Studies on an Oncogenic Avian Adenovirus (CELO)
I. Biophysical Characterization (35587)

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The oncogenic activity of chick-embryo-lethal-orphan (CELO) virus has been described by various authors (1–4). Since CELO is commonly found in chicken eggs (5) and can transform both hamster (6, 7) and human (8) cells *in vitro*, its possible implication in human neoplasia warrants further study. In addition, some avian adenoviruses differ both immunologically (9) and in heat stability (10) from the adenoviruses in general (11). Therefore, we have attempted to determine whether the biophysical properties of CELO virus distinguish it from other members of the adenovirus group.

Materials and Methods. Virus. The Phelps strain of CELO virus (5) was used. Stocks were produced by inoculating 10^6 PFU/0.1 ml of virus into the allantoic cavity of 10-day-old embryonated CELO-free eggs (Spafas, Inc., Norwich, Conn.). The eggs were incubated at 37° for 3 days. Before harvesting, the eggs were chilled overnight at 4°; the allantoic fluids were pooled and frozen at —20°. The virus pools used in these studies contained approximately 10^9 PFU/ml.

Plaque assay. This technique was described (12). Plaques were counted after incubation for 11 days.

Complement-fixation tests. Our adaptation of the micro-complement-fixation (CF) test (13) was used. Fractions from density gradients were diluted 1:10 with Hanks' balanced salt solution (HBSS) and frozen at—20° until tested. Antiserum to CELO (14) was produced in adult LSH/LAK hamsters (15) by inoculation of density gradient purified virus. Antigens were detected using 4–8 units of antiserum.

Human adenovirus group and control antigens, as well as the human reference serum, were purchased from Microbiological Associates, Bethesda, Maryland.

Microhemagglutination (HA) test. Rat erythrocytes (Microbiological Assoc.) were washed 3 times with phosphate buffered saline (PBS), pH 7.2. The 10-drop fractions from density gradient experiments were diluted with 0.1 ml of PBS. Twofold dilutions were prepared in 0.05-ml aliquots in microtiter U plates (Cooke Engineering Co.). The diluent contained 1% normal rabbit serum. One drop (0.025 ml) of 1% (v/v) rat erythrocytes was added to each dilution. Plates were incubated at 37° until the cells in the control wells settled.

Results. Purification of CELO virus. In preliminary experiments to visualize CELO virus by electron microscopy, we found that clarification of the allantoic fluid by light centrifugation (500*g*) removed most of the virus particles. Since titration of the virus consistently showed a titer of 10^9 PFU/ml, it was suspected that much of the virus was embedded in debris present in the allantoic fluid. Previous studies by Black *et al.* (16) indicated that SV40, also closely associated with cellular material, could be purified by treatment with sodium deoxycholate and trypsin. A combination of the methods of Black *et al.* (16) for SV40 and Green and Pina (17) for adenovirus was used in the purification of CELO virus.

The allantoic fluid was treated with a final concentration of 1% sodium deoxycholate and 0.01% trypsin at 37° for 30 min. The fluids were clarified for 30 min at 15,000 rpm in a Sorvall SS-34 rotor at 4°; and the supernatant fluid was removed. Since electron microscopic examination of virus sedimented against the wall of a tube revealed many distorted particles, the cushion technique of

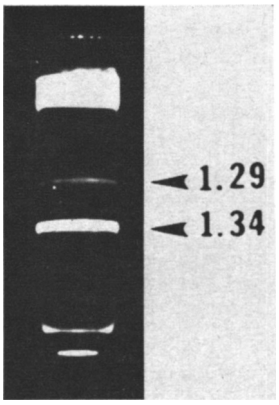

FIG. 1. Density gradient of CELO virus after centrifugation at 35,000 rpm for 24 hr in 40% rubidium chloride. Two light-scattering bands are seen. The band at 1.34 g/cm^3 contains highly infectious, intact virions; the lighter band, at 1.29 g/cm^3, contains incomplete forms of low infectivity.

Crawford (18) was utilized. The virus was sedimented on a 60% potassium tartrate cushion in 0.01 *M* Tris–HCl buffer, pH 7.3, using a Spinco SW 25.1 rotor. The centrifugation was performed for 3 hr at 25,000 rpm and the opalescent band at the interface of the two solutions was removed. Bands from 3 tubes were pooled, dialyzed overnight at 4° against 2 changes (1000 ml) of 0.01 *M* Tris buffer, and concentrated to a volume of approximately 3 ml with Carbowax (Union Carbide Corp.). One ml of virus was layered on 4.0 ml of 40% (w/w) rubidium chloride (density 1.38) and centrifuged at 35,000 rpm for 24 hours at 4° in a Spinco SW 39 rotor to form an isopycnic gradient.

Two bands were consistently found in the rubidium chloride gradients (Fig. 1). The higher, more diffuse band occurred at a density of 1.27–1.31 g/cm^3 and consisted of broken segments and empty or distorted virus particles (Stenback *et al.*, in preparation). The lower, compact band was found at a density of 1.33–1.34 g/cm^3 and consisted of uniform, well-structured adenovirus particles (Stenback *et al.*, in preparation).

Recovery of infectivity following purification. To determine the infectivity recoverable after purification, 25 ml of stock virus were processed by the method described. The virus band at a density of 1.34 g/cm^3 was assayed by the plaque technique. Table I shows the efficiency of recovery of infectivity. After purification, more than twice as many PFU were found in the banded material than were calculated to be present in the starting material. Presumably, the treatment with deoxycholate and trypsin disrupted aggregates of virus present in the untreated allantoic fluids.

Distribution of infectivity after density gradient centrifugation. To determine the distribution of infectious virus after density gradient centrifugation, 80 ml of stock virus were purified as described. Ten-drop fractions were collected from the gradient by puncturing the bottom of the tube. The density of each fraction was determined by weighing 20-μl aliquots. The infectivity of the fractions was assayed by the plaque technique. Results are summarized in Fig. 2. The fractions containing complete virus (viz., 16 and 17) had approximately 10^4- to 10^5-fold more infectious units than the fractions comprising the band of incomplete and distorted virus particles (viz., 23 through 29).

Distribution of CF antigenicity after density gradient centrifugation. When fractions were tested for CF activity against CELO immune hamster sera (Fig. 2), the results coincided with the infectivity titrations. The largest amounts of CF activity resided in the fractions at densities of 1.34 and 1.28–1.29. However, considerable amounts of viral soluble antigens remained at the top of the gradient.

Distribution of HA activity after density gradient centrifugation. Tests of fractions from density gradients indicated that the HA activity was associated with the bands at densities of 1.34 and 1.29 (Fig. 3). Therefore, hemagglutinin was associated with in-

TABLE I. Recovery of CELO Infectivity Following Purification.

Total PFU[a]	Expt. 1	Expt. 2
Before purification[b]	2.5×10^{10}	3.1×10^{10}
After purification	5.3×10^{10}	1.2×10^{11}

[a] 25 ml of stock virus were purified as described. The virus band at a density of 1.34 g/cm^3 was recovered and assayed by the plaque technique.

[b] Based on PFU/ml of untreated allantoic fluid.

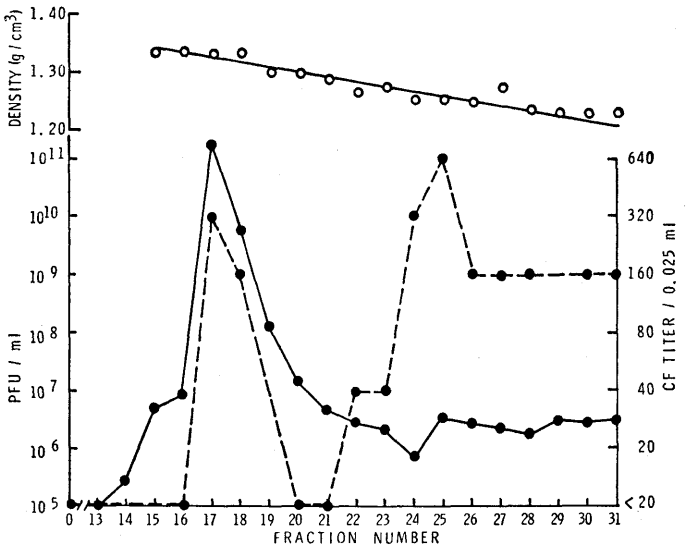

FIG. 2. The correlation of density, infectivity (PFU), and CF antigenicity throughout a RbCl gradient. Infected allantoic fluid (80 ml) was treated with 1% sodium deoxycholate and 0.01% trypsin. The fluid was clarified at 15,000 rpm for 30 min. The virions were concentrated on a cushion of 60% potassium tartrate. After isopycnic centrifugation in RbCl, 10-drop fractions were collected and assayed by the plaque technique and for CF activity. (○—) density (g/cm³); (●—) infectivity (PFU/ml); (●- - -) CF titer/0.025 ml.

tact virions, as well as with incomplete particles.

Temperature sensitivity of purified CELO virus. A stock of CELO virus was purified by the above procedure. The virion band at a density of 1.34 g/cm³ was removed and diluted with 10 ml of HBSS, pH 7.2. The diluted virus was dialyzed against HBSS for 4 hr to remove the rubidium chloride. One ml of the virus suspension was placed in test tubes, which were sealed with rubber stoppers. Tubes were placed in a 56° water bath and at various intervals a tube was removed for assay of virus. An unheated virus preparation served as control. The results of this experiment are summarized in Table II. CELO virus, unlike the human adenoviruses (11, 19) is quite stable to heat. Kawamura *et al.* (10), utilizing unpurified virus, found no inactivation by heat after 1 hr at 50°.

Attempts to detect adenovirus group specific antigens in CELO virus. When CELO

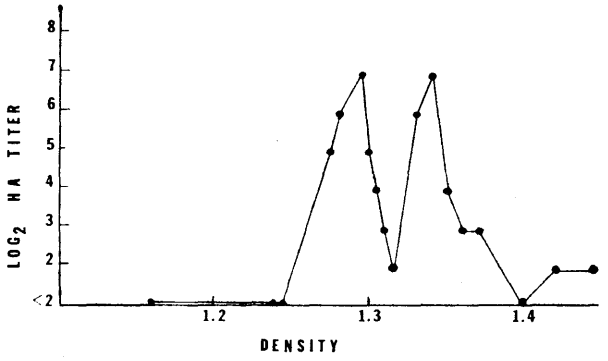

FIG. 3. Distribution of hemagglutinating activity throughout a density gradient. Virus (25 ml) was treated as described in Fig. 2. Fractions (10 drops) were collected and assayed for hemagglutinating activity.

TABLE II. Heat Stability of Purified CELO Virus at 56°.

Time at 56° (min)	Virus titer (PFU/ml)
Unheated	9.9×10^8
15	10.2×10^8
30	7.8×10^8
60	6.2×10^8

preparations were tested for the presence of the adenovirus group antigen, no reactivity could be demonstrated in either infected allantoic fluids or in purified virions (Table III). The adenovirus group antigen also did not fix complement in the presence of hamster anti-CELO serum.

Discussion. This paper describes the purification of large quantities of CELO virus from the allantoic fluids of embryonated eggs. Aggregated virions were liberated from cellular debris with trypsin and sodium deoxycholate and were subjected to density gradient centrifugation in 40% rubidium chloride. Virus stocks purified in this manner yielded 2–3 times as many total PFU as were present in the untreated starting material. Although CELO virus can be produced in chick kidney cells grown in tissue culture (20), the allantoic cavity of embryonated eggs produces higher titered virus preparations for use in purification procedures (Anderson, unpublished observations).

In a density gradient, CELO virus behaved similarly to the human adenoviruses (21). The infectious, complete virus banded at a density of 1.33–1.34 g/cm^3; the incomplete particles banded at a density of 1.27–1.30 g/cm^3. There was 10,000 times more PFU in the lower band than in the upper band. The highest concentrations of CF and HA activity were also found to be associated with these bands. CELO virus has hemagglutinating properties that are similar to those of Rosen's subgroup II for human adenoviruses (22), *i.e.*, producing complete agglutination of rat erythrocytes. CELO does not agglutinate human type O or guinea pig erythrocytes (McCormick *et al.*, in preparation). The association of the complete hemagglutinin with the virion is also characteristic of most human adenoviruses of subgroup II (23).

The remarkable heat stability of the CELO virion (10) has been confirmed using purified virus particles. The stability of purified virions indicates that this property is not the result of protective substances in the suspending medium. Antigenic studies (Table III, 24) have indicated that CELO virus, like GAL virus (9), does not contain the adenovirus group antigen. The lack of this antigen, as well as the heat stability of the virion, indicates that CELO is an aberrant member of the adenovirus group. However, its biophysical properties and its ultrastructural characteristics (Stenback *et al.*, in preparation) are typical of the adenoviruses in general.

Summary. CELO virus, from infected allantoic fluids, was treated with 1.0% sodium deoxycholate and 0.01% trypsin and centrifuged to equilibrium in a rubidium chloride gradient. Two bands, at densities of approximately 1.34 and 1.29 g/cm^3, were consistently found. Plaque titrations indicated that the band at 1.34 g/cm^3 contained the majority of infectious virus. Both hemagglutinating and CF activities were associated with these bands; however, soluble CF antigens were

TABLE III. Lack of Group Reactive Adenovirus Antigens in CELO Virus.

	Reciprocal of CF antigen titer[a]		
	Human reference serum	Hamster anti-CELO serum	Normal hamster serum
CELO-infected allantoic fluid	<32	≥256	<32
Normal allantoic fluid	<32	<32	<32
CELO, purified virus from density gradient	<2	128	<2
Human adenovirus group antigen	≥16	<2	<2
Group antigen control	<2	<2	<2

[a] Tested versus 4–8 units of antibody.

also present in the less dense regions of the gradients. The infectivity of purified CELO virions was heat stable and the virions did not contain the adenovirus group antigen.

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