TABLE V. ECHO, Coxsackie, and Poliomyelitis CF Antibody Titers of Sera Collected from Persons in Acute and Convalescent Phase of Type 1 Paralytic Poliomyelitis.

Patient	Date serum collected	ECHO antigens E2	E3	E4	E6	E7	E11	Coxsackie B antigens B1	B3	B5	Poliomyelitis antigens P1	P2	P3
DF	8/ 2/56	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
	27	"	"	"	"	"	"	"	"	"	32	"	"
RC	9/10	8	8	"	32	16	"	"	"	"	<8	"	"
	10/17	8	8	"	"	"	"	"	"	"	32	"	"
ED	7/25	8	<8	"	<8	<8	"	"	"	"	<8	"	"
	8/13	8	"	"	"	"	"	"	"	"	32	"	"
TG	8/17	8	"	"	16	32	"	"	"	"	<8	"	"
	9/ 7	<8	"	"	8	"	"	"	"	"	16	"	"
DG	8/ 3	"	"	"	<8	<8	"	"	"	"	<8	"	"
	25	"	"	"	"	"	"	"	"	"	16	"	"
RP	9/25	16	8	"	"	16	"	32	32	32	<8	"	"
	10/10	"	8	"	"	"	"	"	"	"	"	"	"

virus type 10 and any of the other enteroviruses.

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1. Committee on the ECHO viruses, Nat. Fn. for Infantile Paral., *Science,* 1955, v122, 1187.

2. Committee on the enteroviruses, Nat. Fn. for Infantile Paral., *Am. J. Public Health,* 1957, v47, 1556.

3. Huebner, R. J., Ward, T. G., Paffenbarger, R. S., Suskind, R. G., unpublished data.

4. Rosen, L., Johnson, J. H., Huebner, R. J., Bell, J. A., *Am. Jour. Hyg.,* 1958, v67, 300.

5. Curnen, E. C., Shaw, E. W., Melnick, J. L., *J. Am. Med. Assn.,* 1949, v141, 894.

6. Huebner, R. J., Risser, J. A., Bell, J. A., Beeman, E. A., Beigelman, P. M., Strong, J. C., *New Eng. J. Med.,* 1953, v248, 267.

7. Cheever, F. S., Daniels, J. B., Hersey, E. F., *J. Exp. Med.,* 1950, v92, 153.

8. Dalldorf, G., Sickles, B. A., in Diagnostic Procedures for Virus and Rickettsial Diseases, *Am. Public Health Assn.,* New York, 1956, 153.

9. Salk, J. E., *J. Am. Med. Assn.,* 1953, v151, 1081.

10. Halonen, P., Huebner, R. J., Turner, H. C., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 530.

11. Bengtson, I. A., *Pub. Health Rep.,* 1944, v59, 402.

12. Johnsson, T., Böttiger, M., Löfdahl, A., *Arch. f. Virusforsch.,* 1958, v8, 306.

13. Zeipel, G. von, Svedmyr, A., *ibid.,* 1957, v7, 355.

14. Hammon, W. McD., Yohn, D. S., Ludwig, E. H., Pavia, R. A., Sather, G. E., McCloskey, L. W., *J. Am. Med. Assn.,* 1958, v167, 727.

15. Hsiung, G. D., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 387.

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Formalin Treated Chicken Erythrocytes as Indicators of Influenza A Virus (Asian) and Its Antibody.* (24897)

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Formalin treated human erythrocytes have been used to measure the hemagglutinating capacity of influenza virus(1,2), detect antibodies against coupled protein(3,4), haptens (4,5) and enterobacterial antigens(6). While such erythrocytes were reported(1) as early as 1948 to be comparable to normal human and

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chicken erythrocytes for adsorbing and eluting influenza A hemagglutinating activity they have not replaced fresh cells perhaps because of the spontaneous agglutination which may occur in the presence of traces of formaldehyde(1,7) or because such cells do not lend themselves well to reading by the usual pattern method(1,3). As there are, however, substantial theoretical advantages to their use, continued efforts have been made toward obtaining preparations that would be suitable for routine hemagglutination and hemagglutination-inhibition tests. Tube titrations of influenza viruses and their antibodies with formalin preserved human erythrocytes recently have been reported by Cox and Pirtle (2) and Ingraham(4) has described the use of treated human erythrocytes for attaching proteins and haptens to detect specific antibodies by means of the tube pattern method. This report will describe the preparation of formalin preserved chicken erythrocytes, their use in titration of the Asian strain of influenza virus A and its antibody and comparison of results obtained in such titrations with preserved and fresh chicken erythrocytes. Similar trials with preserved human and sheep cells proved to be unsatisfactory because of incomplete settling even after overnight incubation.

Material and methods. The diluent used throughout consisted of 0.137 M NaCl in distilled water buffered at pH 7.2 with 0.01 M Na_2HPO_4 and 0.003 M KH_2PO_4 (PBS). Chicken blood was drawn into modified Alsever's solution(8) and stored at 4°C as a 50% suspension which was permitted to stabilize for one week before being used. *Preparation of preserved chicken erythrocytes (PCE).* The procedure described by Cox and Fingerhut(9) as altered by Feeley, Sword and Manclark(6) was followed except for minor modifications. Fifty ml of a 50% suspension of whole blood in Alsever's solution was diluted with an equal volume of PBS. This was rapidly mixed with 40 ml of 50% formalin diluted in twice concentrated PBS. The mixture was incubated for 2 hours in a 37°C water bath. The flask was agitated vigorously every 10 minutes. During incubation, the cells gradually changed in color from red to dark brown. They were then washed 6 times with not less than 40 volumes of PBS. Centrifugation was performed in an International Model V centrifuge (size 2), head #239, at 2,000 rpm for 10 minutes. Since the formaldehyde caused marked clumping of the cells, they were resuspended with a magnetic stirrer for 5 minutes during the washing procedure. Finally, the cells were resuspended in 400 ml of PBS and stored at 4°C for 48 hrs; the supernatant was then decanted, the PBS replaced and the cells resuspended. After a second 48 hour period, the erythrocytes were suspended in PBS to a final concentration of 10% packed cells. To this stock suspension, formaldehyde (final concentration 0.3%) was added and the suspension stored at 4°C until used. These cells were found to retain their original degree of reactivity with A/Asian/influenza virus for at least 3 months.

Before use, however, the cells in the stock suspension required several washings to prevent spontaneous agglutination. Subsequently it was learned that a 10% stock suspension of PCE preserved with merthiolate 1:10,000(3) and stored in small aliquots at 4°C was superior. After 2 months' storage, the cells in such stock suspensions could be adjusted to a final concentration of 0.325% in 0.005% bovine albumin‡ in PBS without additional washing. Both positive and negative patterns were as distinct and the end points as sharp as those observed with PCE in 0.3% formalin. Although we still had to use 0.005% bovine albumin in PBS to prevent occurrence of spontaneous agglutination in the hemagglutination tests, preservation of stock suspensions of stabilized erythrocytes in merthiolate offers a distinct advantage in that it is not necessary to wash the cells before use. *Antigen.* The hemagglutinating antigen used throughout was pooled allantoic fluid, *Influenza A/Asian/Japan/305/57.*§ *Sera* were obtained from medical student volunteers before and 4 and 14 days after receiving 0.1 ml of monovalent 400 CCA Asian/A/Influenza vaccine intradermally (to be published). Each serum was treated with periodate(10) before testing. *Hemagglutination (HA) and hemagglutina-*

‡ Bovine albumin, Fraction I, Armour and Co.

§ We are indebted to Dr. Morris Schaffer, P.H.S. for this antigen.

tion inhibition (HI) tests. The Communicable Disease Center standard technique(10) using 4 units of antigen was employed. Tests were carried out in hemagglutination tubes (Vontes) and read by the pattern method (11), after incubation for one hour at room temperature. Antigen and sera were diluted in PBS. Virus titrations (HA) were performed with 0.5 ml of antigen dilution to which 0.5 ml of either preserved (PCE) or fresh (FCE) chicken erythrocyte suspensions were added. The reciprocal of the initial dilution of virus in the last tube showing complete agglutination was considered to be the end point in the HA tests. Lowest serum dilution measured was 1:10. The HI end point was the reciprocal of the initial dilution of serum in the last tube showing no agglutination (complete inhibition). Before use, the fresh chicken erythrocytes were washed 3 times with PBS and a 0.5% suspension of packed cells made in PBS with or without bovine albumin. Small amounts of the stock 10% suspension of PCE were washed 5 times with at least 10 ml of PBS and final suspensions were prepared either in PBS or serial dilutions of bovine albumin, or in normal rabbit serum inactivated at 56°C for 30 minutes and diluted with PBS.

Results. Optimal concentration of preserved chicken erythrocytes. Spontaneous agglutination and irregular agglutination patterns were observed consistently in the HA tests with PCE when PBS was used to suspend the cells. This was not the case, however, in the HI tests, in which patterns comparable to those obtained with FCE were noted. From this, it was inferred that incorporation of the test sera into the system prevented spontaneous agglutination of the PCE. Therefore, preliminary titrations were performed to test the effects of rabbit serum and bovine albumin on agglutination patterns of PCE in presence of influenza virus. Rabbit serum inactivated at 56°C for 30 minutes was found to inhibit agglutination of both PCE and FCE in the HA tests, when used in either 1%, 0.5% or 0.25% concentration. Spontaneous agglutination of PCE did not occur, however, in the presence of the rabbit serum, the negative patterns being similar to those observed with FCE.

Titrations were performed simultaneously with both FCE and PCE suspended in bovine albumin. A series of 5-fold dilutions of bovine albumin in PBS (0.001 to 0.5%), was used to suspend the PCE in a final concentration of 0.5% by volume, of packed cells. Similar suspensions of FCE were prepared in 0.5%, 0.05% and 0.005% bovine albumin in PBS. A parallel titration of FCE in saline also was performed. Albumin was observed to produce a marked effect on the agglutination patterns of PCE. When the highest dilution (0.001%) was used, both positive and negative patterns were indistinguishable from those in the control titrations of FCE in PBS. Furthermore, numerous, replicate titrations of the virus' hemagglutinating activity with fresh cells (FCE) suspended either in PBS, 0.5%, 0.05% and 0.005% bovine albumin showed no significant differences in their end points. Nonetheless, HA tests with 0.5% suspensions of PCE failed to show distinct end points. When these could be determined, they usually were different from those detected with FCE, the difference frequently being more than one tube. Subsequently, the 0.5% PCE suspensions were found to contain more cells per ml than those of FCE because of shrinkage‖ and to pack more completely during centrifugation. Therefore, it was considered that the differences in end points might be due to different numbers of cells in each suspension. In Table I is summarized an example of an experiment designed to test the validity of this assumption. The variations in end points of PCE agglutination by the Asian strain of influenza virus seem to be related to number of cells in the suspension. The agglutination patterns exhibited by the preserved cells in the 0.325% suspension of PCE in 0.005% bovine albumin were usually found to be in close agreement with those observed with the 0.5% suspensions of FCE. When the cells were suspended in concentrations of 0.3% or less, the tests were unsatisfactory because they were difficult to distinguish from the control tubes. In 27 of 32 hemagglutination ti-

‖ Ratio between arithmetic averages of 8 enumerations of PCE and FCE was 1.33.

TABLE I. Effect of Cell Concentration on Agglutination of Formalin Preserved Chicken Erythrocytes by Asian / A / Influenza Virus.

Erythrocytes			Agglutination‡ dilution								
Kind*	% conc.	Diluent†	50	75	100	150	200	400	800	Control	Titer
FCE	.5	PBS	4	4	4	4	1	0	0	0	150
"	.5	BA	4	4	4	4	0	0	0	0	150
PCE	.5	"	4	3	2	1	1	1	0	0	50
"	.4	"	4	4	2	2	1	1	0	0	75
"	.35	"	4	4	4	3	1	0	0	0	100
"	.325	"	4	4	4	4	0	0	0	0	150
"	.3	"	4	4	4	4	4	4	—	—	—
"	.275	"	—	—	—	—	—	—	—	—	—

* FCE = Fresh chicken erythrocytes. PCE = Preserved chicken erythrocytes.
† PBS = Phosphate buffered saline. BA = 0.005% bovine albumin in PBS.
‡ Agglutination patterns were graded from 0 (no agglutination) to 4 (complete agglutination). — = not readable or not obtained.

trations, performed simultaneously with 0.5% FCE and 0.325% PCE, the end points coincided. Furthermore, when discrepancies did occur, in only one instance did the disagreement amount to more than one tube. The end points of titrations with 0.325% PCE in 0.005% bovine albumin were, as a rule, as easily read as those exhibited by FCE.

Accuracy in replicate titrations of antibody. To determine whether the sensitivity of PCE was comparable to that of FCE when used to titrate Asian/A/influenza antibody, experiments such as that summarized in Table II were performed. Four individuals were selected at random from among 20 volunteers who had received, intradermally, 0.1 ml of 400 CCA monovalent Asian/A/Influenza vaccine (not yet published). From each of these, 3 samples of sera obtained just before (A), 4 days (B) and 14 days (C) after vaccination, were used. Simultaneous titrations with FCE and PCE were performed in paired samples of each serum but the order of titration with each serum was not randomized. These titrations were repeated on 2 different days, using antigen and erythrocytes suspensions from the same pools. The data (Table II) show good agreement between end points when tests were performed with FCE and PCE.

Discussion. That hemagglutination and hemagglutination inhibition procedures are highly sensitive and accurate biological reactions is well established(11,12,13) and supported by the present experience. A major difficulty associated with the HA and HI procedures with freshly prepared erythrocytes, however, is the instability of the red blood cells. Loss of agglutination activity of as much as 50% has been demonstrated after storage of cell suspensions for as short a period as 7 days(12).

Use of formalin preserved cells should be advantageous in reducing the requirement for a constant supply of blood and the necessity for frequent, laborious preparations of cell suspensions. That no detectable loss of hemagglutinating activity of these cells was observed after storage for at least 3 months, is in accordance with other reports(2,4). The high degree of accuracy of replicate titrations, attainable under the conditions described, is comparable whether freshly prepared or formalin preserved cell suspensions were employed. The spontaneous agglutination which is said to occur(1,7) with formalinized cells was observed in the HA tests but not in the HI reactions. Addition of small amounts of bovine albumin to the phosphate buffered saline was sufficient to prevent this difficulty. When the experiments reported herein were nearing completion, a paper by Ingraham(4) on a new method for preparing formalinized human erythrocytes appeared. Cells prepared according to this report are said to be insensitive to traces of formaldehyde. We have prepared chicken and human cells according to this method but the results of titrations of the Asian influenza A virus and its antibody were not superior to those performed with the other cells prepared in the present study. Furthermore, spontaneous agglutination was observed to occur in the HA tests when these cells were suspended in buffered saline. We were, however, dealing with a somewhat dif-

TABLE II. Replicate Hemagglutination-Inhibition Titrations of Asian Influenza A Antibody with Fresh and Formalin Preserved Chicken Erythrocytes in Sera Obtained from Immunized Human Volunteers.

Subject	Serum*	Erythrocytes†	Hemagglutination-inhibition titrations‡ Determination 1	2	3
59-07	A	FCE	10	0§	10
		PCE	"	10	"
	B	FCE	160	160	80
		PCE	"	"	160
	C	FCE	320	640	640
		PCE	"	320	"
60-01	A	FCE	10	0	0
		PCE	"	0	0
	B	FCE	"	0	0
		PCE	"	0	0
	C	FCE	40	40	40
		PCE	"	"	"
60-06	A	FCE	0	0	0
		PCE	0	0	0
	B	FCE	0	10	0
		PCE	0	"	0
	C	FCE	80	40	80
		PCE	"	80	160
60-16	A	FCE	0	0	0
		PCE	0	0	0
	B	FCE	40	40	20
		PCE	"	"	"
	C	FCE	"	"	40
		PCE	"	"	20

* Samples of serum obtained from subjects, before (A); 4 days (B), and 14 days (C) after receiving monovalent Asian influenza vaccine.

† FCE = Fresh chicken erythrocytes. PCE = Preserved chicken erythrocytes.

‡ Reciprocal.

§ 0 = <10.

ferent system. Ingraham(4) had used 1:100 rabbit serum in saline to suspend the cells. That the absence of spontaneous agglutination is related to the rabbit serum is suggested by the present observations that either rabbit serum or small amounts of bovine albumin prevented spontaneous agglutination in the HA tests. The human sera used in the HI reactions have a similar effect. Rabbit serum, however, is not useful in influenza virus titrations, since it often contains non-specific inhibitors(14). When the formalinized cells were adjusted volumetrically to concentrations similar to 0.5% fresh chicken cells, different end points were obtained and the preserved cells usually produced less distinct patterns than fresh cells. The end points of titrations with preserved cells tended to be lower than those employing fresh cells. Since the suspensions of preserved cells were found to contain more cells than the corresponding concentration (by volume) of fresh cells, it appeared as though the lower sensitivity exhibited by the preserved cells was related to the larger number of cells in suspension. If this is the case, then the behavior of cells which have been stabilized by formaldehyde does not differ from fresh cells in this regard either, for it has been observed that with the latter, a simple inverse proportion exists between the highest dilution of the virus preparation causing complete agglutination and the quantity of cells used in the indicator system (11).

Summary. Formalinized chicken erythrocytes were equivalent to fresh cells for measurement of influenza A virus (Asian) and its antibody. The preserved cells were smaller than fresh ones, requiring some volumetric adjustment. It was necessary, also, when titrating HA ability of treated cells to incorporate small amounts of protein into the diluent.

1. Flick, J. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 448.

2. Cox, C. D., Pirtle, E. C., *ibid.*, 1956, v93, 373.

3. Cole, L. R., Farrel, V. R., *J. Exp. Med.*, 1955, v102, 645.

4. Ingraham, J. S., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 452.

5. Lawlis, J. F., Jr., *ibid.*, 1958, v98, 300.

6. Feeley, J. C., Sword, C. P., Manclark, C. R., *Am. J. Clin. Path.*, 1958, v30, 77.

7. McKenna, J. M., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 591.

8. Bukantz, S. C., Rein, C. R., Kent, J. F., *J. Lab. Clin. Med.*, 1946, v31, 394.

9. Cox, C. D., Fingerhut, B., *ibid.*, 1956, v48, 298.

10. Communicable Disease Center Influenza Rep., U. S. Dept. of Health, Educ., Welfare, No. 8, 1957.

11. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

12. Miller, G. L., Stanley, W. M., *J. Exp. Med.*, 1944, v79, 185.

13. Stavitsky, A. B., *J. Immunol.*, 1954, v72, 360.

14. Henry, C., Younger, J. S., *ibid.*, 1957, v78, 273.

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