organs. Five of these isozymes occurred in developing liver while the 6th type was one of 3 isozymes found in brain and spleen. Brain and spleen exhibited only slight changes in isozyme content during maturation while more extensive changes occurred in liver. The demonstration of LDH isozymes of liver transplanted to the chorioallantoic membrane revealed that there was a reduction in the number of isozymes suggestive of a return to a less differentiated state.

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Factors Affecting Hemagglutination by Enteroviruses. (28961)

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The initial observation that the prototype strains of ECHO virus types 3, 6, 7, 11 and 12 and of Coxsackie virus group B type 3 could agglutinate human erythrocytes(1) was followed by other reports(2,3,4,5,6,7) in which the property of hemagglutination was described for prototype or non-prototype strains of ECHO virus types 13, 19, 20, 21 and 25, Coxsackie virus group A types 7, 20, 21 and 24, and Coxsackie virus group B types 1 and 5. Since JV-10 virus, known variously as Enterovirus type 59(8), Unclassified Picornavirus type 1 (U.S.)(9) or ECHO virus type 29(10) also has been found to hemagglutinate(11), at least one strain of 18 of the 59 enterovirus serotypes recognized in 1962(8) had been found to possess this biological property. Although hemagglutination has been reported for still other enterovirus serotypes(12,13,14), those mentioned above are the only ones for which it was demonstrated clearly that the hemagglutination reaction was specifically inhibited by homologous antiserum. The hemagglutination-inhibition (HI) test has been used successfully for virus identification and for demonstrating serologic responses following infection with hemagglutinating serotypes(6,7,13, 15,16,17,18,19,20,21).

To define further some of the factors that influence hemagglutination by enteroviruses, we studied representative strains of nearly all of the human enterovirus serotypes known to hemagglutinate as well as hemagglutinating strains of newly recognized, but as yet unclassified, enteroviruses. The results of these investigations are the subject of this report.

Materials and methods. Virus strains. Hemagglutinating enteroviruses studied included

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ECHO virus types 3 (Morrisey), 6 (D-1), 7 (Wallace), 11 (Gregory), 12 (Travis), 13 (Del Carmen), 19 (Burke), 20 (Gillis), 21 (Foreman), 29 (JV-10) and 30 (Frater) (10); Coxsackie virus group A types 21 (Bouma) and 24 (Joseph) and Coxsackie virus group B types 1 (Toluca R1049), 3 (strain obtained from Dr. M. Goldfield) and 5 (Toluca R1709). The unclassified hemagglutinating enteroviruses of human origin included Toluca-1 and JV-9. Other strains studied included ECHO virus prototypes 1, 2, 4, 5, 8, 9, 14 to 18, 22 and 24 to 28; Coxsackie virus group B prototypes 1 to 6 and the Texas Pleurodynia(22) and Powers(23) strains of Coxsackie virus group B types 3 and 4 respectively; the Russian ABIV strain of Coxsackie virus group A type 7 and the prototype strains of Coxsackie virus group A types 9, 10, 11, 13 to 18, 20, 20A, 20B and 21, and Polio types 1 (Mahoney), 2 (Lansing) and 3 (Leon).

Tissue cultures. Primary rhesus kidney (MK) and human embryonic kidney (HEK) monolayer tissue cultures in screw-cap tubes and MK cultures in 32 oz. prescription bottles were obtained from Microbiological Associates, Bethesda, Md. All cultures were washed free of growth medium with Hanks' balanced salt solution(24) and, except where noted otherwise, MK tube cultures received 0.9 ml of synthetic Medium No. 199(25) without a serum supplement and containing 500 units of penicillin and 500 μg of streptomycin per ml. Bottle cultures received 40 ml of the same maintenance medium. The medium for HEK cultures, unless noted otherwise, was 1.0-1.5 ml of Medium No. 199 with antibiotics as above and with an additional 0.06% of sodium bicarbonate.

Preparation of hemagglutinating virus pools. Stock pools of the following hemagglutinins were prepared in MK tube or bottle cultures: All ECHO virus types except for the Foreman strain of ECHO virus type 21, Coxsackie virus group B types 1, 3 and 5 and Toluca-1. The ECHO type 21 strain was grown in HEK cultures maintained with 1.5 ml of Medium No. 199 without added bicarbonate. HEK tube cultures were used to prepare pools of JV-9 and of the Coxsackie virus group A types 21 and 24 viruses. All cultures were inoculated with 0.1 ml, and bottles with 0.5 to 1.0 ml, of seed virus either undiluted or diluted 1:10. MK tubes were incubated at 37°C in a stationary position while HEK tubes with the exception of those infected with the Foreman strain of ECHO virus type 21 were incubated at 37°C in a roller drum revolving at approximately 12 revolutions per hour. Cultures of the Foreman strain were incubated in a stationary position. All cultures were frozen at approximately —20°C when the cytopathic effect had involved the entire monolayer or when the tissue had fallen from the glass. After rapid thawing in water slightly above room temperature, the contents of tubes were pooled. Without further treatment, each of the fluids was distributed in either a 0.5 or 1.0 ml volume (depending upon the titer of hemagglutinin) in 1 dram screw-cap glass vials. The vials were refrozen and stored at approximately —20°C.

Erythrocytes. Human erythrocytes from adults or from the umbilical cords of newborn infants were collected and standardized for use in hemagglutination and HI tests as has been described previously(26). The blood group of each lot of cells was determined by conventional macroscopic slide agglutination tests. Group O erythrocytes were used exclusively. Prior to use in titrations, all cell suspensions prepared in the test diluents were screened for their ability to sediment at both 4°C and 37°C as compact "buttons" in the absence of virus. This cell control procedure was also employed simultaneously with all titrations.

Hemagglutination. The procedure used was identical with that described for reoviruses (26), except for the use of phosphate-buffered saline in addition to unbuffered saline. The diluent designated "saline" was 0.85% NaCl. Phosphate-buffered saline diluents of pH 5.8, 6.9 and 7.4 were prepared by the appropriate combination of 0.01 M NaH_2PO_4 and Na_2HPO_4 stock solutions; the latter were prepared by dissolving the buffer salts in saline. References to other diluents are

TABLE I. Grouping of Enteroviruses by Optimal Sedimentation Temperatures.

Maximum HA titer at		
4°	37°	Same at both
ECHO 3	ECHO 6	ECHO 7
" 11	" 24	" 12
" 13	" 29	" 20
" 19	" 30	" 21*
Coxsackie A-21	Coxsackie B-1	Coxsackie B-3
" A-24	" B-5	
JV-9		
Toluca-1		

* Results obtained with non-prototype Foreman strain; hemagglutination previously reported with the prototype Farina strain(6).

made in the text. Hydrogen ion concentration was determined with a Beckman pH meter, model G.

Results. Effect of sedimentation temperature and type of erythrocyte on hemagglutinin (HA) titers. We reported(6) that higher HA titers were almost always obtained with hemagglutinating strains of Coxsackie group B types 1, 3 and 5 viruses when cord erythrocytes were used instead of those from adult donors. In the present investigation the HA titers for a number of additional hemagglutinating serotypes were determined with both cord erythrocytes and with those from adult donors. Titrations were performed at both 4°C and 37°C to determine the optimal sedimentation temperature for demonstration of hemagglutination for each of the erythrocyte types. As seen in Table I, it was possible to separate the serotypes into 3 groups on the basis of the HA titers obtained at the 2 sedimentation temperatures. The divisions were identical with both types of human erythrocytes and comparable results were obtained with at least 2 pools of each serotype. However, the data in Table II show that 4-fold to 8-fold higher HA titers of ECHO virus type 13 and of Coxsackie group A type 24 virus were obtained with cord cells as compared with adult cells at 4°C. At 37°C, the titers of ECHO virus type 20 and of the Coxsackie virus group B strains were 4-fold higher with cord cells. Although 2-fold differences in the titers obtained at 4°C or 37°C were not usually considered significant in any titration, higher HA titers with various pools of the D-1 strain of ECHO virus type 6 were consistently obtained with adult and cord erythrocytes at 37°C. For this reason, this serotype was placed in the "37°C group" of Table I.

Effect of hydrogen ion concentration on HA titer. Preliminary observations of enterovirus hemagglutinins indicated that, although they were active over the pH range of 5.5 to 8.0, higher titers with certain serotypes were obtained at one or the other pH extreme. This effect of hydrogen ion concentration was further studied at pH 5.8, 6.9 and 7.4 after preliminary tests had indicated that standardized suspensions of adult and cord erythrocytes agglutinated spontaneously or lysed in 0.01 M phosphate-buffered saline of higher or lower pH at either 4°C or 37°C, or at both temperatures. At least one pool of each hemagglutinating virus serotype which reacted positively in saline was studied at each pH. Four replicate titrations of each virus were carried out at each pH with standardized suspensions of adult and cord erythrocytes prepared at the corresponding pH. For each buffered diluent, adult erythrocytes were used for 2 titrations and cord erythrocytes were used for the 2 remaining. One

TABLE II. Representative Enterovirus HA Titers* Obtained at 4°C and 37°C with Adult and Cord Erythrocytes.

	Titers			
	4°C		37°C	
Virus	Adult	Cord	Adult	Cord
ECHO 3	256	256	<8	<8
" 6	32	64	64	128
" 7	256	256	256	256
" 11	512	512	<8	<8
" 12	512	512	512	1024
" 13	32	128	<8	<8
" 19	1024	1024	32	32
" 20	128	128	64	256
" 21	512	512	256	512
" 24	<8	<8	<8	8
" 29	64	32	128	128
" 30	<8	<8	16	16
Cox A-21	256	256	<8	<8
" A-24	64	512	<8	<8
" B-1	8	64	64	256
" B-3	32	64	16	64
" B-5	16	128	64	256
Toluca-1	128	64	<8	<8
JV-9	32	16	<8	<8

* Reciprocal of dilution.

TABLE III. Effect of pH on Enterovirus Hemagglutinin Titers at 4°C and 37°C with Adult and Cord Erythrocytes.

	4°C						37°C					
	Adult (pH)			Cord (pH)			Adult (pH)			Cord (pH)		
	5.8	6.9	7.4	5.8	6.9	7.4	5.8	6.9	7.4	5.8	6.9	7.4
ECHO 11	256	512	1024	256	1024	1024	<8	<8	64	<8	32	32
" 19	1024	1024	1024	2048	1024	2048	128	64	32	128	64	64
" 20	256	64	64	512	256	128	512	64	32	512	256	64
" 21	512	512	256	1024	512	256	1024	256	128	1024	512	256
" 24	<8	<8	<8	<8	<8	<8	<8	<8	<8	16	8	8
" 30	<8	<8	8	<8	<8	16	128	32	<8	64	32	<8
Cox B-1	<8	8	16	<8	64	64	<8	64	64	32	256	256
" B-3	64	32	64	256	256	128	128	16	16	256	128	64
" B-5	<8	32	64	<8	128	128	<8	32	128	8	256	256
JV-9	256	64	32	256	64	64	<8	<8	<8	<8	<8	<8

titration with each type of red cell was allowed to settle at 4°C and the other at 37°C. The effects of pH on HA titers are shown in Table III. Maximal HA titers of ECHO virus type 11 and Coxsackie virus group B types 1 and 5 were obtained at pH 7.4, while ECHO virus types 20 and 21, Coxsackie virus group B type 3 and JV-9 viruses showed maximal titers at pH 5.8. Agglutination of adult or cord cells with JV-9 virus was not observed at 37°C under any condition. The Frater strain of ECHO virus type 30, in contrast to the report by Duncan(27), specifically agglutinated both types of red cells. At 37°C with saline as diluent, a titer of 1:16 was obtained with both types of red cells. However, with a diluent of pH 5.8 at this temperature, 4- to 8-fold increases in titer were noted. A reversal of the pH optimum was observed at 4°C; titers of 1:8 to 1:16 were obtained only at pH 7.4.

The strain of JV-9 virus tested was one of 3 strains which had been compared by hemagglutination tests in saline and in buffered diluents. A more detailed investigation was undertaken since these strains were similar to a large group of enteroviruses isolated in our laboratory and by others(28) which caused a typical enterovirus CPE in HEK and HeLa but not in MK cell cultures. Maximal HA titers were obtained at pH 5.8 and the reaction could be demonstrated only at 4°C. Experiments were carried out to determine the effect of alteration of hydrogen ion concentration and the buffer molarity on the HA titer obtained with this virus. Phosphate buffered saline diluents were prepared in the pH range of 5.7 to 7.5 in increments of approximately 0.2 pH unit. Molarity was held constant at 0.005. A second series of diluents was prepared at pH 5.7 or 5.8, in which the molar concentration of buffer salts decreased from 0.1 M through 0.05, 0.025, 0.01, 0.0075 to 0.005 M. Citric acid-sodium citrate buffered saline, 0.01 M, also was used at pH 5.8. Adult and cord erythrocytes were employed in all titrations with a sedimentation temperature of 4°C. The maximal HA titer of 1:128 was obtained at pH 5.7 with titers decreasing 4-fold at pH 6.7 and 8-fold at pH 7.3. Neither the alteration of buffer molarity at pH 5.7-5.8 nor the use of a citrate in place of a phosphate buffer system significantly affected the HA titers obtained with this virus.

Partial sedimentation patterns. In titrations of Coxsackie virus group B hemagglutinins with adult erythrocytes, we reported (6) that a partial type of red cell sedimentation pattern was seen which was similar to that noted in the agglutination of rat erythrocytes by certain adenovirus serotypes. The use of cord erythrocytes in titrations of these hemagglutinins in a saline diluent usually resulted in complete erythrocyte sedimentation patterns, although in a few cases, partial patterns were still seen. A series of titrations of Coxsackie group B type 3 virus was carried out with several lots of cord and adult cells in order to determine the influence of pH and of length of storage time of erythrocytes on the occurrence of partial patterns. Unbuffered saline and saline buffered at pH 5.8 were em-

ployed as diluents at a temperature of 37°C. It was observed that when buffered saline was used as test diluent, complete sedimentation patterns with both red cell types were observed. With unbuffered saline as diluent, all cell preparations but one resulted in the formation of partial patterns beginning with the lowest test dilution of 1:8. No significant difference in HA titer attributable to storage of the cell preparations for one week was observed. However, the titers obtained with all cord cell preparations were consistently 2-fold higher than with adult cells.

Detection of new enterovirus hemagglutinins. When it was noted that HA titers of several enterovirus serotypes were improved when cord instead of adult cells and/or buffered diluents were employed, those serotypes for which hemagglutination has never been previously demonstrated (see *Materials and methods*) were tested in the following manner. All viruses were tested for activity at a 1:4 or 1:8 dilution in unbuffered saline and in 0.01 M phosphate-buffered saline of pH 5.8, 6.9 and 7.4. Adult and cord erythrocytes were used with sedimentation temperatures of 4°C and 37°C. Pools of viruses that were prepared with tissue cultures maintained with media containing serum were tested only at a 1:8 dilution. Hemagglutination was observed only with ECHO virus type 24 (strain De Camp). Since this property had never been reported for this serotype, it was further investigated.

HA titrations of ECHO virus type 24 were carried out with cord red cells at 4°C and 37°C in diluents of pH 5.8, 6.9 and 7.4 and in unbuffered saline. Hemagglutinating pools of 2 other ECHO type 24 strains, Jones (JV-3) and Toluca (S13) were included for comparison. The results, given for the prototype strain, are shown in Tables I, II and III. Maximal HA titers for the non-prototype strains were also obtained at pH 5.8 and were 1:16 and 1:32 respectively.

Discussion and summary. Some of the factors that are known to affect the agglutination of various erythrocytes by viruses are the age of the donor animal, the hydrogen ion concentration of the diluent and the temperatures at which erythrocytes are allowed to sediment. The results reported here indicate that these factors, either singly or in combination, also affect the agglutination of human erythrocytes by enteroviruses known to possess this property.

Cord erythrocytes or diluents of specific hydrogen ion concentration are not usually necessary for demonstration of enterovirus hemagglutinins. However, the use of these cells and of diluents other than those buffered at or above pH 7.0 has enabled us to detect hemagglutinating activity where it existed in what might be termed a "borderline state." This was exemplified by our demonstration of hemagglutination by ECHO virus type 24. Other reports(15,29,30) have indicated that the pH optimum for hemagglutination reactions with certain enteroviruses was other than at pH 7.0 to 7.3. Temperature has been shown to be the determining factor in whether or not hemagglutination is demonstrable in an unbuffered diluent with ECHO virus types 3, 11, 13 and 30, with Coxsackie group A types 21 and 24 and with Toluca-1 and JV-9 viruses.

While several reports have appeared concerning utilization of hemagglutination and HI reactions to identify enteroviruses, there has been little effort to determine the optimal environmental conditions necessary to propagate enteroviruses in order to produce maximal titers of their hemagglutinins. We have been able to demonstrate hemagglutination regularly with tissue culture fluids of nearly all the serotypes utilized in these experiments, but difficulty has been encountered with a few serotypes such as the prototype strain of ECHO virus type 24. We have consistently been able to produce hemagglutinins with other strains of this same serotype. The reason(s) for the varying capacity of different strains of the same enterovirus serotype to agglutinate human red cells is(are) not apparent.

It has been recommended(8) that new candidate enteroviruses which have been shown to be antigenically distinct from all established types by neutralization tests be tested for hemagglutinating activity. From

the data presented here, it is suggested that such viruses be tested for hemagglutinating capacity at 4°C and 37°C with human cord erythrocytes in diluents of both high and low pH as an integral part of their characterization.

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Deoxyribonucleic Acid Polymerase Activity During Azo Dye Carcinogenesis.* (28962)

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Many investigations have been made of the carcinogenesis which occurs in the livers of rats fed azo dyes. Changes in tissue and cell morphology(1), alterations in subcellular components(2), and variations in certain proteins(3,4) have been studied. During the period of azo dye carcinogenesis, some enzymes of liver tissues have been examined both histochemically(5), and in extracts(6). Intense cellular proliferation has been observed to characterize several stages of this carcinogenesis. This observation prompted an

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