6*c*) and to the E-T complex (Fig. 6*d*). The reaction of the L antigen-toxin complex, which eluted maximally at 0.16 *M* NaCl with anti E-T (Fig. 6*d*) suggests that the reaction of toxin with specific antiserum also resulted in fixation of complement.

Chromatography of E antigen-toxin (E-T) complex at pH 5.2: Although chromatography of crude type 5 virus at pH 5.2 presented a method of obtaining E antigen

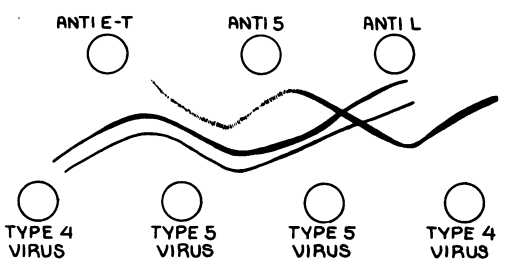

FIG. 4.—The reaction of certain antigen-specific antisera with crude types 4 and 5 virus as revealed by agar-diffusion.

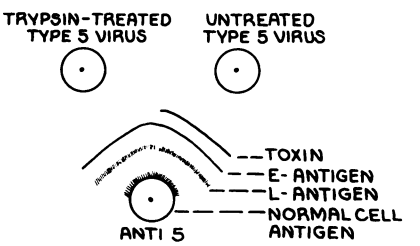

FIG. 5.—Diagrammatic representation of the effect of trypsin on the agar-diffusion pattern of crude type 5 adenovirus and suggested identities of precipitin lines.

free from toxin, the separation of toxin from contaminating material, in this case the L antigen, was not accomplished. Two observations, however, furnished a basis for separation and purification of the E antigen and toxin: (1) an E antigen-toxin mixture essentially free from L antigen and virus could be obtained from the chromatography of crude type 5 virus on DEAE at pH 7.2, and (2) E antigen was sharply separated from toxin by chromatographing E-T complex material on DEAE at pH 5.2. This method is exemplified in the following experiment. A pool of eluates containing a mixture of E antigen and toxin from a preliminary separation of type 5 antigens on a DEAE column at pH 7.2 was dialysed against 0.01*M* acetate buffer at pH 5.2. The dialysate was centrifuged to remove a light precipitate which formed during dialysis, and the clear supernatant fluid was added to a DEAE column at pH 5.2 as described in the previous section. Each eluate obtained from this column was titrated for toxin and for complement-fixing antigens employing various antisera.

The pattern of reaction obtained with type-specific rabbit antiserum revealed two peaks of complement-fixing activity (Fig. 7*a*). The second of these peaks corresponded with elution of toxin at the expected molarity of 0.16 *M* NaCl. Antisera to heterologous virus types, in this case anti-2 and anti-4, reacted only with the 0.16 *M* NaCl eluting material (Fig. 7*b*). These data support the concept that toxin is a common adenovirus antigen and as such is responsible for the lack of total specificity hitherto noted with the E-T complex (Figs. 2*b* and 2*c*). The CF

antigen recovered in the original effluent was devoid of toxic activity and reacted only with antisera produced in response to type 5 antigens (Figs. 7*a* and 7*c*). This material represented the E antigen and behaved in the manner expected of a type-specific antigen.

These experiments clearly indicate that type 5 adenovirus-infected cells produce in addition to infectious virus a group-specific antigen (the L antigen), a type-specific antigen (the E antigen), and a third soluble component possessing toxin-like properties which reacts as a second group-specific or common antigen.

The relationship of virus-induced antigens to normal host cell antigens: To ascertain whether the virus-induced antigens were contaminated with host cell antigens or, alternatively, were antigenically related to normal cell materials, each of the eluates from a chromatographic separation of type 5 antigens was tested for

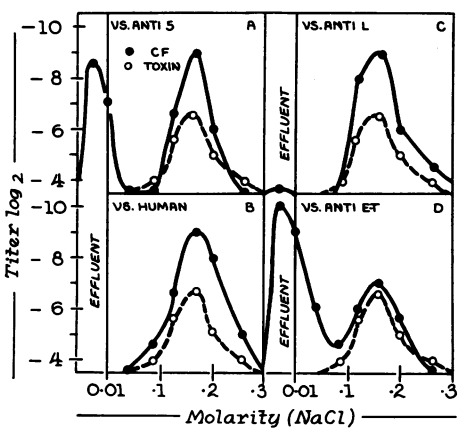

FIG. 6.—Complement-fixation patterns revealed by the reaction of various antisera with eluates obtained from chromatography on DEAE of homogenates of type 5-infected HeLa cells at pH 5.2. The elution pattern of toxic activity is included for purposes of comparison.

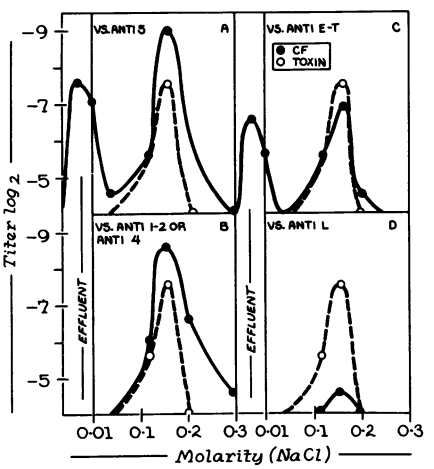

FIG. 7.—Separation of E antigen and toxin by chromatographing E-T complex material on DEAE at pH 5.2. Each eluate was tested with various antisera to reveal the nature of the CF antigens. E-T complex material was obtained from the chromatography at pH 7.2 of an homogenate of type 5 adenovirus-infected HeLa cells.

complement-fixation with antiserum directed against normal cell lysates (anti-NCE) as well as with antiserum specific for type 5 virus. The results of a typical experiment are shown in Figure 8. It is evident that the quantity of complement-fixing antigen in these eluates as measured with anti-NCE did not correspond with the elution patterns of virus-induced antigens. Only a single small peak of host-cell complement-fixing antigen was observed, eluting maximally at a molarity of 0.04 *M* NaCl. The slight contamination of E antigen with normal cell components was reduced below detectable quantities by a second chromatography of the E antigen peak.

Agar diffusion tests employing these same eluates revealed a single precipitin band shared by the 0.04 and 0.08 *M* NaCl eluates. This line, however, was not continuous with either of the precipitin bands characteristic of the E-T complex (Fig. 3).

These data were further verified by chromatographing normal cell homogenates at pH 7.2 under the same conditions used for homogenates of virus-infected cells. Each eluate was tested for normal cell antigens by employing agar diffusion as well as complement-fixation techniques. Antiserum to normal host cell materials (anti-NCE) was utilized in these tests. Both techniques indicated the presence of only a single peak which exhibited complement-fixing as well as precipitating activity. This antigen or antigens eluted maximally at a molarity of 0.04 *M* NaCl as anticipated from the preceding tests.

Virus-induced antigens in adenovirus-infected monkey kidney (MK) and Hep 2 cells: The antigens produced by type 5–infected HeLa cells were characteristic relative to their chromatographic and immunologic behavior. Despite the observation that these antigens do not appear to be related to normal host cell materials, it seemed pertinent to ascertain whether the production of these antigens was controlled by the type of cell infected. In order to accomplish this aim, two questions were asked: first, do other cell lines infected with type 5 adenovirus synthesize antigens identical to those produced in infected HeLa cells; and secondly, do such antigens, if produced, possess the same chromatographic characteristics as those obtained from type 5–infected HeLa cells?

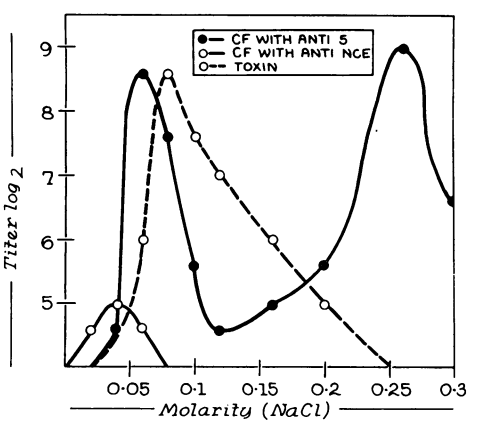

FIG. 8.—Comparison of the elution characteristics of host cell antigens and virus-induced antigens. Eluates obtained from the chromatography at pH 7.2 of type 5 adenovirus-infected HeLa cell homogenate were tested as indicated above.

Crude cell homogenates from type 5–infected monkey kidney (MK)[32] and Hep 2 cells were chromatographed at pH 7.2 in the manner previously described. As before, each eluate was titrated for complement-fixing antigens, toxin, and infectious virus. Elution patterns so obtained were identical with those noted with homogenates of type 5–infected HeLa cells (Fig. 1*a*). No immunologic differences between the E, L, and toxin antigens of type 5 virus produced in MK, Hep 2, or HeLa cells could be detected by complement-fixation or agar-gel diffusion techniques. Type-specific rabbit antiserum and antisera specific for each antigen were employed in these experiments.

It may be deduced from these data that insofar as the metabolic competence of the infected cell permits, the physical and immunologic characteristics of the soluble adenovirus antigens are rigidly determined by the virus genome rather than by the type of host cell. For this reason it would appear that the synthesis of these antigens is specifically associated with virus propagation.

Comparison of the chromatographic behavior of type 4 and type 5 virus preparations: It has been suggested that the lower-numbered adenoviruses may be divided into two subgroups, on the basis of cytologic changes induced by these viruses and the kinetics of neutralization and multiplication of the infectious agents.[16] On this basis, types 1, 2, 5, and 6 comprise one group and types 3, 4, and 7 the other. If

the differences between these groups were sufficiently marked, it might be predicted that suspensions of the latter group (types 3, 4, and 7) would exhibit similar chromatographic characteristics, which were, however, different from those of the agents of the first group. In this regard, it is pertinent to note that the results of Klemperer and Pereira indicate that the elution characteristics of types 2 and 5 virus on DEAE were identical.[13]

To test this hypothesis, homogenates of types 4 and 5 adenovirus-infected HeLa cells were chromatographed at pH 7.2 under identical conditions. Appropriate homotypic rabbit antisera as well as human convalescent serum were used to test duplicate samples of each eluate for complement-fixing antigens. The results of these experiments are shown in Figure 9. Unlike type 5 virus, homogenates of

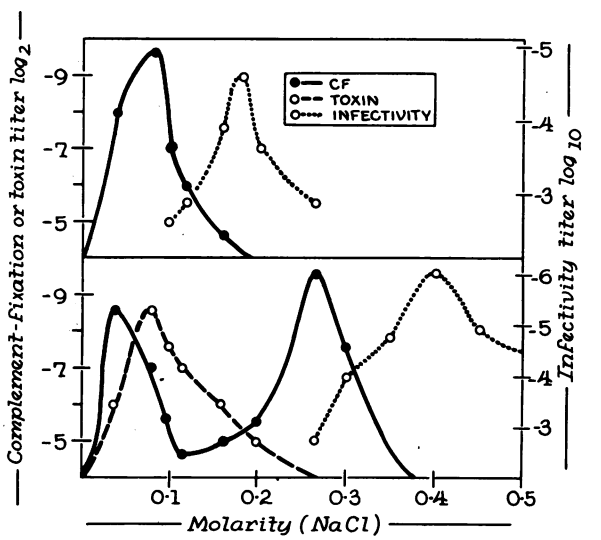

FIG. 9.—Comparison of the chromatographic behavior of homogenates obtained from type 4 and type 5 adenovirus-infected HeLa cells (upper figure, type 4; lower figure, type 5). The appropriate homotypic rabbit antiserum was used in each case to test for complement-fixation.

type 4–infected cells exhibited only a single peak of CF activity (Fig. 9) which correspond roughly with the E antigen of type 5 virus as regards its elution characteristics (Figs. 2*a* and 9). Even more striking was the difference in the chromatographic behavior of type 4 infectious particles, which eluted maximally at 0.16 *M* NaCl, and the type 5 agent which was recovered maximally in the 0.4 *M* NaCl eluates. Because type 4–infected HeLa cells produce only small amounts of toxin, it could not be detected in eluates obtained by the chromatographic procedure employed.

In order to gain some insight into the immunologic composition of the single CF peak, each of the eluates obtained from the chromatography of crude type 4 virus was tested for complement-fixing antigens employing antisera to heterologous virus types (anti-5 and anti-2) and antigens (anti-L and anti-E-T). With the exception of anti-E-T, each of these antisera reacted strongly with the 0.08 *M* NaCl eluates to reveal a pattern identical to that obtained with type-specific

rabbit antiserum as shown in Figure 9. These eluates comprising the type 4 CF peak may therefore be considered to contain a common or group-specific antigen immunologically similar to the L antigen of type 5 virus–infected cells. Antiserum prepared in response to the E-T complex of type 5 virus (anti-E-T) reacted very weakly with the material of the CF peak, a finding which was not surprising since the T antigen has been shown to be group-specific. Although one may consider from this evidence that the single peak obtained by chromatography of type 4 adenovirus was homogenous and a group antigen, other studies have demonstrated that this agent can be identified by complement-fixation with type 4–immune rabbit serum.[29, 30] It is probable, therefore, that the single peak of antigenic material contains type-specific antigen as well as the common adenovirus group antigen.

Chromatography of type 4 virus at pH 5.2: The success achieved in dissection of the antigens of type 5 virus by means of chromatography under acidic conditions suggested that similar procedures might be utilized to determine whether the single type 4 virus complement-fixation peak (Fig. 9) contained more than one antigen. Figure 10 illustrates the reaction of various antisera with eluates obtained when chromatographic separation of crude type 4 virus was carried out at pH 5.2. Under acidic conditions the single peak of CF activity seen at pH 7.2 (Fig. 9) was resolved into two well defined complement-fixing antigens (Fig. 10*a*). The use of antisera specific for heterologous virus types indicated that the first of these peaks was a common or group-specific CF antigen (Figs. 10*b* and 10*c*). This interpretation was substantiated by results obtained when antiserum specific for the common antigen (L antigen) from type 5 virus–infected cells was employed (Fig. 10*d*). In contrast, eluates comprising the second peak of complement-fixing activity eluting maximally at a molarity of 0.12 *M* NaCl, reacted only with antiserum directed against type 4 virus (Fig. 10*a*). These results indicate that like type 5 virus–infected cells, those infected with the type 4 agent produced a specific as well as a common CF antigen.

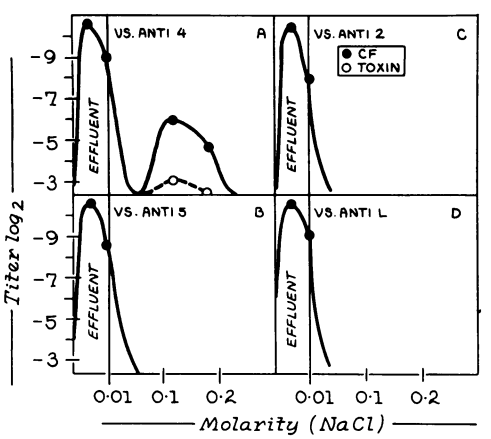

FIG. 10—Complement-fixing patterns obtained following chromatography of crude homogenates from type 4–infected HeLa cells at pH 5.2. Aliquots from each of the eluates were tested for complement-fixation with the various antiserum preparations shown above.

Discussion.—It is clear from the preceding data that a simple yet efficient separation of adenovirus-induced antigens may be effected by chromatographic procedures. By extending these methods, it was possible to obtain relatively pure preparations of the various soluble antigens associated with adenovirus-infected cells. In terms of quantitative recovery following a single cycle of chromatography, infectivity ranged from 30 to 80 per cent of original values, toxic activity from 80 to 100 per cent, whereas recovery of the complement-fixing antigens was consistently less than 30 per cent of the original amount.

When one combines the results of the experiments just described with those reported by Klemperer and Pereira,[13] it appears likely that in addition to virus, the lower-numbered adenoviruses characteristically elicit the production of three soluble antigens in infected cells. Two of these antigens were shown to be group-specific or common adenovirus antigens while the third was type-specific. The separation of these materials, either by chromatography,[12, 13] or by immunoelectrophoretic techniques,[33, 34] indicated that these antigens differed in certain physical characteristics. It is important to emphasize that similar antigens from types 4 and 5 virus–infected cells differed widely in their characteristics of elution from DEAE. These differences in chromatographic properties were unexpected in the case of the common or group complement-fixing antigens of these agents, since by immunological criteria these materials appeared to be closely related if not identical. Types 4 and 5 infectious virus particles also exhibited considerable differences in their elution optima.

Examination of the chromatographic characteristics of adenovirus antigens indicate two major patterns of behavior, one typified by the antigens of adenovirus types 2 and 5,[13, 14] the other by the antigens of type 4 virus. These data furnish an additional criterion to indicate that it may be possible to divide the lower-numbered adenoviruses into two sub-groups: adenovirus types 1, 2, 5, and 6 and virus types 3, 4, and 7. This evidence is compatible with the previously suggested division of the lower-numbered adenoviruses into two sub-groups on the basis of multiplication kinetics, neutralization by antiserum, and cytological changes induced by these agents.[16]

In several instances, a relationship has been shown to exist between soluble virus-induced antigens and certain components of the virus particle proper.[6, 7, 35] These data may be interpreted to indicate that the antigens in question were virus precursor materials or, less likely, degradation products originating from newly synthesized virus. At present there is no precise information regarding the relationship of virus to soluble antigens in the adenovirus-host cell system. There is, however, a certain amount of indirect evidence which serves as a basis for speculation about the nature of these antigens. When the relationship between adenovirus-induced antigens and the host-cell was examined, two findings were prominent: (1) adenovirus-induced antigens could not be shown to be related immunologically to normal host cell antigens, and (2) the immunological and physical nature of the soluble antigens and the infectious virus particles was determined solely by the type of infecting virus rather than by the origin of the host cell. These findings imply that the soluble adenovirus antigens are not merely products of host cell injury but rather are very specifically associated with the viral synthetic processes. It is possible, therefore, that at least certain of these antigens may be virus components existing either as a pool of precursor materials or as breakdown products originating from newly synthesized virus particles. When the acridine dye, proflavine, was added in the proper concentration to cultures of infected cells, it was possible to cause a 70 to 80 per cent reduction in the synthesis of infectious virus with no significant decrease in titer or CF antigens.[35] These data are consistent with the concept that toxin and CF antigens are not derived by the degradation of newly synthesized virus. This hypothesis is supported by evidence which indicates that the primary effect of proflavine is not on the synthesis of viral com-

ponents or precursors but rather is on the maturation or assembly of these materials into infectious virus.[36-39]

A picture of the assembly of virus components may be obtained from a consideration of the studies of Horne *et al.*[40] who concluded that the type 5 virus particle is an icosahedron with a shell constructed from 252 subunits. The diameter of a sub-unit is approximately 70 Å so that each may therefore represent a single protein molecule. These investigators suggested that the icosahedral structure could be obtained if three chemically different sub-units were employed to build the outer shell. In view of these findings, it is important to reiterate that type 5 adenovirus–infected cells characteristically produce three soluble antigens, which are postulated to represent virus precursor materials. The concept of virus maturation proceeding as an assembly of sub-units[6, 41, 42] leads to the possibility that two complement-fixing antigens and toxin combine with specific DNA to produce mature infectious adenovirus particles. In this regard, it is notable that adenovirus-infected cells produce a unique DNA[43] in addition to complement-fixing antigens and toxin. There is some evidence to indicate that this DNA represents precursor virus DNA.[44] It may be that the synthesis of excess viral DNA and protein, which appears to be characteristic for phage synthesis,[43-45] may also be common for multiplication of animal viruses in host cells.

Summary.—A chromatographic method for the separation and isolation of adenovirus-induced antigens from homogenates of infected cells is described. This procedure, in conjunction with immunological techniques, has been employed to identify, characterize, and compare the soluble antigens of types 4 and 5 adenovirus–infected HeLa cells. By such means it was shown that adenovirus-infected cells of various origins produce three soluble antigens in addition to infectious virus. These include a highly type-specific complement-fixing antigen, a group-specific or common complement-fixing antigen, and a toxin-like material which, when added to normal cells, causes rapid degenerative changes. The immunological behavior of the toxin is that of a group-specific or common adenovirus antigen. It was further shown that similar antigens from types 4 and 5 adenovirus–infected HeLa cells exhibited strikingly different chromatographic characteristics.

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SIGNIFICANT LIQUID STRUCTURES, VI. THE VACANCY THEORY OF LIQUIDS

BY HENRY EYRING AND TAIKYUE REE

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF UTAH

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The liquid state is stable in a temperature range intermediate between that of solid and vapor. Usually, the liquid density is also intermediate. Exceptional substances such as water involve a structural shrinkage superposed on the usual expansion of the solid to liquid transition. It is natural to seek an explanation of the intermediate liquid state as an intimate mixture of solid and vapor. All the imperfections of the solid state should be even more abundant in the liquid. However, those imperfections which yield a large ratio of entropy increment to enthalpy increment will be correspondingly more abundant in the liquid and will be the significant structures in a quantitative theory.

The Liquid Model.—Holes of molecular size are assumed overwhelmingly abundant because (*a*) they confer gas-like properties on a neighboring molecule jumping into the hole and (*b*) a solid-like molecule obtains a positional degeneracy equal to the number of neighboring vacancies. Neglecting increase in volume due to holes of other than molecular size, the number of holes per mole of molecules is $(V - V_s)/V_s$, where V and V_s are the molal volumes of liquid and solid, respectively. The chance that a vacancy confers gas-like properties on a neighboring molecule is assumed proportional to the fraction of neighboring positions populated by molecules. This fraction is V_s/V if molecules and vacancies are randomly distributed. Thus, for random distribution of vacancies, the mole fraction of gas-like molecules is $(V_s/V)\ (V - V_s)/V_s \equiv (V - V_s)/V$. The remaining mole