reduction neutralization test employed.

The plasma aliquot from which the A-1 virus was presumably recovered had been heated for 1 hour at 60°C, yet the tissue culture-adapted isolate is inactivated by similar treatment even when suspended in undiluted normal human plasma. The significance of this observation is undetermined.

Summary. 1. A virus has been recovered in tissue cultures inoculated with the 1951 NIH icterogenic plasma pool. 2. This agent produces cytopathogenic effects in a wide variety of cell cultures, but induces no overt disease in common laboratory animals. It produces inapparent infection in embryonated eggs. 3. Serologic studies have failed to show any relation between the A-1 virus and a number of recognized viruses. 4. Heat inactivated sera from normal human beings and animals did not neutralize the virus. 5. Volunteers infected with serum hepatitis virus from 6 different sources have, with a high degree of consistency, developed antibodies against the A-1 agent. 6. Sera from patients with infectious hepatitis have not neutralized the virus with comparable regularity.

The technical assistance of Alfred Segee is gratefully acknowledged.

1. Symposium on the laboratory propagation and detection of the agent of hepatitis. Washington, D.C.: Nat. Acad. Sci., Nat. Research Council, 1954, Publication 322, pp. 1-115.

2. Rightsel, W. A., Keltsch, R. A., Tekushan, F. M., McLean, I. W., *Science,* 1956, v124, 226.

3. Murray, R., Oliphant, J. W., Tripp, J. T., Hampil, B., Ratner, F., Diefenbach, W. C. L., Geller, H., *J.A.M.A.,* 1955, v157, 8.

4. Melnick, J. L., Chap. IV in *Diagnostic Procedures for Virus and Rickettsial Diseases,* Am. Public Health Assn., N. Y., 1956, 116.

5. Enders, J. F., Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

6. Barile, M. F., Yaguchi, R., Eveland, W. C., *Am. J. Clin. Path.,* 1958, v30, 171.

7. Havens, W. P., Jr., *New England J. Med.,* 1958, v259, 1202.

8. Theiler, M., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 380.

9. Hiatt, C. W., *Trans. N. Y. Acad. Sci.,* 1960, v23, 66.

10. Murray, R., Diefenbach, W. C. L., Ratner, F., Leone, N. C., Oliphant, J. W., *J.A.M.A.,* 1954, v154, 1072.

11. Porter, J. E., Shapiro, M., Maltby, G. L., Drake, M. E., Barondess, J. A., Bashe, W. J., Stokes, J., Jr., Oliphant, J. W., Diefenbach, W. C. L., Murray, R., Leone, N. C., *J.A.M.A.,* 1953, v153, 17.

12. Ward, R., Krugman, S., Giles, J., Jacobs, M. A., *Hepatitis Frontiers,* edited by Hartman, F. W., *et al.,* Little, Brown and Co., Boston, 1957, 227.

13. Hughes, T. P., *J. Immunol.,* 1933, v25, 275.

Received July 26, 1961. P.S.E.B.M., 1961, v108.

Inactivation of Vacuolating Virus (SV_{40}) by Formaldehyde. (26892)

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Sweet and Hilleman(1) recently reported discovery of a new virus of rhesus and cynomolgus monkey origin. Unlike other simian viruses this agent fails to cause detectable cytopathogenic changes in cell cultures of rhesus and cynomolgus monkey kidneys where it multiplies to relatively high titers. In kidney cell cultures derived from the African Green monkey, *Cercopithecus aethiops,* however, the virus causes cytopathic effects characterized by cytoplasmic vacuolations which coalesce with eventual destruction of the cells. For this reason the virus has been named vacuolating virus and has been designated SV_{40} by Hull.

The present report describes studies on formaldehyde inactivation of SV_{40} and its detection in some lots of poliomyelitis and adenovirus vaccines.

Materials and methods. Tissue cultures. Kidneys obtained from African Green monkeys were trypsinized and grown at 37°C in

Melnick's(2) lactalbumin hydrolysate medium containing 5% heat-inactivated calf serum. The maintenance medium consisted of medium 199(3) pH 7.8 with 2% heat-inactivated chicken serum. The antibiotics used in the media were 100 units of potassium penicillin and 100 μg of streptomycin sulfate per ml.

Virus. Strain 776 of SV_{40} was used in these studies.* Virus pools were prepared in cell cultures of African Green monkey kidneys in serum-free medium 199. The virus pools were stored at −60°C.

Vaccines. Samples of formalinized poliomyelitis and adenovirus vaccines prepared by domestic manufacturers were used during this study.

Virus titrations and serum neutralization tests. Roller tube cultures of monkey kidney cells containing 1.3 ml of maintenance medium were inoculated with 10-fold dilutions of virus. Four tubes were used per dilution and each tube received 0.2 ml of virus. The cultures were observed for cytopathic changes during 14 days of incubation. Neutralization tests were performed by incubating for 2 hours at 37°C a mixture of equal volumes of 200 $TCID_{50}$ of virus per 0.2 ml and heat-inactivated homologous rabbit antiserum.* The virus serum mixture was inoculated in 0.2 ml amounts into monkey kidney culture tubes which were held for 14 days at 37°C.

Formaldehyde treatment of vacuolating virus. Two hundred ml of vacuolating virus suspension were filtered through a Selas 03 filter by negative pressure. The pH was adjusted to 7.0 by addition of 0.1N hydrochloric acid. The pool was divided into 2 portions of 90 ml each and was pre-heated in a water bath to 37°C. To one portion 10 ml of a 1:400 dilution of Formaldehyde Solution USP, was added with constant mixing to produce a final concentration of 98 μg formaldehyde per milliliter as determined by assay. The other portion which received an equivalent volume of balanced salt solution in place of the formaldehyde solution served as a control fluid. The 2 portions were incubated in tightly stoppered flasks in a water bath at 37 $\pm$ 0.5°C for a period of 14 days. At various time intervals 5 ml samples were withdrawn and treated immediately with 0.15 ml of 1% solution of sodium bisulfite. The samples were stored at −20°C until they were assayed for virus content.

Detection of SV_{40} in vaccines. Each sample of vaccine was dialyzed at 4°C for 24 hours against 20 volumes of buffered saline pH 7.4 and an additional 24 hours against 20 volumes of medium 199. Two test techniques were employed. In the one, 400 ml of vaccine were concentrated by centrifugation at 30,000 RPM for 3 hours in the Model L Spinco centrifuge with the No. 30 rotor. Gelatin in a final concentration of 0.06% was added before centrifugation to facilitate recovery of the virus(4). The pellets were resuspended in 18 ml of medium 199 and the suspension was inoculated into 3 milk dilution bottles of monkey kidney cell cultures containing 12 ml of maintenance medium. The bottles were incubated at 37°C for 14 days at which time 3 ml from each bottle was subcultured into 6 roller tubes of monkey kidney cells, 0.5 ml per tube containing 1.5 ml of maintenance medium. The tubes were observed for 14 days for any cytopathic changes. In the other technic, 400 ml of vaccine were inoculated into 16 Blake bottle cultures of monkey kidney cells, each receiving 25 ml of vaccine and 50 ml of maintenance medium. After 14 days incubation at 37°C fluids from each bottle were subcultured as described for the concentrated test. In all tests uninoculated cell cultures of the same cell lot were included as controls. The milk dilution and the Blake bottles received 15 ml and 75 ml of maintenance medium respectively. They were observed for 14 days and the fluids were subcultured in the same manner as described for test cultures.

Because of the careful microscopic observation and the high magnification required to detect the characteristic CPE, only the subculture tubes were examined at regular intervals for evidence of the presence of the virus. The primary culture bottles were examined only to assure satisfactory cell maintenance. Final identification of agents encountered in subcultures was done by neutralization tests.

* Kindly made available by Dr. Hilleman.

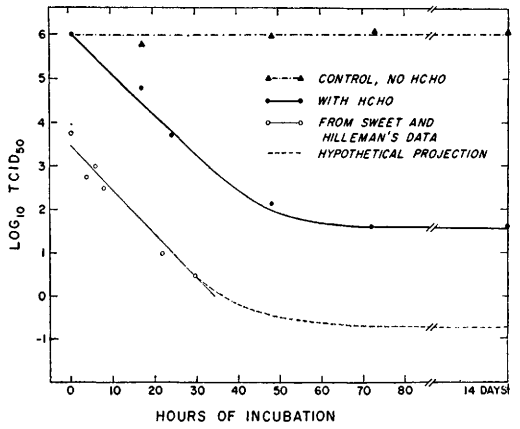

FIG. 1. Inactivation of SV_{40} by 1:4000 formaldehyde solution USP, at 37°C and pH 7.0.

Results. Susceptibility of SV_{40} to formaldehyde. The course of formaldehyde treatment of a serum-free filtrate of SV_{40} suspension is presented in Fig. 1. The slope of the inactivation curve runs approximately parallel to the curve published by Sweet and Hilleman(1) indicating similar rates of inactivation as shown by the solid lines. However, in contrast to the straight line shown by those authors, the results obtained indicate a flattening of the curve with a residual viable fraction remaining throughout the entire 14-day period of observation. A hypothetical projection from the data of Sweet and Hilleman has been included as a dotted line in order to show the probable course of inactivation when lower starting titers of virus are employed. Of further interest is the remarkable thermostability of this agent as evidenced by the full retention of infectivity during heating without formaldehyde at 37° C for a period of 14 days.

Detection of SV_{40} in poliomyelitis and adenovirus vaccines. Randomly selected samples of poliomyelitis and adenovirus vaccines were tested for the presence of viable SV_{40}. The results are summarized in Table I. Four of 8 poliomyelitis vaccines and 3 of 3 adenovirus vaccines were found to produce characteristic cytopathic changes in all subculture tubes. An isolate from each positive vaccine was identified by means of serum neutralization tests. The repeat tests shown were done with different lots of kidney cell cultures and gave similar results. Uninoculated cell controls were included in each test and in addition vaccine D was used as a negative control in the repeat isolations. In no instance was SV_{40} isolated from the negative vaccine control or from the uninoculated cell controls.

To evaluate the sensitivity of the concentrated and unconcentrated test methods all positive vaccines were tested by both methods. No significant difference was observed. With both methods all subcultures derived from positive vaccines showed characteristic cytopathic changes between the 7th-10th day.

Quantitation of SV_{40} in poliomyelitis vaccines. It was of interest to measure the relative amounts of viable SV_{40} in the samples of some vaccines. Ten-fold serial dilutions of 22x concentrated vaccines D, E and F were titrated in tube cultures of monkey kidney cells. At the end of 14 days incubation, fluids from "negative" tubes of each dilution

TABLE I. Detection of SV_{40} in Poliomyelitis and Adenovirus Vaccines.

Vaccines					
Polio	Adeno	Test method	Results	Uninoculated cell controls	Identification‡
A, B, C, D*		Conc.	0/3 †	0/2	
E		Unconc.	16/16	0/3	SV_{40}
E (repeat)		Conc.	3/3	0/2	ND
F		Unconc.	16/16	0/3	SV_{40}
F (repeat)		Conc.	3/3	0/2	ND
G		Conc.	3/3	0/2	SV_{40}
G (repeat)		Unconc.	16/16	0/3	ND
H		Conc.	3/3	0/2	SV_{40}
H (repeat)		Unconc.	16/16	0/3	ND
	I, J, K*	Conc.	3/3	0/2	SV_{40}

* Results shown were obtained with individual vaccine samples.
† No. of positive bottles/Total bottles inoculated.
‡ Isolates identified by neutralization tests.

TABLE II. Titration of Poliomyelitis Vaccine for SV_{40} Content.

Vaccine	Dilutions of concentrated vaccines—$\log_{10}$				Subcultures of pooled negative tubes of each dilution group				Estimated virus particles/ml†
	1	2	3	4	1	2	3	4	
D	0/4*	0/4	0/4	0/4	0/4	0/4	0/4	0/4	None
E	1/4	2/4	1/4	0/4	4/4	0/4	4/4	4/4	2000
F	1/4	0/4	0/4	0/4	0/4	4/4	4/4	0/4	200

* No. of tubes showing CPE/Total tubes inoculated.
† Calculated for unconcentrated vaccine.

group were pooled and subcultured without further dilution into 4 tube cultures of monkey kidney cells. Primary titration of formalin-inactivated poliomyelitis vaccine revealed cytopathic changes in only few of the culture tubes (Table II). The results of subcultures from "negative" primary tubes indicated a 10-100-fold higher concentration of SV_{40}. Assuming that only one virus particle was needed to initiate infection of the subculture tubes the number of virus particles in each vaccine was estimated as shown in the last column of Table II.

Discussion. The results obtained in this study indicate that the course of treatment of SV_{40} with 1:4000 formaldehyde was characterized by a biphasic reaction. The major portion of the viral population was inactivated progressively at a slightly slower rate than poliovirus. The second phase of the curve indicated the persistence of a residual fraction which resisted inactivation. The slope of the linear portion of the curve approximately paralleled the one described by Sweet and Hilleman(1). Their failure to detect viable virus after 46 hours of inactivation is undoubtedly due to the fact that the titer of the virus suspension at the start was 2.25 logs lower than the one used in this study. Therefore, the infectivity of the samples taken after 46 hours of inactivation fell below detectable levels when 0.2 ml volumes were used for assay tests. This has been indicated by the hypothetical projection (dotted line Fig. 1) of their curve. Furthermore, Sweet and Hilleman observed their assay tests for a period of only 10 days. In our experience there was a marked delay in the appearance of cytopathic effects caused by formaldehyde-treated SV_{40}. In most instances initial CPE did not appear until the 11th day and the final infectivity titers were reached on the 13th and 14th day. This observation is similar to the findings by Böttiger *et al.* (5) with formaldehyde-treated poliovirus. Hull's(6,7) data on rate of formaldehyde inactivation of the simian viruses known at that time revealed that all of these agents were inactivated more rapidly than poliovirus, with the exception of SV_{12} and SV_{27} which were destroyed at a rate similar to that for poliovirus.

Further studies are required to determine if there is variation in the viral population with regard to susceptibility to formaldehyde or, alternately, if reactivation of "inactivated" particles occurs as described for bacteriophage(8).

In view of the results obtained in the inactivation studies, the presence of viable SV_{40} in killed poliomyelitis and adenovirus vaccines was not entirely unanticipated. The probability that a vaccine lot contains viable SV_{40} in detectable amounts, depends of course on the concentration of this agent before formaldehyde treatment.

In studies designed to estimate the amount of viable SV_{40} present in vaccines, use of a sensitive assay technic was essential. Through subpassage of the "negative" titration tubes at the end of 14 days the sensitivity of the assay was increased up to 100-fold as compared with the primary titration. The estimated quantity of SV_{40} per milliliter of vaccine is in agreement with the results shown in Fig. 1. Since the incidence of SV_{40} infection in kidney cell cultures has been shown to vary significantly with the species of monkeys (1), this factor could influence the presence of detectable SV_{40} in formalinized vaccines. In addition, the degree of contamination of the seed virus inoculum with SV_{40}, the total

incubation period of the kidney cell cultures during vaccine production and specific variations in the manufacturing process may also be contributing factors.

Preliminary results(9) of studies on antibody to SV_{40} in normal monkey sera show that rhesus monkeys were infected with SV_{40} as far back as 1956. This suggests that kidney cell cultures and hence Salk poliomyelitis vaccine may have contained the virus in viable form. The finding(1) that a high percentage of persons following injection of 2 doses of poliomyelitis or adenovirus vaccine developed relatively high antibody titers to SV_{40}, prompted Sweet and Hilleman to comment on the apparent high potency of SV_{40} antigen in these vaccine preparations. In retrospect, therefore, one can assume that large groups of the population in this country and abroad must have been injected with varying amounts of SV_{40} during the course of immunization with formalinized poliomyelitis or adenovirus vaccine. Careful clinical studies on selected groups of vaccinees suggest that no harmful effect can be attributed to vacuolating virus within the limits of the observation period. Furthermore, oral† or intranasal(10) administration of SV_{40} in man results in viral multiplication without causing illness. While short term studies of the pathogenicity of SV_{40} in laboratory animals have been reported as negative(1), the long term effect in newborn animals remains to be determined.

Summary. Inactivation of SV_{40} with formaldehyde followed a biphasic curve with a levelling off after about 40 hours at 37°C leaving a residual fraction of virus which was resistant to further inactivation for the remainder of the 14-day observation period. Viable SV_{40} was detected in some lots of formalinized poliomyelitis and adenovirus vaccines.

† Melnick, J. L., personal communication.

1. Sweet, B. H., Hilleman, M. R., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 420.

2. Melnick, J. L., *Diagnostic Procedures for Virus and Rickettsial Diseases,* Am. Pub. Hlth. Assn., N. Y., 2nd ed., 1956, 97.

3. Morgan, J. F., Morton, H. J., Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

4. Baron, S., *ibid.,* 1957, v95, 760.

5. Böttiger, M., Lycke, E., Melen, B., Wrange, G., *Arch. Ges. Virusforsch.,* 1958, v8, 267.

6. Hull, R. N., Minner, J. R., Smith, J. W., *Am. J. Hyg.,* 1956, v63, 204.

7. Hull, R. N., Minner, J. R., Mascoli, C. C., *ibid.,* 1958, v68, 31.

8. Heicken, K., Spicher, G., *Zbl. Bakt. Orig. Abt., I.,* 1956, v167, 97.

9. Gerber, P., unpublished data.

10. Morris, J. A., Johnson, K. M., Aulisio, C. G., Channock, R. M., Knight, V., *Fed. Proc.,* 1961, v20, 436.

Received July 14, 1961. P.S.E.B.M., 1961, v108.

Plasma and Tissue Electrolytes of Rats Given Excess Sodium Chloride in Food.* (26893)

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Elevated blood pressure and changes in electrolytes have been reported in rats given increased quantities of sodium chloride(1). The present study further characterizes the distribution of sodium, potassium, and water in plasma and tissues of rats ingesting excess sodium chloride.

* This work was supported in part by grants from Michigan Heart Assn. and American Heart Assn.

Methods and materials. Sodium chloride was administered as 2.8, 5.6 and 8.4% of a synthetic diet(2) to 3 respective groups totaling 35 rats. Rats were Sprague-Dawley strain males, each weighing 160 to 235 g at onset of the experiment. Tap water was provided *ad libitum.* Blood pressures were determined by the tail-cuff method(3) under light ether anesthesia and body weights re-