Data obtained in these studies suggest that in the alligator erythrocyte survival is temperature dependent and inferentially dependent on metabolic rate. The Q_{10} for almost all enzyme systems lies between 1.5 and 5, with the majority being under 3. A change of red cell life span of a comparable order of magnitude was achieved by a 14 to 15°C change in environmental temperature.

Summary. Immature American alligators were maintained at temperatures of 16°-17° C or 31°-32°C for periods of 6 to 7 months. Red cell life spans were measured with P^{32} or tritium labelled diisopropylfluorophosphate. Calculated red cell life spans corrected for changes in total red cell volume were prolonged by a factor of at least 3 at the lower temperature. Erythropoiesis was markedly depressed at low environmental temperature.

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Persistent Infection of Human Carcinoma and Primary Chick Embryo Cell Cultures with Simian Virus 40. (27902)

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Newborn hamsters injected with extracts of cell cultures prepared from the kidneys of apparently healthy rhesus monkeys developed subcutaneous neoplasms at the site of inoculation several months later(1). The oncogenic substance in the rhesus monkey kidney cell cultures was subsequently identified as the vacuolating virus or simian virus 40 (SV-40)(2,3). Before means for detecting the virus were known, SV-40 had been administered to man as a contaminant of poliomyelitis and adenovirus vaccines and as a contaminant of the respiratory syncytial virus tested experimentally in man. Antibody to SV-40 developed in some of the recipients (4-6). The serum titers were low and conclusive evidence that the virus could grow in human cells was lacking until Schein and Enders(7) demonstrated that primary cultures of a number of human tissues supported growth of the virus. No cytopathic changes were noted on first passage to these cultures but after transfer in kidney cell cultures, proliferating foci that became progressively larger were seen. Koprowski *et al.*(8) also cultured SV-40 in primary human buccal mucosa and skin organ cultures and noted morphologically abnormal changes in the cells accompanied by distinct chromosomal differences.

This paper describes the shedding of SV-40 in the fluid of 2 continuous and presumably transformed lines of human carcinoma, HeLa and HEp-2, and in primary chick embryo cell cultures, over periods of time ranging from 6 to 8 months.

Materials and methods. SV-40 virus. SV-40 strain A426 was recovered from a tumor induced by injecting a newborn hamster with an extract of rhesus monkey kidney cell culture(2).

Cercopithecus monkey kidney (CMK) and chick embryo (CE) cell cultures. Primary monolayers of these cells were prepared in

the Tissue Culture Section of the Division of Biologics Standards in 2-oz. prescription bottles or in 15 × 150 mm tubes. Each lot of CMK was prepared from the trypsinized kidney tissue of a single *Cercopithecus aethiops* monkey. The cultures were maintained with Eagle's medium(9) containing 2% horse serum.

The CE cultures were prepared from a pool of 24 minced and trypsinized chick embryos 10 or 11 days of age. The cultures were maintained in Eagle's medium with 2% horse serum.

HeLa and HEp-2 continuous line cultures. HeLa, originally derived from a carcinoma of the uterus(10) and HEp-2 from an epidermoid carcinoma of the larynx(11) had been propagated *in vitro* in different laboratories for a period of approximately 9 years prior to receipt. All cultures were prepared in this laboratory from trypsinized cells grown in Eagle's medium containing 10% calf serum and they were maintained in Eagle's medium containing 5% calf serum. Test cultures were not trypsinized but were refed with nutrient medium at weekly intervals. Sera used in all culture media were heated to 56°C for 30 minutes and all media contained 100 units of penicillin and 100 mg of streptomycin per ml. Cultures were incubated at 37°C.

Hamsters. Pregnant hamsters, *Cricetus auratus,* were obtained from the Animal Breeding Section of the Nat. Inst. of Health and housed in individual cages. The newborn hamsters when less than 1 to 3 days of age were injected subcutaneously with 0.2 ml of fluid from virus infected cell cultures to determine whether the virus retained its oncogenic property. The animals were observed for tumor formation or death every day or every other day. Dead hamsters were routinely autopsied.

Inoculation of test cell cultures with SV-40. Two-oz. prescription bottles containing confluent sheets of HeLa, HEp-2 and CE cultures were inoculated serially with 1 ml each of 10-fold dilutions of SV-40 that had been propagated in CMK. At weekly intervals the cultures were examined microscopically and the nutrient fluids were replaced. On occasions the fluids removed from the cultures were checked for presence of SV-40 by transfer of 1 ml of the fluid to 2-oz. bottles of CMK. These cultures were examined microscopically for cytopathic changes after 7 and 14 days incubation. The cytopathic titers of some of the positive fluids were determined in CMK roller tube cultures using 0.2 ml inoculum per tube and 2 tubes per dilution of each of a series of 10-fold dilutions. The cytopathic titer was the greatest dilution of virus to cause characteristic cytoplasmic vacuoles and ultimate destruction of the cells after 14 days incubation.

Results. Multiplication of SV-40 in test cultures. SV-40 was found to multiply in each of the cell lines under study as shown in Table I and the cells continued to shed virus into the fluid during periods of observation ranging from 178 to 241 days. No specific cytopathic changes occurred in the cells that adhered to the glass but depending on the amount of virus in the inocula the HeLa, and to a lesser extent the HEp-2 cell cultures underwent a series of macroscopic changes. For the first few weeks the cells in all the cultures were clear and in uniform sheets, but there were more spaces between the cells in the culture that had been inoculated with undiluted SV-40 or with SV-40 diluted 10^{-1} and 10^{-2} than in the control uninoculated cultures. The cultures inoculated with SV-40 diluted 10^{-3}, 10^{-4} and 10^{-5} appeared to be somewhat heavier than the control cultures. As time went on the cultures that had been inoculated with the more concentrated virus, undiluted through 10^{-6}, became uniformly heavy while in the uninoculated control cultures and cultures diluted with 10^{-7} and $>10^{-7}$ SV-40, many cells no longer adhered to the glass and those that remained were in heaped-up masses (Fig. 1). At the end of each 7-day period when the HeLa and HEp-2 cells were refed, many cells in both the uninoculated control cultures and the SV-40 infected cultures were present in the culture fluid.

The virus infected CE cultures remained as solid sheets of clear cells that were similar to the uninoculated control cultures

TABLE I. Multiplication of SV-40 in HeLa, HEp-2 and Chick Embryo Cell Cultures as Determined by Cytopathogenicity in Cercopithecus Monkey Kidney Cell Cultures.

Cell culture	Fluid from infected cell cultures incubated (days)	Dilution of virus used as inoculum										
		Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	None
HeLa (1st exp)	15	>Undil.	>Undil.	>Undil.	>Undil.	>Undil.	>Undil.					0
	36	*Idem*	*Idem*	*Idem*	*Idem*	*Idem*	*Idem*					0
	77						6.5*					
	120						9.0					
	241						8.5					
" (2nd exp)	15	"	"	"	"	"	>Undil.	>Undil.	0	0	0	0
	57	"	"	"	"	"	*Idem*	*Idem*	>Undil.	0	0	0
	178	8.5						9.5	9.2			
HEp-2	15	>Undil.	"	"	"	"	"	0	0	0	0	0
	57	*Idem*	"	"	"	"	"	>Undil.	0	0	0	0
	178							6.0				
Chick embryo	15	"	"	"	"	"	0	0	0	0	0	0
	57	"	"	"	"	0	0	0	0	0	0	0
	164				"							
	178				<1.0							

* Expressed as reciprocal log dilution of endpoint per 0.2 ml fluid.

FIG. 1. HeLa cells incubated for 169 days. The sheet of cells in the cultures that had been inoculated with SV-40 undiluted through 10^{-6} were confluent; cultures that were uninoculated or inoculated with SV-40 diluted 10^{-7} through 10^{-9} showed patches of heaped-up cells.

throughout the period of observation. Few cells sloughed into the fluid in 7 days and the titers in CMK were low.

The virus propagated in the HeLa cultures was oncogenic for hamsters. The fluid from one culture inoculated with 10^{-5} SV-40 and incubated for 94 days induced subcutaneous neoplasms in 7 of 10 hamsters 83 to 125 days later. Fluid from another culture inoculated with 10^{-6} virus and incubated for 77 days induced neoplasms in 10 of 11 hamsters 75 to 118 days later.

No neoplasms were produced in 10 hamsters by fluid from a chick embryo culture inoculated with 10^{-3} SV-40 and incubated for 91 days during a 160-day observation period.

Amount of SV-40 necessary to induce tumors in hamsters. It had previously been noted that extracts of rhesus monkey kidney cell cultures with infectivity titers of 10^{-7} in CMK induced neoplasms within a shorter period than extracts with titers of 10^{-5}. To determine if failure of the fluid from the CE culture to induce neoplasms in hamsters was due to insufficient virus concentration in the fluid, the following experiment was carried out. SV-40 that had been propagated in CMK was diluted to contain $10^{7.5}$, $10^{5.5}$, $10^{3.5}$ and $10^{1.5}$ ID_{50} virus per 0.2 ml and each dilution was used to inject a litter of newborn hamsters. Twelve of the 13 hamsters injected with $10^{7.5}$ virus developed neo-

TABLE II. Effect of Dose of Simian Virus 40 on Development of Neoplasms in Hamsters.

Concentration of virus in inoculum ID_{50}*	No. inj	Hamsters: Time of appearance of neoplasms in litter (days) First animal	Hamsters: Time of appearance of neoplasms in litter (days) Last animal	With tumors	Without tumors	Observation time (days)
$10^{7.5}$	13†	89	110	12	1	110
$10^{5.5}$	12	110	159	11	1	159
$10^{3.5}$	12	none		0	12	215
$10^{1.5}$	9	"		0	9	215

* 50% infective dose determined in cercopithecus monkey kidney cell cultures.
† Each hamster was injected subcutaneously when newborn with 0.2 ml of diluted virus.

plasms 89 to 110 days later, and 11 of the 12 hamsters injected with $10^{5.5}$ virus developed neoplasms after 110 to 145 days. None of the hamsters that were injected with fluid containing $10^{3.5}$ or $10^{1.5}$ virus developed neoplasms during a 215-day observation period (Table II).

Discussion. Sweet and Hilleman(12) reported that SV-40 did not multiply in HeLa, human heart, human amnion and Chang liver but they kept their cultures only 8 days. The results reported here indicate that SV-40 multiplies in both HeLa and HEp-2 and to a lesser extent in CE cells, and they confirm the observations of Schein and Enders(7) and of Koprowski *et al.*(8) that the virus is capable of growing in human cells. Cytoplasmic vacuoles characteristic of SV-40 in CMK were not seen in either the HeLa, HEp-2 or CE cells. There were changes in the gross appearances of the HeLa and to a lesser extent of the HEp-2 cells that could be correlated with virus inocula. First there was a diminution of cells adhering to the glass in the cultures that received the largest virus inocula and this was followed by increased proliferation of cells that adhered to the glass as compared with uninoculated cell controls or with cultures that had been inoculated with smaller doses of virus. In the latter cultures many cells dropped off the glass and those that remained were in heaped-up masses. Both HeLa and HEp-2 cells were derived from cancerous tissue, thus it was of considerable interest that they could be stimulated to increased proliferation by SV-40.

The changes in the gross appearance of the cultures could not be correlated with the amount of SV-40 being produced in the HeLa cultures, at least in the test carried out after 178 days incubation. The titer of virus in the fluid of a culture inoculated with 10^{-6} SV-40 which appeared as a solid confluent sheet of cells was not essentially different from the virus titer of fluid from a culture that had been inoculated with 10^{-7} SV-40 and which showed heaped-up patches of cells. Both gave titers of over 10^{-9}.

The difference in the appearance of the cultures depending on the amount of virus in the inocula could be due 1) to a selective process in which the high concentration of SV-40 destroyed less hardy cells, leaving the sturdier cells able to adhere to the glass, or 2) to interference of a second latent and unidentified virus in the HeLa and HEp-2 cells capable of causing syncytia.

SV-40 infection of CE cells also occurred but the virus titers were low and no neoplasms developed when newborn hamsters were injected with the culture fluid and kept for 160 days. This was in accord with the observation that approximately 10,000 ID_{50} SV-40 were necessary to induce neoplasms in hamsters.

It can be assumed that the CE cells were also infected with the lymphomatosis virus (13). They were prepared from 24 chick embryos from a regular commercial flock. Whether the lymphomatosis virus will interfere with the propagation of SV-40 is unknown.

The high concentration of SV-40 required to induce neoplasms in hamsters could have a corollary to infection of man with SV-40. The SV-40 present as a contaminant of some lots of poliomyelitis and adenovirus vaccines

was generally of low titer. Future study must determine whether small amounts of SV-40 stimulate antibodies which effectively inhibit further propagation of SV-40 or whether the virus is capable of continued multiplication until it reaches a concentration sufficiently high to cause a neoplasm.

Summary. Continued multiplication of SV-40 has been demonstrated in 2 continuous lines of human cancer cells, in HeLa for approximately 8 months and in HEp-2 cells for a period of 6 months. Infection of the HeLa cells and to a lesser extent the HEp-2 cells was accompanied by changes in the gross appearance of the cultures depending upon the concentration of SV-40 in the inocula. The concentration of SV-40 in the fluid from the human cell cultures was high and neoplasms were induced in hamsters by fluid from 2 different cultures. SV-40 was also shed into the nutrient fluid of CE cultures kept for 6 months. The infectivity titers were low as determined in CMK and hamsters injected with fluid from a 91-day-old infected CE culture failed to develop neoplasms within a 160-day period. It was determined that approximately $10^{5.5}$ ID_{50} virus was necessary to induce neoplasms in hamsters within a period of 215 days.

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Mechanism of the Effect of Trypsin on Specific Hemagglutination with Incomplete Rh Antisera.* (27903)

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Certain Rh antisera, as well as those of other blood group systems, react with but do not agglutinate homologous cells suspended in saline. Among the methods utilized to detect these incomplete (cryptagglutinoid) Rh antibodies are (1) suspension of RBC in colloidal medium, (2) Coombs antiglobulin reaction, (3) complement fixation, (4) agglutination inhibition, and (5) enzyme treatment of cells. This report is concerned with the mechanism by which trypsin, one of the active enzymes, produces this serologic effect.

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Materials and methods. Crystalline trypsin was obtained from Nutritional Biochemicals Corp. Incomplete anti-C (rh′), anti-D (Rh_o), anti-E (rh″), and anti-c (hr′) were obtained from Wadley Research Inst. and from Ortho Pharmaceutical Corp. The experimental methods largely parallel those employed in studies with papain(1). Serologic tests were performed using the 2 minute procedure previously described(2). Cells are treated with enzyme for 2 min at 37°C,