a part in the inactivation of EPF. The present studies illustrate again that both plasma and urine EPF levels commonly reach higher levels in phenylhydrazine anemia than at comparable degrees of anemia due to bleeding. Why levels of urinary EPF, as compared with corresponding plasma levels, are lowest in bled anemic, intermediate in phenylhydrazine anemic, and highest in hypoxic animals is not immediately apparent. Further chemical characterization of both plasma and urinary EPF is needed to establish whether or not their structure is the same.

Summary and conclusions. Simultaneous assays of plasma and urinary EPF were done in animals with varying degrees of anemia induced by bleeding or phenylhydrazine. Likewise comparisons of plasma and urine, collected after similar intervals of hypoxia from the same rabbits, were carried out. Diverse results were seen in the rabbits with induced anemia. a) Bled anemic rabbits showed distinct EPF elevations in plasma at hematocrit levels of 15 and 10% Only minimal corresponding elevation appeared in the urine, occurring only at the 15% hematocrit level. b) Phenylhydrazine anemic rabbits showed marked elevation of plasma EPF at all hematocrit levels from 25 to 10%. Urinary EPF was also present, although in lesser amounts, at these same hematocrit levels. At no time, in either the bled or phenylhydrazine anemic groups, were EPF levels as high in urine as in plasma.

The time of appearance and potency of EPF in plasma and urine of hypoxic rabbits were strikingly similar. Highest levels were present in both plasma and urine at 24 and 48 hours. Return to control levels was reached at 120 hours in both plasma and urine. At no time were EPF levels higher in urine than in plasma. In our opinion the above results suggest more the similarity than the separate identity of plasma and urinary EPF. They are compatible with the concept that urinary EPF represents partial excretion of plasma content.

The authors appreciate the helpful assistance of Andre Bulba, Ruth Deckert, Edward Dywinski, Helen Fox, Joyce Romano and William Sywenkyj.

1. Gordon, A. S., *Physiol. Rev.,* 1959, v39, 1.

2. Brecher, G., Stohlman, F., Jr., *Advances in Hematology,* 1959, v2, 110.

3. Mirand, E. A., Prentice, T. C., Slaunwhite, W. R., *Ann. N. Y. Acad. Sci.,* 1959, v77, 677.

4. Prentice, T. C., Mirand, E. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 231.

5.———, *ibid.,* 1961, v106, 501.

6. Fried, W. L., Plzak, L. F., Jacobson, L. O., Goldwasser, E., *ibid.,* 1957, v94, 237.

7. Gurney, C. W., *Semiannual Report to Atomic Energy Commission* (Argonne Cancer Research Hosp.), 1960, p7.

8. Borsook, H., Graybiel, A., Keighley, G., Windsor, E., *Blood,* 1954, v9, 734.

9. Hodgson, G., Fisher, S., Perretta, M., Eskuche, L., Araya, G., Dinamarca, M., *ibid.,* 1960, v16, 1398.

10. Van Dyke, D. C., *Hemopoiesis,* Ciba Found. *Symposium,* 1960, p397.

11. Slaunwhite, W. R., Prentice, T. C., Mirand, E. A., Unpublished data.

12. Jacobsen, E. M., Davis, A. K., Alpen, E. L., *Blood,* 1956, v11, 937.

Received November 16, 1961. P.S.E.B.M., 1962, v109.

Loss of Neurotropic Pathogenicity and Hemagglutinating Property of Columbia SK Virus by *in vitro* Cultivation in Sarcoma 180 Ascites Cells.* (27224)

EIICHI FURUSAWA AND WINDSOR CUTTING
Dept. of Medical Microbiology, Stanford University, Calif.

We have recently reported that Columbia SK (Col SK) virus adapted to Ehrlich ascites tumor cells *in vivo*(1,2) could also be adapted to Sarcoma 180 (S180) ascites cells both *in vivo* and *in vitro*(3). After 30 passages of the virus in S180 ascites cells *in vi-*

* Supported in part by California Division Am. Cancer Soc.

TABLE I. Effects of Serial Passages and Refrigeration on Hemagglutinating Property of Columbia SK Virus.

	Original harvests				Subcultures			
	Before freezing		After freezing		First		Second	
Generation	HA	Infectivity	Days	HA	HA	Infectivity	HA	Infectivity
T 1	1024	$10^{6.5}$ LD_{50}	158	1024	1024	$10^{7.2}$ ID_{50}		
T 3	512		138	512	512	$10^{6.5}$		
T 7	256		124	256	32	$10^{6.5}$	512	
T12	1024		108	1024	32		512	$10^{6.5}$ ID_{50}
T15	1024		82	1024	16	$10^{6.2}$	1024	
T20	256		60	256	64	$10^{6.5}$	1024	$10^{7.2}$
T24	128	$10^{6.5}$ ID_{50}	52	<2	<2	$10^{5.8}$	<2	$10^{6.5}$
T25	128		49	8	<2		<2	
T26	128		46	<2	<2	$10^{6.2}$	<2	$10^{6.8}$
T27	128	$10^{6.2}$	43	8	<2	$10^{6.5}$	<2	$10^{6.5}$
T28	<2	$10^{6.5}$	40	<2	<2	$10^{6.5}$	<2	$10^{6.5}$
T29	<2		36	<2	<2	$10^{6.5}$	<2	
T30	<2	$10^{6.5}$	32	<2	<2		<2	$10^{6.8}$
T31	<2	$10^{6.7}$						
T34	<2	$10^{7.5}$						
T37	<2	$10^{6.5}$						
T46	<2	$10^{7.2}$						
T48	<2	$10^{7.5}$						
T55	<2	$10^{7.5}$						

tro, we have obtained an attenuated virus which has completely lost neuro-virulence in mice and the hemagglutinating property. This new strain of Col SK virus is effective as a live vaccine against Col SK infection in the mouse.

Materials and methods. 1). *Virus passage* in vitro. Details have been described (3). After washing, 2 to 4 $\times$ 10^6 S180 ascites cells freshly harvested from mice were suspended in 1 ml of culture medium per tube. The medium for virus passage from the first generation (T1) to the twenty-ninth (T29) was composed of 80% of Medium 199 and 20% of non-inactivated S180 ascites serum from which the fibrin had been removed by standing at 4°C for 1 week. From T30 to T55 and for a second series of virus (Table III) ascites serum inactivated at 60°C for 30 minutes was used. At each generation, 0.1 ml of 10^{-3} dilution of infected supernatant after centrifugation was used for the next passage. The cytopathic effect (CPE) appeared 3 days after incubation, ascertained by erythrosin staining, and was used for virus titration *in vitro*. For titration of hemagglutinins (HA), 0.2% sheep red cells suspended in potassium barbital buffer was used(4). 2). *Virus titration.* Infectivity of the culture fluids was determined at each 10-fold dilution, *in vitro* by CPE and HA (average of 3 tubes), and *in vivo* in 9 mice, 3 of which were inoculated intracerebrally (ic), 3 intraperitoneally (ip), 3 ip accompanied by S180 ascites cells. 3). *Ultraviolet-inactivated brain-Col SK virus* (*UV Col SK*). A WL782-30 Sterilamp (Westinghouse, 118 volts, 60 cycles, 0.5 line ampere) was used for inactivation of Standard Brain Col SK virus. Two ml of virus material (supernatant of one brain homogenate in 10 ml of broth saline, containing about 10^6LD50/0.1 ml of virus by ip route; HA titer 1:1280) was placed in an open petri dish 9 cm in diameter and irradiated at a distance of 20 cm for 3 minutes, with shaking by hand. This time was the minimum for complete inactivation of the virus.

Results. 1. *Effects of serial passage and refrigeration on HA property of Col SK virus.* As reported previously(3), the HA titer of harvests of *in vitro* cultures varied through serial virus passage, as compared with the more consistent infectivity. After the twenty-eighth generation (T28) HA could not be demonstrated, but infective titers were still high, as shown in the second and third columns of Table I. However, harvests from earlier generations which had been stored at −20°C for 1 to 5 months still showed positive HA (Table I). The original harvests from T1 to T20 showed completely maintained HA

TABLE II. Non-specific HA Inhibitor in Normal S180 Ascites Serum.

	Non-inactivated				56°C, 30 min. inactivated			60°C, 30 min. inactivated		
	Ascites serum dilution									
HA units	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:2	1:4	1:8
4	—	—	—	+	—	—	+	+	+	+
8	—	—	—	+	—	+	+	+	+	+
16	—	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+

HA reaction: + indicates positive reaction; — indicates negative reaction.

titers and 2 serial subcultures also produced high titers of HA. However, by contrast, the original harvests from later generations (T24 to T27) had lost HA titer in the freezer in 2 months and the next subcultures could not reproduce HA in spite of high multiplication of infective viruses.

2. *Non-specific HA inhibitor in normal S180 ascites serum.* To explain the loss of HA in the later cultures, it was considered possible that the S180 ascites serum in the culture medium might interfere with production of HA or cover the surface of HA as reported for vaccinia virus by Cassel and Fater (5,6,7) and Japanese B encephalitis virus by Diercks *et al.*(8). To test this, the HA inhibition test was done with non-inactivated and heat-inactivated ascites serum by the usual method(1). Each 0.25 ml of 2-fold dilution of the ascites serum was mixed with 0.25 ml of a definite amount of HA from T2, and after standing at room temperature for 1 hour, 0.5 ml of sheep red cells was added. Table II shows that normal ascites serum produces some HA inhibition which could be inactivated by heating at 60°C for 30 minutes. In spite of the existence of inhibitor, HA production in earlier generations (before T20) was not interfered with by 20% of ascites serum in culture medium (Table I). However, the effect of the inhibitor was considerable on the HA of generations after T24. Therefore, cultures since T30 have been maintained with inactivated ascites serum. The results (Table I) demonstrate that virus which has lost the HA property does not regain it in such medium.

3. *Second series of virus passages from T20 with inactivated ascites serum.* With the expectation that virus maintaining the HA property could be passaged in a culture medium containing inactivated ascites serum without loss of this property, a second series of virus passages was made, starting with T20, in which HA activity was maintained even after storage of 2 months (Table III). Unexpectedly, HA was not detectable in T27, and when new virus passages from T24 or T25 of the second series were repeated, again no HA was detectable in T27.

4. *Comparison of neurotropic pathogenicity between HA positive and negative viruses.* To examine further the changes in cultured viruses during serial passages, infectivity titrations were performed *in vitro* and *in vivo*. The results are shown in Table IV. The earlier passages (T4 to T14, HA positive viruses) showed high neurotropic virulence by the ic route without S180. T22 still showed high virulence by the ic, but rather low by the ip route (with S180). T28, which had lost the HA property, showed low virulence and loss of oncolytic effect on S180 in mice. Passages after T34 showed complete loss of virulence in mice, with only the CPE *in vitro* maintained.

5. *Comparison of prophylactic effect between the attenuated virus and UV Col SK*

TABLE III. Second Series of Virus Passage from T20 with Inactivated Ascites Serum.

Generation	HA	Infectivity
T20	256	
T21	64	$10^{6.5}$ ID_{50}
T22	1024	$10^{7.5}$
T23	512	
T24	512	$10^{6.5}$
T25	1024	
T26	256	$10^{6.5}$
T27	<2	$10^{6.5}$
T28	<2	$10^{7.2}$
T29	<2	
T30	<2	$10^{7.5}$
T31	<2	

TABLE IV. Comparison of Neurotropic Pathogenicity between HA Positive and Negative Viruses.

Generation	HA	Infectivity S180 *in vitro* per .1 ml	ic route per .03 ml	ip route per .2 ml	S180 *in vivo* per .2 ml
T 4	512	$10^{6.5}$ ID_{50}	$10^{6.2}$ LD_{50}	$10^{2.5}$ LD_{50}	$10^{6.5}$ LD_{50}
T14	512	$10^{6.5}$	$10^{6.5}$	$10^{2.5}$	$10^{6.2}$
T22	1024	$10^{7.2}$	$10^{7.2}$	$10^{2.2}$	$10^{4.5}$
T28	<2	$10^{7.2}$	$10^{4.2}$	$10^{0.5}$	$<10^{0.0}$
T34	<2	$10^{7.5}$	$<10^{0.0}$	$<10^{0.0}$	$<10^{0}$
T38	<2	$10^{6.5}$	$<10^{0}$	$<10^{0}$	$<10^{0}$
T46	<2	$10^{7.2}$	$<10^{0}$	$<10^{0}$	$<10^{0}$
T49	<2	$10^{8.2}$	$<10^{0}$	$<10^{0}$	
T52	<2	$10^{7.5}$	$<10^{0}$	$<10^{0}$	
T55	<2	$10^{7.5}$	$<10^{0}$	$<10^{0}$	

virus. In several preliminary experiments, attenuated virus (after T30) seemed to have a definite prophylactic effect by either ic or ip route against later challenge with virulent Col SK brain virus. This virus was then compared with UV irradiated Col SK virus. Ten-fold dilutions of both attenuated and UV virus were injected ip into groups of 5 mice; the injection was repeated one week later in one of the UV groups and in one of the T55 groups. After 10 days, about 10^2LD50 of brain virus was injected ip into all mice. The results are shown in Table V. A single injection of an attenuated virus (T34, T38, T46, T55) was completely protective against the challenge of virulent virus even at the 10^3 dilution. On the contrary, UV Col SK virus did not protect completely unless 2 injections of 10^{-1} dilution had been made.

Discussion. Cassel and Fater(5) reported that there was a non-specific inhibitor of vaccinia HA in the ascites serum of Ehrlich tumor cells and it is of interest to speculate whether or not there is a connection between this inhibitor and the fact that, on serial passage in the tumor, the IHD-E strain of vaccinia virus loses the capacity to produce HA. They also reported(7) that the IHD-T strain of vaccinia, which lacks both HA and HA-inhibiting property, was obtained by exposure to the inhibitor. If such an explanation is to be applied to our virus-host system, it would seem that HA positive virus should be cultivable continually in the culture medium in which the HA inhibitor has been inactivated by heat. Unfortunately, we could not succeed in the serial cultivation of HA positive virus beyond 27 generations. The HA inhibitor did not affect the multiplication of the Col SK virus; the same was noted for vaccinia virus by Cassel(6) and for Japanese B encephalitis virus by Diercks *et al.*(8). Therefore, it seems that the HA inhibitor has no important action on induction of HA negative virus in our system. It is of interest that the HA negative virus had at the same time completely lost neurotropic pathogenicity, and it should be important to clear the relation between HA property and virulence in the central nervous system.

TABLE V. Comparison of Prophylactic Effect of Attenuated Virus and UV-Killed Brain Virus.

Inoculum	Injection, .2 ml ip	Virus dilution 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
UV-killed brain Col SK	Once	3/5*	3/5	1/5	0/5	0/5		
	Twice	5/5	5/5	2/5	0/5	0/5		
T34	Once	5/5	5/5	5/5	5/5	3/5	1/5	0/5
T38	"		5/5	5/5	5/5	2/5	0/5	
T46	"		5/5	5/5	5/5	3/5	0/5	
T55	Once	5/5			5/5	2/5	0/5	
	Twice	5/5			5/5	3/5	0/5	0/5

* Numerator = No. of mice surviving after challenge with virulent virus; denominator = No. of mice used.

In comparing the immunogenic properties, the attenuated virus seems to be superior to UV Col SK virus in dosage and number of injections to immunize. From the fact that a single injection of 10^{-4} dilution of the attenuated virus was enough to immunize mice, it is considered possible that the virus may have propagated subclinically. Similarly, Sabin(9,10) reported that attenuated poliovirus which had been cultured in rhesus monkey cells lost its virulence by intraspinal injection in the chimpanzee but still retained it for rhesus and cynomolgus monkeys. Berge, Banks and Tigertt(11) used guinea pig heart cells to attenuate Venezuelan encephalomyelitis virus in mice. Findlay and Mac Callum(12) passed a pantropic yellow fever virus in mouse carcinoma 63 and found that the passed virus lost pathogenicity for the monkey but retained virulence for mice. From these reports it seems that attenuation of the viruses was induced by use of the heterologous hosts. On the other hand, in our case, attenuation of Col SK virus was obtained during cultivation in homologous mouse tumor cells and in a homologous ascites medium. It is of interest that the virus, now adapted to growing in tumor cells *in vitro*, is unable to grow in normal cells and in the same tumor cells in the homologous animal.

Summary. Attenuation of Columbia SK virus by *in vitro* cultivation in 30 serial passages in Sarcoma 180 ascites cells is described, during which the virus lost neurotropic virulence and the HA property. Nonspecific HA inhibitor in the culture medium has no connection with the changed virus nature. The attenuated virus could be used as a live vaccine for prophylaxis of Columbia SK mouse infection.

1. Furusawa, E., Cutting, W., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 618.
2. ———, *ibid.*, 1960, v103, 851.
3. ———, *ibid.*, 1961, v108, 244.
4. Horvath, B., Jungeblut, C. W., *J. Immunol.*, 1952, v68, 627.
5. Cassel, W. A., Fater, B., *Science*, 1957, v126, 301.
6. Cassel, W. A., *Virology*, 1957, v3, 514.
7. ———, *ibid.*, 1958, v5, 571.
8. Diercks, F. H., Kundin, W. D., Porter, T. J., *Am. J. Hyg.*, 1961, v73, 164,
9. Sabin, A. B., *Ann. N. Y. Acad. Sci.*, 1955, v61, 924.
10. ———, *ibid.*, 1955, v61, 1050.
11. Berge, T. O., Banks, I. S., Tigertt, W. D., *Am. J. Hyg.*, 1961, v73, 209.
12. Findlay, C. M., Mac Callum, F. O., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1937, v30, 507.

Received November 20, 1961. P.S.E.B.M., 1962, v109.

Overwintering of Western Equine Encephalomyelitis Virus in Garter Snakes Experimentally Infected by *Culex tarsalis*. (27225)

LEO A. THOMAS AND CARL M. EKLUND (Introduced by John J. Munoz)
United States Department of Health, Education, and Welfare, P.H.S., Nat. Inst. of Health, Nat. Inst. of Allergy and Infect. Dis., Rocky Mountain Laboratory, Hamilton, Montana

Previous studies have shown that garter snakes are readily infected with western equine encephalomyelitis (WEE) virus either by bites of infected mosquitoes or by the intraperitoneal (IP) injection of infected mouse brain tissue(1). Snakes inoculated by the IP route with 1 million suckling mouse LD_{50} of WEE virus have been shown to hibernate overwinter under simulated natural conditions and to circulate virus as long as 70 days the following spring and summer(2). *Culex tarsalis* became infected by feeding upon these post-hibernating snakes and transmitted the virus to young chicks.

Although garter snakes inoculated IP were shown to be capable of serving as an overwintering reservoir of WEE virus, a natural means of introducing the virus into snakes would have perhaps altered the picture. The present study was undertaken to determine