of Iliffe and Myant(11). These workers used a cell-free preparation from rat liver in which LCFA were synthesized from acetate by way of the biotin-dependent malonyl-CoA pathway. This preparation contained phosphorylating mitochondria and did not require the cofactor additions necessary for synthesis in soluble, cell-free systems. Under these conditions, which more closely resemble the intact cell, malonate had no effect on LCFA synthesis.

Summary. Biotin deficiency in intact chicks resulted in lower total carcass fatty acid deposition and altered fatty acid composition as compared to normal chicks. Dietary malonic acid stimulated total fatty acid deposition in the carcass of biotin-deficient chicks but did not alter the fatty acid composition of the carcass. In normal chicks, malonic acid had no effect on total fatty acid deposition but altered fatty acid composition such that it approached the composition of deficient chicks. Growth and biotin-deficiency dermatitis were unaffected by malonic acid in deficient chicks, but malonic acid depressed the growth of normal chicks.

The author gratefully acknowledges the technical assistance of Mrs. Faye Suggs and Mr. Paul Ziglar and the comments and suggestions of Drs. Gennard Matrone and C. H. Hill.

1. Donaldson, W. E., Proc. Soc. Exp. Biol. and Med., 1964, v116, 622.
2. Spencer, A. F., Lowenstein, J. M., J. Biol. Chem., 1962, v237, 3640.
3. Dils, R., Popjak, G., Biochem. J., 1962, v83, 41.
4. Donaldson, W. E., Poultry Sci., 1964, v43, 784.
5. Wiese, H. F., Hansen, A. E., Coon, E., J. Nutrition, 1962, v76, 73.
6. Marion, J. E., Edwards, H. M., ibid., 1962, v77, 23.
7. Bortz, W., Abraham, S., Chaikoff, I. L., J. Biol. Chem., 1963, v238, 1266.
8. Hulsmann, W. C., Biochim. et Biophys. Acta, 1963, v77, 502.
9. Kallen, R. G., Lowenstein, J. M., Arch. Biochem. Biophys., 1962, v96, 188.
10. Wakil, S. J., Bressler, R., Metabolism, 1962, v11, 742.
11. Iliffe, J., Myant, N. B., Biochem. J., 1964, v91, 369.

Received October 26, 1964. P.S.E.B.M., 1965, v118.

Hemagglutination by Reoviruses Propagated in Various Cell Lines.* (29873)

JOHNG S. RHIM,† JAMES I. KATO AND WILLIAM PELON
(Introduced by G. J. Buddingh)
Virology Section, Louisiana State University, New Orleans, and International Center for Medical Research and Training, San José, Costa Rica

Although primary cultures of renal cells from the Rhesus monkey have been shown to be the most sensitive host system for the study of reovirus types(1), both expense and availability limit the use of tissue from this animal in the virus laboratories of many countries. An alternate solution for this problem would be the use of cells in continuous passage for the study of reoviruses. This report describes the results obtained with respect to hemagglutinin production and development of infectious virus in 3 such cell systems.

* This investigation was supported by USPHS Grant GM 11330-20, Office of International Research.

† Present address: Laboratory of Tropical Virology, Nat. Inst. of Allergy & Infect. Dis., N.I.H., Bethesda, Md.

Materials and methods. Virus. The 3 prototype reovirus strains, type 1 "Lang," type 2 "D-5" and type 3 "Dearing," employed in these studies were supplied by Dr. A. B. Sabin, Children's Hospital Research Foundation, Cincinnati. In addition, the "Abney" strain (type 3) was obtained from Dr. L. Rosen, Pacific Research Station, NIAID; Honolulu, strain "48/4" (type 2) from Dr. C. Moscovici, University of Colorado Medical Center, Denver; strain "988" (type 2) from Dr. A. M. Lerner, Wayne State Uni-

versity College of Medicine, Detroit and strain "HEV" (type 3) from Dr. N. F. Stanley, University of Western Australia, Perth. Strain "716" (type 1) was also used in this study.

Tissue culture methods. All viruses were propagated in either primary monolayer cultures of Rhesus monkey kidney (MK) cells, in monolayer cultures of the diploid human embryonic lung cell, WI-39(2), received from Dr. L. Hayflick, Wistar Institute, Philadelphia and in the established cell lines, LLC-MK_2(3), received from Dr. R. N. Hull, Eli Lilly and Co., Indianapolis and BS-C-1(4), received from Dr. J. E. Hopps, Division of Biologics Standards, Nat. Inst. Health, Bethesda. The latter 2 cell strains were derived from renal cells of the Rhesus and the African green monkey respectively.

Trypsin-dispersed Rhesus MK cells were propagated in MK-lactalbumin hydrolysate medium (M-HLAH)(5) containing 2.0% agamma calf serum‡ during the first 4 days of incubation. The outgrowth medium was then replaced with M-ELAH medium(5) containing 2.0% agamma calf serum. The cultures were inoculated when confluent, using M-ELAH medium described above for maintenance.

WI-38 cells were grown in Eagle's Basal Medium§ prepared in Earle's balanced salt solution (EBME)(6) containing 10% agamma calf serum and were maintained in EBME containing 5.0% agamma calf serum. The outgrowth medium employed with the LLC-MK_2 cell line consisted of double strength EBME (2 × EBME) and 10% agamma horse serum,‡ while 2 × EBME containing 1.0% agamma horse serum was used for maintenance. The BS-C-1 cell line was propagated in 2 × EBME containing 10% agamma calf serum and maintained in 2 × EBME containing 5.0% agamma calf serum.

Antigen preparation. Antigens for hemagglutination (HA) were prepared by inoculating appropriate quantities of undiluted virus-containing tissue culture (TC) fluids into Blake bottle or tube cultures of the cells described above. Infected TC fluids were harvested when the cell sheets became completely disrupted. Such fluids were stored at —20°C and used as antigens without further treatment(7).

HA titrations. The procedures employed were basically those for reoviruses described by Rosen(7) except that human type "O" erythrocytes were washed 3 times in 0.85% NaCl instead of in dextrose-gelatin-veronal solution(8). The test was carried out at room temperature unless otherwise stated. The end-point was taken to be at the dilution showing strong partial agglutination.

Infectivity titrations. Serial 10-fold dilutions of virus were made in the maintenance medium of the particular cells employed. The cell cultures in tubes were inoculated with 0.2 ml of diluted virus and were examined for development of cytopathic effects (CPE). The final reading was made on the 8th day following inoculation and the 50% infective end-point titer calculated.

Experimental results. Use of different host cell systems for preparation of reovirus HA antigens. Experiments were carried out to determine if variations in the potencies of the reovirus type HA antigens would result when such viruses were propagated in different host cell systems. Aliquots of each prototype of the virus stocks as received from the

TABLE I. Titers of Reovirus HA Antigens Prepared in Different Host Cell Systems.

Reovirus type	Strain	Virus stock passed once into	HA titer	Infectivity titer* Log TCD 50/ml
1	Lang	WI-38	128	7.0
		Rh-MK	128	7.5
		LLC-MK_2	64	7.5
		BS-C-1	32	not done
2	D-5	WI-38	128	7.5
		Rh-MK	64	7.5
		LLC-MK_2	64	7.0
		BS-C-1	64	not done
3	Dearing	WI-38	8	7.5
		Rh-MK	4	7.5
		LLC-MK_2	8	7.0
		BS-C-1	4	not done
	Abney	WI-38	16	7.5
		Rh-MK	16	7.0
		LLC-MK_2	8	7.5
		BS-C-1	8	not done

* Determined in homologous cell system.

‡Hyland Laboratories, Los Angeles, Calif.

§ Microbiological Associates Inc., Bethesda, Md.

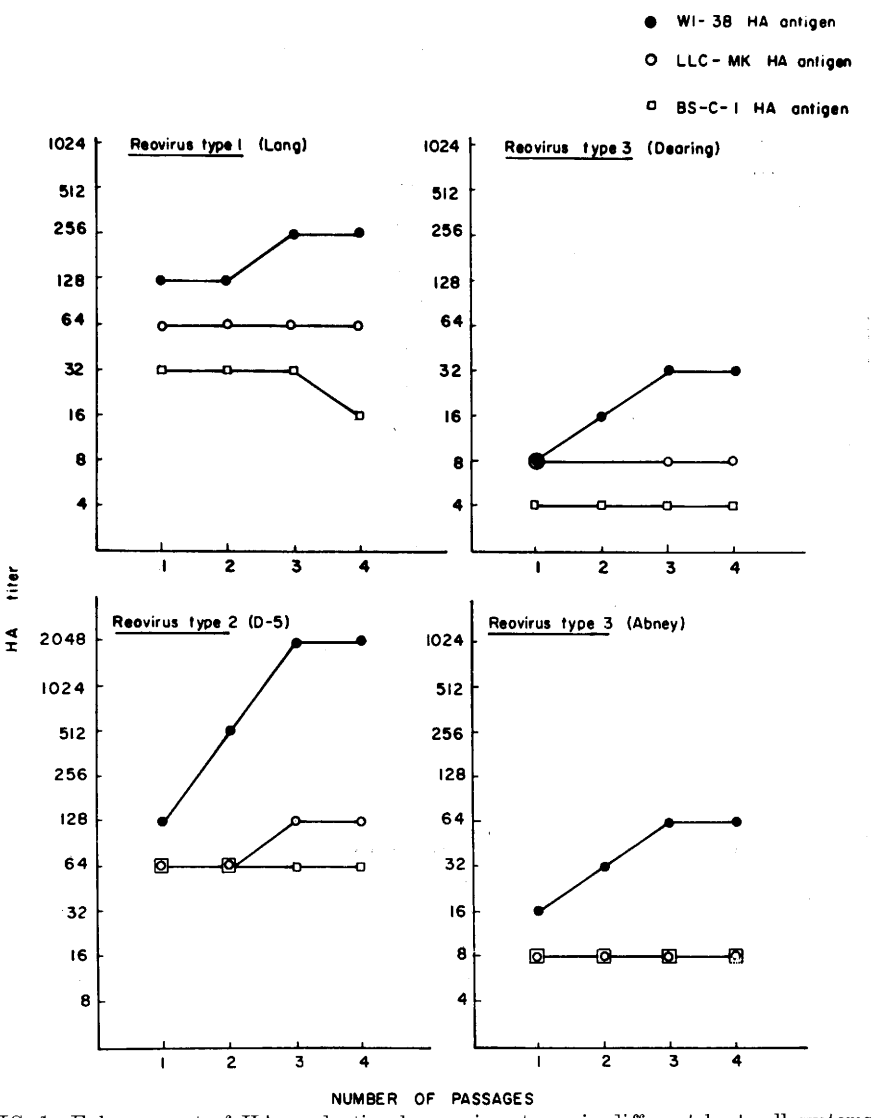
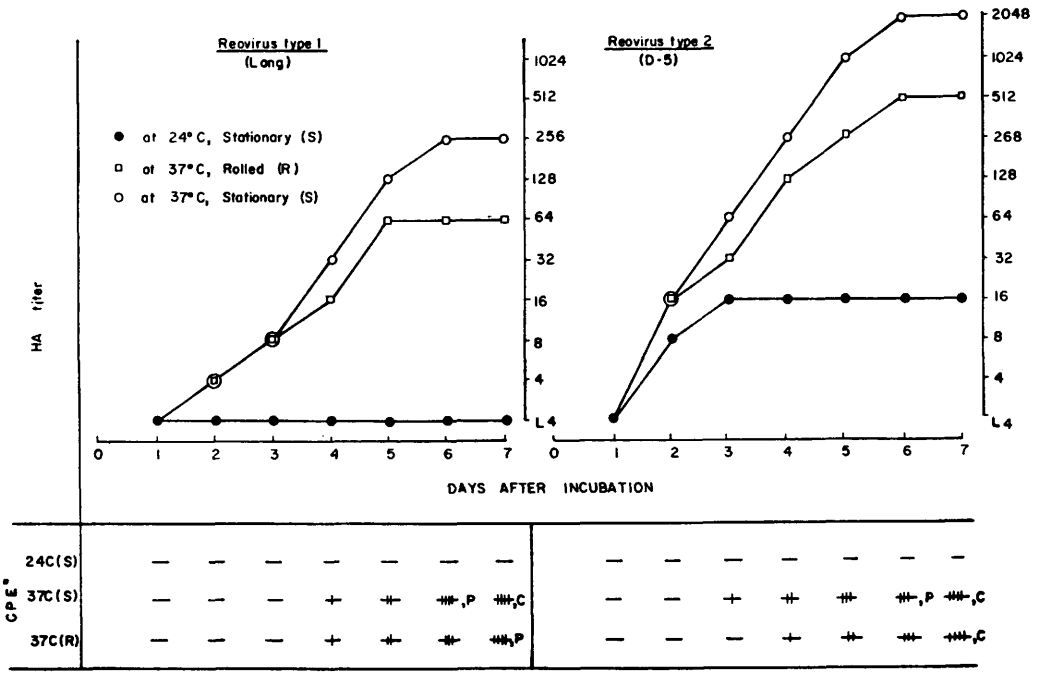


FIG. 1. Enhancement of HA production by reovirus types in different host cell systems by passage.

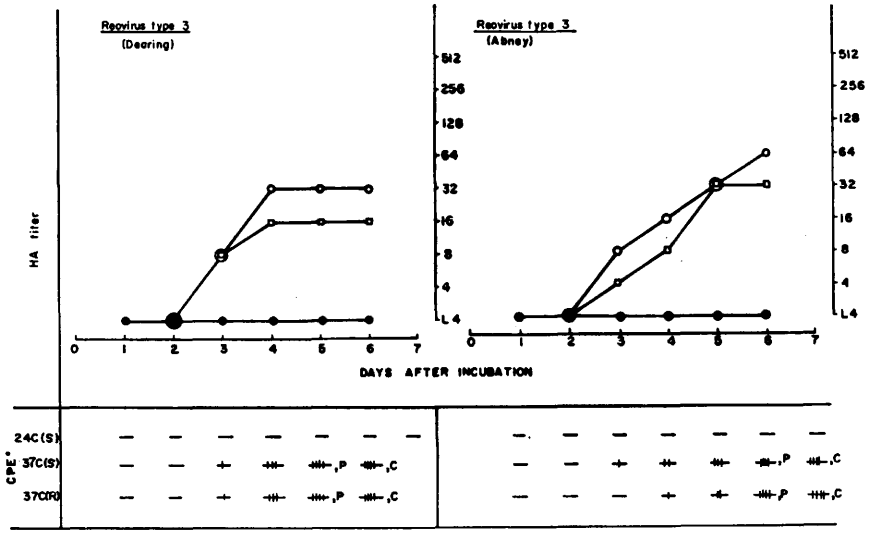
sources described earlier were inoculated without additional passage into cultures of Rhesus MK, WI-38, LLC-MK_2, and BS-C-1 cells. Infected TC fluids were harvested as described above, then titrated simultaneously.

The results presented in Table I show that the titers of the reovirus type HA antigens prepared in cultures of WI-38 cells were equal to or at times possibly greater than those obtained from antigens prepared in primary cultures of Rhesus MK cells. Antigens produced in LLC-MK_2 cell cultures appeared to be almost as satisfactory as those from Rhesus MK. The BS-C-1 cells appeared to be less suitable for type 1 HA antigen production than Rhesus MK; however, type 2 and 3 HA antigens prepared in these cells was found to be as satisfactory as those prepared in Rhesus MK cells. The potencies of the type 3 HA antigens were found to be much lower than those of types 1 and 2 despite the different host cell systems employed.

Effect of virus passages upon HA production. Since there were no significant differences observed between HA titers of antigens



2



3

FIG. 2, 3. Influence of incubation temperature and method of incubation upon HA production by reovirus types in WI-38 cells. * CPE: — = no cellular change, + = 25%, ++ = 50%, +++ = 75%, ++++ = 100% cell affected. P = Cells partially off. C = Cells completely off glass.

propagated in the various cell lines (Table I), studies were undertaken to determine the effect of virus passage in such cell systems upon HA production.

The results obtained are shown in Fig. 1. With each reovirus type, titers of HA antigens prepared in cultures of WI-38 cells were 2- to 16-fold greater than those obtained with antigens prepared in LLC-MK_2 and BS-C-1 cells. As may be seen, the optimal HA titer for each type was attained following 3 passages in WI-38 cells.

Comparison of HA titers and CPE of reoviruses propagated in stationary and rolled WI-38 cell cultures at 37°C in stationary WI-38 cell cultures at 24°C. Recently, it was reported that an increase in production of HA and an enhancement of CPE was observed when cultures of Rhesus MK cells inoculated with reoviruses were rolled during incubation(9). Experiments were conducted to determine whether similar effects would be obtained when cultures of WI-38 cells inoculated with reoviruses were incubated under similar conditions. In addition, reoviruses were propagated in stationary cultures of WI-38 cells at a temperature of 24°C and the subsequent effect upon HA production investigated.

Each of the reovirus types employed was inoculated undiluted (0.2 ml) into each of a group of 42 tube cultures of WI-38 cells. Each group of inoculated cultures was divided into 3 sets of 16 cultures each. Two of the 3 sets were incubated in a stationary position at temperatures of 24 and 37° respectively. The third set was incubated at 37°C in a roller drum apparatus, rotating at 12 revolutions per hour. All cultures were observed for the development of CPE. Two cultures were removed from each set daily, the culture fluids pooled and stored frozen without further treatment. Harvested cultures fluids were simultaneously titrated for HA content.

It can be seen in Fig. 2 that incubation of reovirus types 1 and 2 in a stationary position ultimately resulted in a higher HA titer than when rotated. Although significant differences were not noted with the 2 strains of type 3 employed (Fig. 3), an apparent enhancement favoring incubation in a stationary position was observed. The development of CPE appeared to parallel the production of HA.

TABLE II. Effect of Temperature of Hemagglutination Upon HA Titers of Reovirus Antigens Passaged Once in WI-38 Cells.*

Type	Strain	HA titer at incubation temp of 4°C	24°C	37°C
I	Lang	128	128	128
	716	16	16	16
II	D-5	128	128	128
	988	128	128	128
	4/48	64	32	16
III	Dearing	<2	8	8
	Abney	<2	32	32
	HEV	<2	8	8

* *Versus* human group "O" erythrocytes.

In those cultures inoculated with type 1, type 2 and "Abney" strain of type 3, the CPE seemed to progress more rapidly when incubated in a stationary position than when rotated. No differences in rates of development of CPE were observed in those cultures inoculated with the prototype strain of type 3.

CPE and HA were not detected in cultures of WI-38 cells inoculated with types 1 and 3 and incubated in a stationary position at 24°C. In contrast, CPE and HA were detected to a limited extent in cultures inoculated with type 2 and incubated under similar conditions.

Effect of temperature upon reovirus HA antigen titer. The temperature during the course of HA has been shown to be a factor in the HA titers obtained with certain ECHO (10), Coxsackie(11) and adenovirus types (12). Studies were conducted to determine if similar differences could be detected between the 3 reovirus types when HA was carried out at temperatures of 4, 24 and 37°C. Several strains of each of the reovirus types were passaged once in cultures of WI-38 cells, and the TC fluids from this passage employed as HA antigens. Table II summarizes results of this experiment. No differences in HA titers were obtained with 2 strains of type 1 virus, despite differences in temperatures employed. Similar results were obtained with 2 strains of type 2; however, 1 strain demonstrated an increase in

HA titer as the temperature of HA was decreased. In contrast to the first 2 reovirus types, 3 strains of reovirus type 3 failed to hemagglutinate human group "O" erythrocytes at 4°C. A significant increase in HA titer by these 3 strains was observed at temperatures of 24 and 37°C. Additional passages of the prototype virus strains in WI-38 cells did not alter the temperature-HA relationship observed with fluids from the initial passage in WI-38 cells.

Discussion. Experiments comparing reovirus HA antigens prepared in various cells indicated that the human diploid cell strain (WI-38) and the established renal cell lines, LLC-MK_2 and BS-C-1 could be satisfactorily employed in preparation of reovirus HA antigens in lieu of primary renal cell cultures of the Rhesus monkey. Of the 3 host cell systems tested, the diploid cell strain (WI-38) proved to be optimum for reovirus HA antigen preparation as shown in Fig. 1. Since all 3 types of reoviruses were found to produce CPE in WI-38, LLC-MK_2 and BS-C-1 cells, such data also provide additional information to the distinctive biological characteristics of reovirus reported earlier(13).

The maximum HA titer for each type was obtained after 3 passages in WI-38 cells, supporting the finding that several passages may be required before suitable concentrations are obtained(7).

It has been reported that proliferation of the 3 reovirus types was enhanced markedly when tube cultures of the Rhesus MK cells were incubated on a roller drum apparatus. This resulted in higher HA and infectious virus titers and in addition, permitted rapid, efficient isolation and identification of these viruses(9).

In this study, CPE appeared either to develop at the same rate in both rolled and stationary cultures, or to be enhanced by incubation in a stationary position. Cultures inoculated with type 2 "D-5" strain and the "Abney" strain of type 3 exhibited CPE a day earlier when incubated in a stationary position. No differences in development of CPE were observed in cultures inoculated with prototype strains of reovirus type 1 and type 3. Of greater interest were the higher HA titers which were obtained in the pools of cultures fluids from a stationary cultures (Fig. 2 and 3). It is apparent that the effect of position during incubation with respect to the enhancement of reovirus CPE, increased HA and infectious virus titers varies with the host cell system employed.

Reovirus types 1 and 3 failed to multiply in WI-38 cell cultures at 24°C as evidenced by the absence of CPE and HA. On the other hand, reovirus type 2 was able to produce some CPE and HA at this temperature. The T markers of reovirus types at 24°, 37°, and 40°C were already reported using Rhesus MK cell cultures(14). The present study confirms these earlier findings.

It has been reported that all 3 reovirus types produce a HA capable of agglutinating human group "O" erythrocytes. In addition, type 3 also produces a HA which can agglutinate bovine erythrocytes at 4°C(15,16). The interaction of type 3 reovirus with bovine erythrocytes was explained by the presence of neuraminic acid-containing receptors on the erythrocytes(15). There is also evidence reported that reovirus 1 and 2 do not react with such receptors(10,17). Even though HA titers of reovirus types propagated in Rhesus MK cells were reported not to be significantly different at 4°C and 37°C(11,17), the 3 strains of reovirus type 3 grown in WI-38 cells and tested, showed significant differences in HA titers at 4°C as compared to those obtained at 24 and 37°C. This appears to be a general characteristic of reovirus type 3 which may possibly be employed for the differentiation of reovirus types.

Summary. 1. Reovirus HA antigens prepared in the human diploid cell strain, WI-38 and in the established renal cell lines, LLC-MK_2 and BS-C-1, may be satisfactorily employed in lieu of primary renal cell cultures of the Rhesus monkey. 2. Of the 3 host cell systems tested, WI-38 cells proved to be the most suitable for preparation of reovirus HA antigens. Maximum HA titers to the 3 reovirus types were obtained after 3 passages in this cell strain. 3. Higher reovirus HA titers were attained in stationary WI-38 cell cultures incubated at 37°C as compared with that obtained from cultures rotated at this

temperature. The enhancement of CPE with respect to the methods of incubation varied with the virus strains employed. At an incubation temperature of 24°C only reovirus type 2 produced CPE and HA to a limited extent in WI-38 cells. 4. Reovirus types 1 and 2 HA antigens prepared in WI-38 cells showed no significant differences in titers when HA was carried out at temperatures of 4, 24 and 37°C. Antigens prepared with 3 strains of type 3 failed to agglutinate erythrocytes at 4°C but demonstrated HA at 24 and 37°C.

1. Rhim, J. S., Melnick, J. L., Tex. Rep. Biol. Med., 1961, v19, 851.
2. Hayflick, L., J. Nat. Cancer Inst., 1962, Monograph No. 7, 63.
3. Hull, R. N., Cherry, W. R., Tritch, O. J., J. Exp. Med., 1962, v115, 903.
4. Hopps, H. E., Bemheim, V. C., Nisalak, A., Tjio, J. H., Smadel, J. E., J. Immunol., 1963, v91, 416.
5. Melnick, J. L., Diagnostic Procedures for Virus and Rickettsial Diseases, Am. Public Health Assn., 1956, 142.
6. Eagle, H., J. Exp. Med., 1955, v102, 595.
7. Rosen, L., Am. J. Hyg., 1960, v71, 242.
8. Rhim, J. S., Pelon, W., ibid., in press.
9. Lerner, A. M., Cherry, J. D., Finland, M., Proc. Soc. Exp. Biol. and Med., 1962, v110, 727.
10. Goldfield, M., Srihongse, S., Fox, J. P., ibid., 1957, v96, 788.
11. Feorino, P. M., Humphrey, D. D., Gelfand, H. M., Public Health Rep., 1963, v78, 349.
12. Rosen, L., Am. J. Hyg., 1960, v71, 120.
13. Hsiung, G. D., Proc. Soc. Exp. Biol. and Med., 1958, v99, 387.
14. Rhim, J. S., Pelon, W., Virology, in press.
15. Gomatos, P. J., Tamm, I., ibid., 1962, v17, 455.
16. Eggers, H. J., Gomatos, P. J., Tamm, I., Proc. Soc. Exp. Biol. and Med., 1962, v110, 879.
17. Lerner, A. M., Cherry, J. D., Finland, M., Virology, 1963, v19, 58.

Received October 30, 1964. P.S.E.B.M., 1965, v118.

Virus-Like Particles Associated with Chloroleukemia in the Rat.* (29874)

RONALD S. WEINSTEIN AND WILLIAM C. MOLONEY
Mixter Laboratory for Electron Microscopy, Massachusetts General Hospital, Boston, and Research Laboratories, Holy Ghost Hospital, Cambridge, Mass.

Many investigators have now succeeded in inducing leukemia in mouse and rat with mouse leukemia virus which was initially isolated from tumors, from mice with spontaneous leukemia or from mice with leukemia induced with carcinogenic agents(1,2). Attempts to isolate leukemogenic viruses from rats with spontaneous or chemically induced leukemias have, however, been unsuccessful. These failures can be due either to an absence of such viruses in the rat or to the use of unsatisfactory techniques. This report deals with the results of an electron microscopic study in which virus-like particles were observed in leukemic rats.

Materials and methods. Chloroma cells, harvested originally in 1958 from a non-inbred Wistar rat with Shay chloroma, have been subtransferred subcutaneously in Wistar rats for the last 7 years(3). Three such rats, two from generation 52 and one from generation 55, were used in this study. Peripheral blood smears, organ imprints and histologic study of the tissues of these animals revealed that each animal had acute myelogenous leukemia with involvement of spleen, thymus and liver at time of sacrifice. Several rats from later generations, also bearing myelogenous leukemia, were examined using negative staining techniques but were not studied with thin sections.

Small pieces of chloromas were fixed in 2% osmic acid buffered with veronal-acetate at pH 7.2. Tissues were then dehydrated with ethanol, passed through graded dimethyl sulfoxide (DMSO) and propylene oxide and

* This investigation was supported by USPHS Research Grant CA-05691 from Nat. Cancer Inst., and by USPHS Research Grant CA-07368-01 from Nat. Inst. Health.