processes which underlie the generation of potential differences. However the possibility of electrical shunting by an external pathway across surfaces that were not sufficiently dry cannot be ruled out.

Measurements were made on 4- and 5-day embryos of the potential across the chorion alone before it became fused to the allantois. In 2 experiments the inner (basal) surface was found to be at a potential of +4 mV relative to the external bathing medium (Earle's solution). If the chorion, once it has fused to the allantois, retains this reversed sign for the potential across it, as compared to that across the combined CAM, it is evident that the interior of the CAM is more positive than either its inner or outer surfaces. In view of the opposite orientation of the cells in the chorionic and allantoic layers of the membrane it can be concluded that both epithelial layers are negative on their free apical surfaces, but that the potential difference across the allantoic layer exceeds that of the chorionic layer, hence imparts its sign to the net difference across the whole membrane.

Conclusions. The experiments discussed here indicate that a potential difference, requiring the expenditure of metabolic energy, is maintained across the chorioallantoic membrane, the inner surface being negative. Its value is somewhat smaller than the potential observed across most other biologically active membranes(1), and it is unlikely that these small electric forces would act directly as organizing agents in a tissue graft on the membrane. Nevertheless the potential difference may be an expression of an underlying metabolic asymmetry, maintained for example by a gradient of CO_2 or O_2 across the membrane, which could be related more relevantly to the observed polar reconstitution of the organs in the experiments of Weiss and Taylor.

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Contamination of Adenovirus Stocks with SV40 (Papovavirus Group)*† (28361)

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Since Sweet and Hilleman(1) demonstrated the presence of vacuolating virus (SV40)—a member of the papovavirus group(2)—in normal rhesus and cynomolgus monkey kidney cell cultures, the biology of the virus has been studied intensively. Although only low grade subclinical infection has been observed in human beings infected with SV40(3,4), the agent is able to produce fibrosarcomas (5,6) and ependymomas(7) in newborn hamsters, and to transform human cells(8,9) and hamster cells *in vitro*(10-12). With SV40 occurring as an adventitious agent of monkey kidney cultures, it is not surprising that it has been discovered in polio- and adenovirus vaccines(13,14).

The above findings led us to search for SV40 contamination in experimental adenovirus vaccines being prepared by means of $MgCl_2$-inactivation of the virus(15). Such contamination of our adenovirus stock and vaccine was found. In this paper we de-

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† Papovavirus was officially accepted as the name for the papilloma-polyoma-vacuolating group of viruses by the International Subcommittee on Viral Nomenclature, International Conference on Microbiology, meeting on Aug. 20, 1962, in Montreal.

scribe the extent of the SV40 contamination as measured by particle counts, infectivity and antigenicity both of adenovirus and of the contaminating papovavirus.

Materials and methods. Monkey kidney (MK) cells. Kidneys from immature rhesus and green monkeys were trypsinized, grown and maintained in M-E lactalbumin medium (16).

Virus. Adenovirus type 7, obtained from Dr. H. S. Ginsberg, was passaged in human amnion cells (FL strain) once and primary human kidney cells twice before making 5 serial passages in rhesus MK cells maintained on M-E medium. Harvests of each MK lot were frozen and thawed 3 times and sonicated in 15 ml amounts in 30 ml lusteroid tubes by a Raytheon sonic oscillator (9 KC, Model S-102A). Cell debris in virus suspension was removed by centrifugation at 2000 rpm for 5 minutes.

Heating methods. Virus fluids were diluted with an equal volume of distilled water or $MgCl_2$ at twice the final concentration desired. Aliquots to be heated were delivered directly to the bottom of the tube in 1.2 ml amounts and the top of each tube was flamed thoroughly to sterilize at least one inch of the uppermost surface of the tube. All test tubes used were uniform in size and thickness, measuring 13 × 100 mm, with an 0.8 mm wall. The tube was then rubber-stoppered and plunged into a water bath covering the tube to ¼ inch from the stopper. At the end of the heating period, the tubes were quickly transferred into an ice water bath.

Virus titration. For titration of adenovirus an equal amount of 1:10 dilution of green monkey anti-SV40 serum (titer 1:1000) was added to each dilution of virus fluid and incubated at 37°C for 2 hours, then 0.2 ml of the virus-antiserum mixture was inoculated into each of 6 rhesus MK cell tubes. Cultures were observed for cytopathic changes (CPE) for 14 days. Prior to titration, $MgCl_2$-treated samples were dialyzed against 200 volumes of M-E medium diluted with an equal volume of 0.85% NaCl for 24 hours to remove the high concentration of the salt. In the titration of SV40 the same procedure as above was applied except that rabbit anti-adenovirus type 7 serum (titer 1:160) and green MK cells were used.

Animal inoculation. Adenovirus stock prepared by 5 serial passages in rhesus MK cells was used for immunization experiments. Young New Zealand rabbits weighing about 2500 g and guinea pigs about 300 to 400 g were obtained from one supplier. Groups of 2 or 3 rabbits and of 3 pigs were used per antigen and each rabbit or pig was inoculated intramuscularly with 2 ml or 1 ml of antigen, respectively. Baseline bleedings were obtained prior to inoculation, and the second injections and subsequent bleedings were performed as described below.

Neutralization test. The tube method was used for titration of antibody against adenovirus, and both tube and plaque reduction (17) methods for SV40. In the tube method, dilution of inactivated serum (56°C for 30 minutes) in 0.5 ml volumes of adenovirus or SV40 containing 100 TCD_{50} per 0.1 ml. Virus-serum mixtures were incubated for 2 hours at 37°C, then 0.2 ml of each sample was inoculated into each of 4 tube cultures of rhesus (for adenovirus) or green (for SV40) MK cells. Cultures were observed for 7 days and antibody titers were calculated by the Reed and Muench method. In the plaque reduction method 100 PFU of SV40 per 0.1 ml were used, and the cultures were observed for 14 days. The antibody titer of a serum was considered to be the highest dilution of the serum which showed 90% reduction of plaques compared to the virus control culture.

Virus particle counting.‡ The agar pseudoreplication technique(18) was used. Validity curves were established by Dr. K. O. Smith to show that as the virus sample was diluted, there was a corresponding reduction in virus particle counts, both for SV40 and for adenovirus.

Results and discussion. Concentration of adenovirus and SV40 in adenovirus stocks. Each undiluted lot of adenovirus stock prepared in MK cells was mixed with an equal volume of (a) distilled water or (b) 2M $MgCl_2$. Samples were heated at 50°C for 30

‡ We are indebted to Dr. K. O. Smith for his help in the counting experiments.

TABLE I. Concentration of Adenovirus and SV40 in Type 7 Adenovirus Stocks.

Treatment of virus	Infectivity units and particle counts	Concentration as $\log_{10}$/ml — Adenovirus P-2*	Adenovirus P-3	Adenovirus P-4	Adenovirus P-5	SV40 P-2	SV40 P-3	SV40 P-4	SV40 P-5
No treatment (live virus)	TCD_{50}	3.4	3.6	3.6	3.6	5.4	6.7	6.4	5.6
	No. of virus particles	<7.3	8.0	<8.0	7.3	8.3	9.3	9.3	8.7
Heated with 1 M $MgCl_2$ (50°C, 30 min)	TCD_{50}	N.D.†	0	N.D.	0	N.D.	4.5	N.D.	3.5
Heated with water (50°C, 30 min)	"	"	0	"	0	"	6.6	"	5.4

* P-2 = 2nd passage of virus stock in rhesus monkey kidney cell cultures; P-3 = 3rd passage, etc.

† N.D. = not done.

minutes and infectivity of adenovirus and SV40 was titrated. As shown in Table I, each lot of virus contained a higher infectious titer of SV40 than that of adenovirus. Sweet and Hilleman(1) reported infectivity titers of SV40 in "normal" rhesus MK cell cultures to be as high as $10^{6.7}$ per ml, and we observed similar concentrations in our material. The adenovirus titers averaged $10^{3.55}$ TCD_{50} per ml.

A precise counting of virus particles in this study could not be made because of the relatively low concentration of adenovirus. Nevertheless each lot of virus contained about 10 times more SV40 particles than adenovirus particles. Because of the low concentration of adenovirus, it was difficult to find both types of particles in a single microscopic field. To demonstrate the ease with which adenovirus and SV40 virus particles may be distinguished morphologically, mixtures of the 2 viruses were made and prepared for counting. An electron photomicrograph of one of these preparations is shown in Fig. 1. It can be seen that 80 mμ adenovirus particles are easily differentiated from the 45 mμ SV40 particles.

After heating, either in 1M $MgCl_2$ or in distilled water, infectious adenovirus could no longer be detected. As regards SV40, no loss in titer occurred in the samples heated in water; however, the samples heated in 1M $MgCl_2$ lost about 99% infectivity.

Immunization of rabbits and guinea pigs with SV40-contaminated adenovirus stock. Groups of 2 or 3 rabbits were inoculated, each rabbit intramuscularly with 2.0 ml (a) of virus heated in distilled water, or (b) of virus heated in M $MgCl_2$ or (c) of live virus in distilled water. After 7 weeks each rabbit received a second injection using the same antigens in the same volumes. Post-immunization serum samples were obtained one week after the first and second injections. The results are shown in Table II. As described previously(15), rabbits immunized with $MgCl_2$-inactivated virus produced almost the same titer of neutralizing antibody against adenovirus as those immunized with live virus. However with SV40, $MgCl_2$-heated virus produced less antibody than the stock containing fully infectious virus. Although

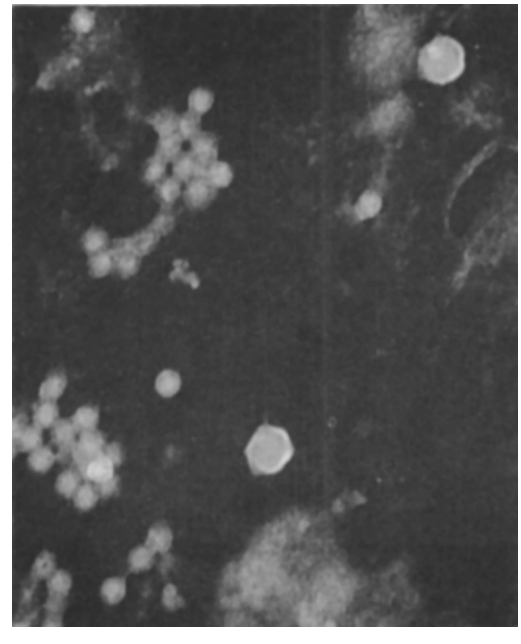

FIG. 1. Mixture of adenovirus and SV40 virus stained with uranyl acetate (approximately 80,000 × magnification).

TABLE II. Neutralizing Antibody Titers of Sera from Rabbits Immunized with SV40-Contaminated Adenovirus.

Treatment of virus	Rabbit No.	Neutralizing antibody titer: Adenovirus, Tube method, Post-1st inoc*	Adenovirus, Tube method, Post-2nd inoc	SV40, Tube method, Post-1st inoc	SV40, Tube method, Post-2nd inoc	SV40, Plaque reduction method, Post-1st inoc	SV40, Plaque reduction method, Post-2nd inoc
Heated with 1 M $MgCl_2$	1	64	256	<10	<10	<10	10
(50°C, 30 min.)	2	32	126	<10	<10	<10	10
	3	16	126	N.D.†	<10	N.D.	10
Heated with water	4	8	32	<10	32	<10	> 10
(50°C, 30 min.)	5	16	40	<10	18	<10	>100
No treatment (live virus)	6	32	256	<10	32	<10	>100
	7	32	158	<10	100	<10	>100

* Serum specimens

† N.D. = not done.

virus stock heated in water produced the lowest titer of adeno-antibody in rabbits, the same stock produced a higher titer of SV40-antibody than did the $MgCl_2$-treated virus.

In the guinea pig series each animal was inoculated intramuscularly with 1.0 ml (a) of live virus or (b) of virus heated in 1M $MgCl_2$. Two weeks after the first and second injections, serum was obtained. The interval between the first and second injections was 4 weeks. As shown in Table III, after 2 injections guinea pigs immunized with $MgCl_2$-treated virus or live virus had similar titers of neutralizing antibody against adenovirus, but again $MgCl_2$-treated virus produced a lower titer of SV40 antibody than did fully infectious virus.

Thus, although adenovirus treated with 1M $MgCl_2$ at 50°C lost its infectivity, it retained its full antigenicity(15). Under the same conditions SV40 lost 99% of its infectivity and a significant amount of its antigenicity. It is noteworthy that SV40 alone or when mixed with poliovirus(19) behaved differently from SV40 grown in cultures together with adenovirus. When the 2 DNA viruses were grown together, SV40 manifested more resistance to $MgCl_2$ enhancement of thermal inactivation.

Summary. Adenovirus stocks prepared by serial passage of the virus in rhesus monkey kidney cells were found to be contaminated with the simian papovavirus SV40. The infectivity titer and number of particles of SV40 in each of 4 virus stocks examined were higher than those of adenovirus. The virus stocks had an average adenovirus titer of $10^{3.6}$ TCD_{50}/ml and an adenovirus particle count of about $10^{7.6}$ particles/ml. The same stocks had an average SV40 titer of $10^{6.0}$ TCD_{50}/ml and an SV40 particle count of about $10^{9.0}$ particles/ml. Antigenicity of the adenovirus and SV40 components was tested by inoculation of rabbits and guinea

TABLE III. Neutralizing Antibody Titers of Sera from Guinea Pigs Immunized with SV-40-Contaminated Adenovirus.

Treatment of virus	Guinea pig No.	Neutralizing antibody titer: Adenovirus, Tube method, Post-1st inoc*	Adenovirus, Tube method, Post-2nd inoc	SV40, Tube method, Post-1st inoc	SV40, Tube method, Post-2nd inoc	SV40, Plaque reduction method, Post-2nd inoc
Heated with	1	0	126	<10	<10	>10
1 M $MgCl_2$	2	0	126	<10	<10	>10
(50°C, 30 min)	3	0	32	<10	<10	N.D.
No treatment	4	16	64	<10	32	>10
(live virus)	5	0	126	<10	32	>10
	6	16	32	<10	<10	>10

*Serum specimens.

pigs, using the untreated stock, and stocks heated in water and in $MgCl_2$. When the virus stock was heated at 50°C for 30 minutes in water, the adenovirus component lost all of its infectivity and a large part of its antigenicity, while the SV40 component retained both infectivity and antigenicity. When the virus stock was heated in M $MgCl_2$ at 50°C for 30 minutes, the adenovirus component again lost all of its infectivity but it retained all of its antigenicity. However the SV40 component was reduced both in infectivity and in antigenicity.

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A Medium Free of Agar, Serum and Peptone for Plaque Assay of Herpes Simplex Virus.* (28362)

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The plaque technique developed by Dulbecco(1) has been applied to many animal viruses and several such techniques have been developed for plaquing of herpes simplex virus. Kaplan(2) developed a plaque assay in primary rabbit kidney cells utilizing an overlay which contained a peptone and serum. Farnham(3), however, was unable to plaque herpes simplex virus under agar in HeLa cells and developed, accordingly, a microscopic plaque method in HeLa cell cultures. Russell(4), employing various cell lines, developed a sensitive method involving the use of carboxymethyl cellulose and antiserum for restricting formation of secondary plaques. The medium utilized by Russell, like that of Kaplan, contained peptones and human serum. Liebhaber and Takemoto(5) reported inhibition of plaque formation of some viruses by an acidic polysaccharide in agar and reversal of the inhibition by protamine sulfate.

To test the direct effects of various drugs on plaque formation by viruses, it is often necessary to exclude from the medium the interfering substances introduced by addition of peptones or serum. In the present report,

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