Effect of pH on Cytopathogenicity of Orphan Viruses.* (22958)

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Orphan viruses represent the third virus group, in addition to poliovirus and Coxsackie virus, isolated from human enteric tract and associated with poliomyelitis-like disease(1). Fourteen serotypes of orphan viruses have been classified as ECHO (enteric cytopathogenic human orphan) viruses(2,3). The role of ECHO viruses in human illness, with special reference to aseptic meningitis (nonparalytic poliomyelitis), was recently discussed(4,5). During the course of an investigation of an outbreak of seasonal aseptic meningitis in western New York in 1955, it was found that the cytopathogenic effect of certain orphan viruses in monkey kidney tissue culture was depressed in the presence of an acid pH in tissue culture fluid.

At the present time, the sole method of isolation and titration of orphan viruses is the detection of their cytopathogenic effect in simian or human tissue cultures. It appeared important therefore to investigate the effects of pH change upon sensitivity of the system. Some of the factors influencing this phenomenon are reported in the present study.

Materials and methods. Tissue culture system and media. Monkey kidney trypsin-dispersed monolayer tissue cultures were used (1). Two nutritionally similar media were employed, one with a low and the other with a high buffer capacity. The low buffer medium consisted of 0.5% lactalbumin hydrolysate, 2% calf serum in a base of Hanks' balanced salt solution(6,7). The high buffer medium substituted Earle's saline(7,8) for the Hanks' balanced salt solution. The pH was determined colorimetrically, using phenol red indicator in the medium, by comparison with color standards. The pH of both media was 7.6-7.8. The cells were implanted in the low buffer medium and this medium was exchanged once, 3-4 days after preparation of the tissue culture. As the cell sheet developed, the pH progressively dropped, so that after 5-7 days, when the monolayer was complete, the pH was 6.6-6.8. The medium was replaced at time of virus inoculation with a maintenance medium consisting of either the high buffer or low buffer medium. Table I compares buffer systems in the 2 media, as used in this study. The essential difference between the 2 media is the greater content of $NaHCO_3$ in the highly buffered medium. Tissue cultures in the high buffer medium maintained a pH of 7.6-7.8 during the 7-9 day observation period. However, when the low buffer medium was employed as maintenance medium, the pH of the culture fluid rapidly declined to 6.6-6.8 after 48 hours incubation, remaining at 6.6 during the remainder of observation period. Cells were readily maintained in good condition in both media. In the low buffer medium, the cells tended to appear spindle-shaped, granular and somewhat contracted, whereas cells maintained in the high buffer medium were flatter, more translucent and squamous in appearance. Virus titration endpoints were calculated according to the method of Reed and Muench(9), and were expressed as log TCD_{50}/0.1 ml. Usually 5 tissue culture tubes were inoculated per dilution, but in a few experiments, 2 or 3

TABLE I. Composition of Buffer Systems of Media.

Constituent	Concentration/100 ml	
	High buffer medium	Low buffer medium
	mg	mg
$NaHCO_3$	187.5	52.5
Na_2HPO_4		4.4
$NaH_2PO_4 \cdot H_2O$	11.1	
KH_2PO_4		5.2

* Presented in part at annual meeting of Canadian Society of Microbiologists, Macdonald College, Quebec, June 1956.

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TABLE II. Comparative Virus Titrations in Low Buffer and High Buffer Media.

Virus	Medium (buffer)	Virus titer — Days — 1	2	3	4	5	6	7	9
ECHO 6									
D'Amori*	L†	3.5‡	5.5	6.5	6.6	6.8	7.2	7.2	7.2
	H	4.5	6.5	6.8	7.0	7.0	7.0	7.0	7.0
Patient No. 8 (Buffalo)	L	.5	1.2	2.5	2.5	2.8	4.0	5.2	5.4
	H	1.2	2.5	4.5	4.6	5.5	5.6	5.6	
14	L	.5	.8	2.6	2.6	3.4	4.0	5.5	5.5
	H	1.4	1.8	4.2	4.8	5.6	5.6	5.8	
18	L	2.4	3.6	4.5	4.6	5.5	5.5	6.2	6.5
	H	3.2	4.5	6.4	6.6	6.8	7.2	7.2	
25	L	.0	.0	.5	1.5	1.5	3.0	3.0	
	H	.0	.5	1.5	2.5	4.0	5.0	5.0	
ECHO 12									
Travis 2-85*	L	2.6	4.8	5.5	5.5	5.5	5.5	5.5	5.5
	H	3.2	4.8	5.5	5.5	5.5	5.5	5.5	5.5
Unidentified orphans									
Patient No. 32	L	1.5	2.5	2.5	3.5	4.2	4.6	5.5	6.4
	H	.8	2.5	4.2	4.5	5.5	5.5	5.8	6.2
801	L		<1.5	<1.5	<1.5	<1.5	<1.5	2.8	
	H		1.8	3.3	4.8	4.8	5.3	5.3	
Poliovirus									
Type I (Mahoney)	L	3.6	6.5	6.8	6.8	6.8		6.8	
	H	3.5	6.3	6.6	6.6	6.6		6.6	
Type II (MEF-1)	L	3.5	6.2	6.5	6.6	6.8		6.8	
	H	3.5	5.8	7.3	7.3	7.3		7.3	

* Prototype strain. † L = Low; H = High. ‡ Log TCD_{50}/0.1 ml.

tubes were used. A virus‡ inoculum of 0.1 ml was added to 0.9 ml of the maintenance medium.

Results. Virus titration endpoints were compared in tissue cultures maintained in low and high buffer media. Depression of virus cytopathogenic effect demonstrated in the low buffer medium as compared with the high buffer medium will be referred to as the "pH effect." The results, using prototype and Buffalo isolates of ECHO 6, prototype ECHO 12, unidentified agents Nos. 32 and 801 and poliovirus types I and II, are presented in Table II. ECHO 6 prototype, as well as the four 1955 Buffalo ECHO 6 isolates, showed a slower rise in titer in the low buffer medium as compared with high buffer medium, with the final titer tending to be lower in low buffer medium. All isolates of ECHO 6 were not equally sensitive to the "pH effect." The unidentified orphan isolate from patient No. 801 showed the greatest "pH effect" of those tested. Poliovirus type I maintained approximately the same titer in both media, whereas type II had a slightly lower titer (0.5 log TCD_{50}) in low buffer medium. Depression of virus titer in low buffer medium was more marked with the more slowly cytopathogenic agents than with viruses which attained high titer rapidly. The "pH effect" was particularly manifest on or about the 5th day of incubation. When incubation was continued sufficiently long, the final titers were frequently similar in both media.

To further clarify the influence of acid pH on virus titer, the low buffer medium was ar-

‡ *ECHO virus prototypes* were obtained through the courtesy of the members of the Committee on ECHO viruses(2). In addition, orphan virus isolates from patients with aseptic meningitis were used, including 4 strains of ECHO 6 and 2 unidentified agents, Nos. 32 and 801. These agents were isolated in Buffalo, N. Y., in 1955(5). Coxsackie B1 (Conn-5) was obtained from Dr. A. J. Pappenheimer, Boston, and poliovirus types I (Mahoney) and II (MEF-1) were obtained from Connaught Medical Research Laboratories, Toronto.

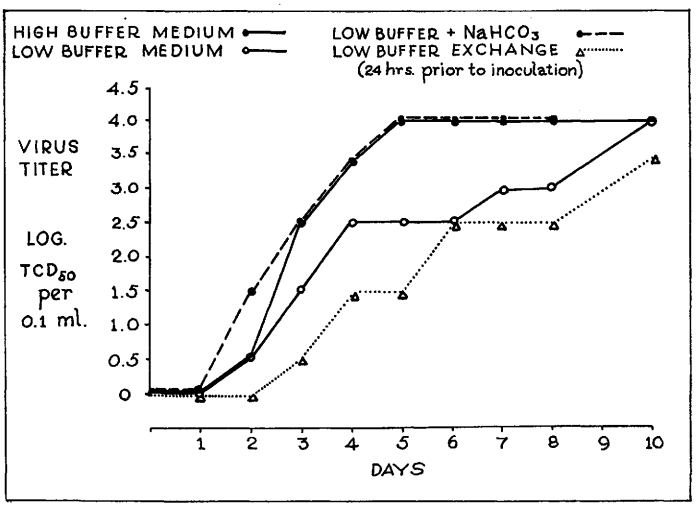

FIG. 1. Influence of alteration of acidity of tissue culture medium upon "pH effect". Virus: ECHO 6 (No. 25).

tificially maintained at pH of 7.6 by addition of $NaHCO_3$ to culture tubes during incubation. After incubation for 12 hours, $NaHCO_3$ (0.5 mg) was added to each tube. The pH was returned from 7.0 to 7.6. After incubation for an additional 36 hours, the pH had dropped to 7.0 and $NaHCO_3$ (0.5 mg) was again added. The pH was thus restored to 7.6, where it remained for duration of experiment. Titration results in the above cultures were compared to those obtained with cells maintained in low buffer medium and high buffer medium. There was no difference in titration endpoints between high buffer and low buffer medium with added $NaHCO_3$, which indicated that the return to an alkaline pH by simple addition of $NaHCO_3$ prevented the "pH effect." Exchange of tissue culture fluid with low buffer medium 24 hours prior to virus inoculation rather than simultaneously with virus inoculation tended to exaggerate the "pH effect." In this case, the pH of medium at time of virus inoculation was 6.8, as a result of 24 hours incubation. Thus, less than 24 hours after virus inoculation, the pH had reached 6.6. Results obtained employing the above methods of altering "pH effect" are plotted in Fig. 1.

It was found that when glucose, which is present in concentration of 100 mg% in both media, was omitted from low buffer medium, the pH did not decline. This suggested that formation of acidity was related to glucose metabolism. Cells maintained in low buffer medium without glucose remained in a satisfactory condition for virus study for 7-9-day period. During the period of incubation, the cells without glucose morphologically resembled those maintained in high buffer medium. ECHO 4 prototype was titrated in low buffer medium and in the same medium devoid of glucose. The results are plotted in Fig. 2. The titers obtained with low buffer medium without glucose were comparable to titers obtained with high buffer medium, which served as a control, and in both systems, the pH did not fall below 7.4. In low buffer medium containing glucose, where pH became 6.6 on incubation, the "pH effect" was quite marked. A similar experiment, performed with pH sensitive No. 801 isolate, showed the same effect. The above results again pointed to the fact that it was the development of acidity in tissue culture medium which was associated with depression of virus cytopathogenicity.

The influence of size of virus inoculum on "pH effect" was examined by studying the effect of varying virus doses upon extent of cell damage, rather than by comparative virus endpoint titrations. Degrees of cell involvement as a result of progressive viral infection

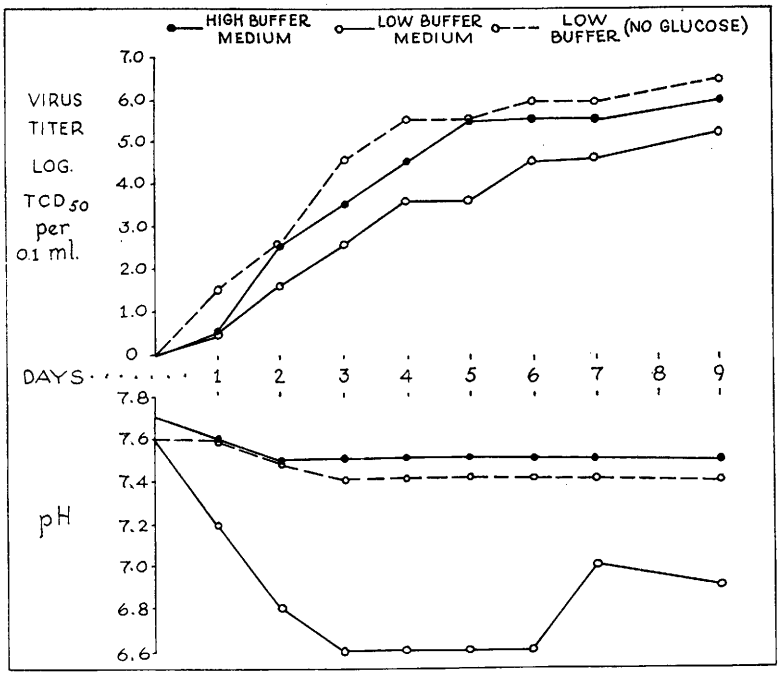

FIG. 2. Effect of omission of glucose from low buffer medium on pH and on growth of ECHO 4 virus.

were arbitrarily rated in 0.5 unit increments from 0.5 (minimal early effect) to a maximum of 3.5 (virtual disappearance of cells). A cytopathogenic score per tube was calculated by dividing the sum of ratings of tubes by the number of tubes in experimental group

$$\left(\text{Cytopathogenic Score} = \frac{\text{Sum of Ratings}}{\text{No. of tubes}}\right).$$

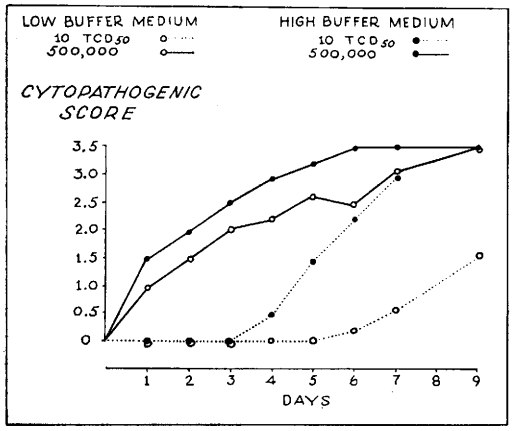

FIG. 3. Influence of virus dose on "pH effect". Virus: ECHO 6 (No. 8). Cytopathogenic score was calculated according to the formula in the text.

When a large dose of ECHO 6 isolate No. 8 was employed, approximately 500,000 TCD_{50}, the "pH effect" was minimal. When a small dose of the same virus was employed, approximately 10 TCD_{50}, the effect was more pronounced (Fig. 3). With the small virus dose, using low buffer medium, there was no evidence of viral cytopathogenic effect until the 6th day. Cell destruction in this medium, as estimated by the cytopathogenic score, was not very marked even after 9 days incubation. In high buffer medium, the cytopathogenic effect was detected on day 4 and was quite marked on day 7.

Employing the cytopathogenic score technic, the effect of an acid environment was studied with 12 of the ECHO prototypes by inoculation of approximately 100 TCD_{50} into tissue cultures maintained in low and high buffer media. The results are summarized in Table III. ECHO 1, 5, 7, 8 and 12 showed minimal "pH effect." ECHO 2, 6, 11 and 13 were moderately affected, whereas 3, 4 and 9 were markedly sensitive. Coxsackie B1 was markedly sensitive, while poliovirus types I and II showed little "pH effect."

TABLE III. Sensitivity of Viruses to "pH Effect" as Measured by Cytopathogenic Score.

Virus*	Medium (buffer)	Days 1	2	3	4	5	6	7	8	9
Minimal										
ECHO 1	L†	.2‡	1.5	2.5	3.0	3.5				
	H	.8	1.5	2.9	3.5	3.5				
5	L	.0	.5	1.9	2.5	3.4	3.5			
	H	.2	1.0	2.1	3.2	3.5	3.5			
7	L	.9	2.0	3.0	3.3	3.3	3.5			
	H	.5	2.5	3.5	3.5	3.5	3.5			
8	L	.5	.8	1.6	3.0	3.4	3.5			
	H	.2	1.2	2.5	3.5	3.5	3.5			
12	L	.2	1.1	2.4	3.0	3.5				
	H	.4	1.4	2.5	3.4	3.5				
Moderate										
2	L	.1	.3	1.0	1.5	2.5	3.0	3.5		
	H	.1	1.0	1.7	2.3	3.5	3.5	3.5		
6	L	.0	.3	1.5	2.2	3.0	3.5			
	H	.0	1.0	1.9	2.8	3.5	3.5			
11	L	.1	.5	.6	1.5	2.5	3.1	3.5		
	H	.0	.9	1.4	2.8	3.5	3.5	3.5		
13	L	.3	1.9	2.5	3.0	3.3	3.5			
	H	1.0	2.0	3.0	3.5	3.5	3.5			
Marked										
3	L	.2	.3	1.0	1.5	2.5	3.0	3.5		
	H	.7	1.5	1.9	2.8	3.5	3.5	3.5		
4	L	.0	.1	.2	.8	.8	1.4	2.3	2.6	3.5
	H	.0	.8	1.9	2.8	3.5	3.5	3.5	3.5	3.5
9	L	.0	.1	.4	1.4	2.3	2.5	3.4		
	H	.0	1.0	1.8	2.4	3.0	3.4	3.5		
Coxsackie B1 (Conn-5)	L	.1	.5	.4	.4	2.0	1.6	2.3	2.5	2.7
	H	.0	.6	1.2	1.8	2.5	2.9	3.5	3.5	3.5
Poliovirus Type I	L	.1	1.5	3.0	3.5	3.5		3.5		
	H	.0	1.5	3.4	3.5	3.5		3.5		
" " II	L	.0	1.0	2.5	3.0	3.5		3.5		
	H	.0	1.0	3.5	3.5	3.5		3.5		

* Virus dose, approximately 100 TCD_{50}. † L = Low; H = High. ‡ Cytopathogenic score (refer text).

The effect of pH of medium upon direct isolation of cytopathogenic enteric viruses from clinical specimens was considered. Known virus-positive stool suspensions were inoculated in parallel into tissue cultures maintained in low buffer and high buffer media. Preparation of stool suspensions and method of inoculation have been described elsewhere(5). The results of this isolation study are presented in Table IV. After a single passage, in high buffer medium, 77% (151/196) of tissue cultures were recorded as positive (*i.e.* showing definitive cytopathogenic effect typical of viral infection), whereas only 39% (87/222) of cultures were positive in low buffer medium. Tissue cultures with any suspicion of possible virus effect were recorded as questionable. Using these criteria of grading, it was felt that many questionable cultures would be regarded as negative in routine diagnosis, particularly if only a single tissue culture passage were employed.

Discussion. Our results have indicated that development of an acid environment in tissue culture fluid may delay or depress the appearance of viral cytopathogenic effect with some human enteric agents. When the acid environment was neutralized by addition of $NaHCO_3$ or prevented from forming by omission of glucose, the "pH effect" was not observed. Recently Gifford *et al.*(10) have reported studies on the effect of pH on metabo-

TABLE IV. Influence of "pH Effect" on Recovery of Orphan Viruses from Stool Suspensions.

Virus isolated	No. stools	Low buffer medium No. of tubes			High buffer medium No. of tubes		
		Pos.	Quest.	Neg.	Pos.	Quest.	Neg.
ECHO 6	19	60	84	42	123	20	25
Unidentified Orphan	3	27	9	0	28	0	0
Total	22	(222) 87	93	42	(196) 151	20	25
%		39	42	19	77	10	13

lism of HeLa cells, which showed that increase in acidity decreased poliovirus production. A small virus dose of 25 TCD_{50} was employed in their study. These investigators found that there was no decrease in virus stability at lower pH levels to account for the decrease in virus production. This would suggest that the "pH effect" is not restricted to monkey kidney cell cultures alone. Although the effect of H^+ ion concentration on viral infection of cells in culture has not been the subject of intensive investigation, Hanks (11), in an excellent account of nutrition of cells in culture, referred frequently to the fact that of all metabolic endproducts resulting from cellular metabolism, only the H^+ ion is known to be toxic. The mechanism of H^+ ion toxicity for cells has not been fully explained.

In our experiments, several factors influencing the "pH effect" became apparent. There were differences in "pH effect," both between ECHO virus serotypes, as well as among different isolates within a single serotype. Rapidly cytopathogenic agents, as typified by the poliovirus group, demonstrated minimal "pH effect." The employment of a large virus inoculum was found to obscure the "pH effect." An explanation for the minimal "pH effect" of rapidly cytopathogenic agents or of large inocula is not entirely clear. Certainly one factor may be the 24-48 hours lag period for production of an acid environment. During this interval a rapidly multiplying virus or a massive virus inoculum may have sufficient time in an environment with a physiological pH in which to cause cell destruction. The enhancement of the "pH effect" by media exchange 24 hours prior to virus inoculation would point in this direction. Preliminary experiments would suggest that the inhibitory effect of lowered pH is on the host cell, rather than on the free virus, and that virus release into the medium is correlated with the degree of cell damage.

Primary isolation attempts, in a single tissue culture passage, using known positive stool suspensions, revealed that acid producing low buffer medium was approximately one half as effective in detecting definitive viral cytopathogenic effect as the high buffer medium. Consequently, clinical sources of virus material, such as throat washings, spinal fluid or stools containing a small number of infectious units of a pH sensitive agent may falsely be considered virus negative if sought for in the presence of a highly acid medium. Since the isolation of orphan viruses rests entirely upon manifestation of a cytopathogenic effect in tissue culture, the influence of the "pH effect" upon sensitivity of this system appears to be worthy of consideration.

Summary. 1. The cytopathogenic titer of certain orphan viruses in monkey kidney tissue culture was depressed or delayed when grown in the presence of a medium which developed an acid pH. This phenomenon has been termed the "pH effect." 2. Sensitivity to the "pH effect" appeared to be strain characteristic. Differences occurred among serotypes as well as within serotypes of the orphan viruses. One Coxsackie group B virus tested also demonstrated the "pH effect." Two poliovirus types tested were minimally affected by pH variation. 3. Neutralization of tissue culture acidity with an excess of $NaHCO_3$, or prevention of acid formation by omission of glucose restored optimal viral cytopathogenicity. 4. The "pH effect" tended to be less evident with rapidly cytopathogenic

agents or in the presence of large inocula. 5. Rate of primary isolation of orphan virus from stool suspensions was substantially decreased when acid producing tissue culture medium was used.

The authors wish to acknowledge the technical assistance of Miss Alicia Bukowski.

1. Melnick, J. L., *Ann. N. Y. Acad. Sci.*, 1955, v61, 754.

2. Committee on the ECHO Viruses, *Science*, 1955, v122, 1187.

3. Editorial addition, ECHO Viruses, in *Diagnostic Procedures for Virus and Rickettsial Diseases*, Am. Public Health Assn., New York, 1956.

4. Viruses in Search of Disease, *Ann. N. Y. Acad. Sci.*, in press.

5. Karzon, D. T., Barron, A. L., Winkelstein, W. Jr., Cohen, S., *J.A.M.A.*, 1956, v162, 1298.

6. Hanks, J. H., Balanced Salt Solutions, Inorganic Requirements, and pH Control, in *An Introduction to Cell and Tissue Culture*, Burgess Publishing Co, Minneapolis, 1955.

7. Melnick, J. L., Tissue Culture Methods for the Cultivation of Poliomyelitis and Other Viruses, in *Diagnostic Procedures for Virus and Rickettsial Diseases*, Am. Public Health Assn., New York, 1956.

8. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

9. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

10. Gifford, G. E., Robertson, H. E., Syverton, J. T., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 321.

11. Hanks, J. H., Miscellaneous Problems in Cell Culture Management, in *An Introduction to Cell and Tissue Culture*, Burgess Publishing Co., Minneapolis, 1955.

Received December 13, 1956. P.S.E.B.M., 1957, v94.

Assay of Properdin in Biological Fluids.* (22959)

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A naturally occurring serum protein, properdin, has been described which participates in certain types of non-specific immunity(1). The known constituents of the properdin system are magnesium ions, complement (C'), and properdin (P)(1,2). In the presence of magnesium ions, properdin complexes with zymosan, a complex of insoluble polysaccharides derived from yeast cell wall, and various other polysaccharides(1-4). The third component of complement (C'3) is specifically inactivated by this properdin-zymosan complex. Various assays for properdin based on inactivation of C'3 have been reported(1-3,5). These employ two reagents, (a) a serum depleted in properdin but containing all components of complement (RP) and (b) a serum lacking properdin and C'3 but containing a large excess of all other components of complement (R3).

Since the source of human sera for preparation of satisfactory test reagents is restricted to 10 to 20% of the population, surveys of large numbers of sera are necessary to obtain reagent sera in practical quantities (1,2). The previously described method for removal of properdin from RP preparation has not been demonstrated to be quantitative in our laboratory and complete removal of C'3 from human sera has also been difficult to achieve. In previously described properdin assay there is no provision for the contribution made by C'3 of the test serum, and variable end points result when large particles of zymosan sediment to bottom of the tube. This paper reports a simple reproducible method for preparation of 2 reagent sera and a more sensitive assay for properdin in biological fluids.

Materials. A. *Barbital Buffer.* The composition of stock pH 7.4 buffer was reported elsewhere(2). The diluted buffer was prepared from stock daily and discarded after 12 hours to ensure constancy of pH. B. *Activated Sheep Cells.* Sheep blood in equal volume of Alsever's solution was obtained

* This investigation was supported by Office of Surgeon General, Department of Army.