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G. W. A. Dick; ... et. al

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BOVINE PLASMA ALBUMIN IN BUFFERED SALINE SOLUTION AS A DILUENT FOR VIRUSES

G. W. A. DICK AND R. M. TAYLOR[1]

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To the investigator working with viruses the question of the suitability of a given diluent for use with these agents is an important one, whether he is engaged in laboratory studies of known active agents or in field work in which attempts are being made to isolate viruses from human, animal or insect sources. The diluent most commonly used hitherto is one prepared with serum.

The stabilizing action of serum on viruses has been studied by various workers, including Bauer and Mahaffy (1), Brodie (2) and Cook and Hudson (3), Zinsser and Tang (4), and Goodpasture and Buddingh (5), who demonstrated the effect of serum on the yellow fever, St. Louis encephalitis, herpes and vaccinia viruses, respectively. For the dilution of the virus of yellow fever it has been the custom to use 10 per cent normal monkey or human serum in physiological saline solution. Animal sera in various concentrations in saline or buffer solution have been used as diluents for other viruses; for example, heat-inactivated horse serum (2–5 per cent) for influenza virus and 10 per cent normal rabbit serum for the encephalitides viruses.

The use of serum in diluents is not entirely satisfactory because of the presence of nonspecific virus-inactivating substances in small amounts in animal and human sera (6, 7, 8, 9). For example, it has been observed that a consistently higher titer of yellow fever virus is obtained if dilutions are made in 10 per cent normal monkey serum than if 10 per cent normal human serum is used as a diluent. Moreover, the titer of the virus may vary with different pools of either normal monkey or normal human serum. Also of importance are the observations that presumably normal sera may develop antiviral properties after storage (9, 11). Because of these hazards the selection of the sera best suited for diluents is not uncommonly a tedious procedure. The virus under study may have to be titrated in diluents containing several different sera before a satisfactory serum is found. Furthermore, in field work involving the isolation of viruses from human, animal or insect it has to be assumed that the serum employed in the diluent contains no antibody for the unknown infectious agents.

Various diluents dispensing with serum have been employed. For influenza virus, broth containing buffers has been recommended (12). Buffered gelatin-saline and nutrient broth have been used as diluents for herpes simplex virus (13). Duffy and Stanley (14) demonstrated that autoclaved 10 per cent skim milk in saline solution could be substituted for rabbit serum in titrations and neutralization tests of Japanese B encephalitis virus.

[1] During the period when these studies were made, GWAD was in receipt of a Rockefeller Foundation Fellowship and on leave of absence from the Yellow Fever Research Institute, Entebbe, Uganda.

Little is known of the kinetics of the inactivation of viruses in suspensions. It has been suggested by Adams (15) that virus particles become inactivated when they reach a gas-liquid interface and that the loss of infectivity may be prevented by saturating the gas-liquid interface with another protein, thereby preventing virus access to the surface. This would appear to be a rational explanation for the use of stabilizing protein substances in the diluents employed in virus tests.

The ideal diluent should (a) contain the best available protein-stabilizing substance which will prevent the denaturation of viruses when the viruses are present in high dilution. In addition, it should (b) have no specific or nonspecific virus-inactivating substances, (c) be readily sterilizable, (d) have a constant composition so that it may be employed in comparative tests, (e) be readily available and simply prepared, (f) contain no suspended particles and (g) form a clear solution.

This paper presents the results of some investigations on bovine plasma albumin in buffered saline solution, which suggest that it may fulfill the requirements of an ideal diluent for influenza and yellow fever viruses. Experiments in which this diluent was used for other viruses (Mengo encephalomyelitis virus and the Lansing strain of poliomyelitis virus) have been limited, but the results have been sufficiently promising to warrant its further trial in other laboratories.

MATERIALS AND METHODS

Preparation of diluents. All diluents, except broth, were made in buffered saline solution (pH 7.4 to 7.8) prepared according to the formula of Mason and Sanford (16).[2] In practice, 13.6 gm Na_2HPO_4[3] and 2.0 gm KH_2PO_4 were dissolved in separate 100 ml quantities of distilled water. Ten milliliters of each of these solutions were added to 980 ml of distilled water containing 7.0 gm NaCl. The bovine-albumin diluent was prepared by dissolving 2.0 gm of crystallized bovine plasma albumin (Armour) in 1 liter of the buffered saline solution; this solution was then passed through a Seitz-E. K. pad. The serum-saline diluents contained heat-inactivated normal monkey or horse serum. Bacto-gelatin (Difco) was used for the gelatin-saline diluent, and the gum arabic employed was acacia, U. S. P. white powder. The nutrient broth was buffered at pH 7.4.

Influenza virus. The PR8 strain of influenza virus in allantoic fluid, stored in glass ampoules at −70 C, was employed. The eggs used were of White Leghorn stock and were inoculated after 10 to 11 days' incubation at 38 C. The inoculum was 0.2 ml per egg. Groups of 4 eggs served, as a rule, to test each dilution. After inoculation, eggs were incubated at 35 C for 3 days, and the allantoic fluids were harvested in the usual manner. Agglutination tests were made by adding 1 ml of 0.5 per cent chick red cells to 1 ml of allantoic fluids. The mixtures were allowed to stand 40 to 60 minutes at 4 C and then examined for agglutination.

Yellow fever virus. The French neurotropic strain of yellow fever virus was used;

[2] NaCl—7.0 gm
Na_2HPO_4, $2H_2O$—1.7 gm
KH_2PO_4—0.2 gm.

[3] 1 gm Na_2HPO_4, $2H_2O$ = 0.7977 gm Na_2HPO_4.

the stock virus was lyophilized and stored at −70 C in glass ampoules. Swiss mice of the Rockefeller Institute stock were inoculated intracerebrally with 0.03 ml of the various suspensions of the virus tested, and were observed for 18 days.

Other viruses. The UR 22 strain of Mengo encephalomyelitis virus (17) and the Lansing strain of poliomyelitis virus were studied.

Shaking machine. A shaking machine was used in the experiments. This had a horizontal reciprocating motion of 316 cycles per minute, and the distance traversed by the carriage was 7 cm. It was fitted with chemically clean and sterile Neutraglas bottles of 35 ml capacity which stood vertically in compartments in the machine and were closed with rubber stoppers.

EXPERIMENTAL

In preliminary experiments influenza virus of the PR8 strain was titrated, in dilutions of 10^{-1} to 10^{-3} in 2 per cent normal horse serum (NHS) diluent. Serial dilutions from 10^{-4} to 10^{-9} were made in either the bovine-albumin or 2 per cent NHS diluent. Eggs were inoculated with virus suspensions in each diluent (a) immediately and (b) after standing at room temperature or in the water bath (37 C) for various periods of time. Similar types of experiments were made with yellow fever virus to compare the titers obtained with bovine albumin and with 10 per cent normal monkey serum in saline solution (NMS). In neutralization tests with Mengo encephalomyelits virus and the Lansing strain of poliomyelitis virus, comparisons were made of the LD_{50} titer obtained when 10 per cent NMS and when bovine albumin were used in the diluent.

From all these preliminary experiments it was found that the 0.2 per cent bovine-albumin diluent was as effective as the serum diluents for influenza and yellow fever viruses. Moreover, no evidence was obtained to indicate that bovine albumin contained specific or nonspecific neutralizing substances for influenza, yellow fever or Mengo encephalomyelitis viruses or for the Lansing strain of poliomyelitis virus.

Shaking experiments. The inactivating effect of shaking on eastern and western equine encephalomyelitis viruses in buffered saline suspensions was reported by McLimans (18). Adams (15) studied the kinetics of the inactivation, by shaking, of bacterial viruses of the T group active against *E. coli.* The fact that this kind of inactivation can be prevented by the presence of proteins in the diluent was pointed out by Adams in his studies on bacteriophage.

The stabilizing action of various proteins on influenza and yellow fever virus was studied in experiments in which varying amounts of these viruses in various diluents were subjected to shaking. This provided a severe test of the stabilizing action of the diluents.

In initial screening experiments, dilutions of PR8 virus from 10^{-1} to 10^{-5} were made in one diluent; 10^{-6} dilutions of the virus were made in the various diluents being tested, by adding 1 ml of the 10^{-5} dilution to 9 ml of the diluent in Neutraglas bottles. Samples were taken from the bottles at various time intervals and tested for infectivity by inoculation of eggs. In this manner the following diluents were tested: 2 per cent NHS; 0.1 per cent and 0.2 per cent bovine albuinm; 0.01,

0.1, 0.2, 0.5 per cent gelatin; 1 and 5 per cent gum arabic, and nutrient broth. It was found that after 28 hours of shaking, active virus was still present in the 0.2 per cent bovine albumin and in the 2 per cent NHS diluents, but not in the other virus suspensions. With the broth and 0.5 per cent gelatin diluents, virus was demonstrable after 18 hours of shaking, but not after 24 hours of shaking. With the gum arabic diluent, virus was present in the 12-hour, but not in the 18-hour, sample. The virus in the 0.01 to 0.1 per cent gelatin-diluent samples had been inactivated by the 6th hour of shaking, and PR8 virus in buffered saline solution was no longer demonstrable after 4 hours.

It appeared that 0.2 per cent bovine albumin and 2 per cent NHS were equally effective in protecting a 10^{-6} dilution of PR8 virus from inactivation and that the two diluents were better than those containing broth, gelatin, or gum arabic.

Further experiments were made in which not only 10^{-6} dilutions of virus but also 10^{-6} to 10^{-9} dilutions in various diluents were shaken for 24 hours. The 10^{-1} to 10^{-5} dilutions of virus were made in one diluent as before, and 10^{-6} to 10^{-9} dilutions were made serially by adding 0.5 ml to 4.5 ml of the diluents under test. The results of 2 such experiments are presented in table 1.

It is seen that in these shaking tests the PR8 virus retained higher ID_{50} titers in the diluent which contained 0.2 per cent bovine albumin than in any of the other diluents tested, except that containing 10 per cent NHS.

A series of similar experiments was made with yellow fever virus. This virus was reconstituted in distilled water, with which 10^{-1} to 10^{-3} dilutions were made. The 10^{-4} and higher dilutions were prepared as in the experiments with PR8 virus. After the preparations had been shaken for 17 hours, each was inoculated into a group of mice. One hundred per cent mortality occurred in groups inoculated with the virus in the 0.2 per cent bovine-albumin diluent; the mortality ratios in groups inoculated with the 1 per cent and 0.5 per cent gelatin diluents were $\frac{2}{5}$ and $\frac{4}{5}$, respectively, and the ratio in mice inoculated with the broth was $\frac{1}{5}$. After 21 hours of shaking, yellow fever virus could still be demonstrated in a 10^{-4} dilution in 10 per cent NMS and in the 5 per cent gum arabic diluent, but not in the 0.2 per cent bovine-albumin suspension.

Accordingly, these 5 diluents were tested for their stabilizing action on virus in higher dilutions: 10^{-1} to 10^{-3} dilutions of yellow fever virus were made in distilled water and 10^{-4} to 10^{-6} dilutions in Neutraglas bottles, following the method used in the experiments with influenza virus. The suspensions were shaken for 24 hours at room temperature and then tested for the presence of virus. The results of one of these experiments are presented in table 2.

It may be seen that the virus showed a slightly higher titer in diluent containing 10 per cent NMS than in diluent containing 0.2 per cent bovine albumin, which in turn held the titer better than the other diluents tested.

Efforts to sterilize bovine albumin by heat were not successful. A neutral solution begins to precipitate when the temperature reaches 68 to 70 C, and while a solution at pH 5 may be heated to 100 C without precipitation, precipitation occurs when the pH is brought to the neutral point. Heating in the dry state gave inconsistent results. In some experiments solution was obtained after heating the

bovine albumin crystals in an oven for one hour at 120 C, but as a rule complete solution was not obtainable following heating at this temperature.

Thus, as with serum, filtration must be relied upon for bacterial sterilization. Naturally, this procedure may not suffice for removing a virus contaminant, but

TABLE 1

Results of shaking dilutions of the PR8 strain of influenza virus for 24 hours in various diluents

DILUENT	HOURS OF SHAKING	AGGLUTINATION RATIO* OF ALLANTOIC FLUIDS FROM EGGS INOCULATED WITH VIRUS AT DILUTION				RECIPROCAL OF LOG OF ID_{50}†	LOG UNITS INACTIVATED
		10^{-6}	10^{-7}	10^{-8}	10^{-9}		
0.2 per cent bovine albumin	0	4/4	4/4	4/4	1/4	8.7	
0.2 per cent bovine albumin	24	4/4	4/4	0/4	—	7.5	1.2
0.1 per cent bovine albumin	24	4/4	3/4	0/4	—	7.3	1.4
5 per cent gum arabic	24	4/4	1/4	0/4	—	6.7	2.0
1 per cent gelatin	24	4/4	0/4	0/4	—	6.5	2.2
2 per cent normal horse serum	24	3/4	0/4	0/4	—	6.3	2.4
0.2 per cent bovine albumin	0	—	—	3/4	2/4	9.0	
10 per cent normal horse serum	24	4/4	3/4	1/4	—	7.5	1.5
0.2 per cent bovine albumin	24	4/4	2/4	0/4	—	7.0	2.0
2 per cent normal horse serum	24	3/4	1/2	0/4		6.6	2.4

* Denominator indicates number of eggs inoculated, the numerator the number of eggs with infected allantoic fluids.

† Calculated by the method of Reed and Muench (19).

TABLE 2

Results of shaking dilutions of yellow fever virus for 24 hours in various diluents

DILUENT	MORTALITY RATIO OF MICE INOCULATED WITH VIRUS AT DILUTION			RECIPROCAL OF LD_{50}
	10^{-4}	10^{-5}	10^{-6}	
10 per cent normal monkey serum	5/6	5/6	0/6	5.3
0.2 per cent bovine albumin	4/6	1/6	1/6	4.5
0.1 per cent bovine albumin	2/6	1/6	0/6	≦3.9
5 per cent gum arabic	2/6	1/6	0/6	≦3.9
1 per cent gelatin	0/6	0/6	0/6	≦3.5
0.5 per cent gelatin	0/6	0/6	0/6	≦3.5

≦ Indicates equal to or less than.

See footnotes, table 1, for explanatory remarks.

the likelihood of bovine albumin containing an unknown virus is certainly no greater than is the case with serum. Filtered bovine albumin solution prepared from several different samples has been used during the last 10 months for routine passage and titration of viruses in embryonated eggs and in animals without encountering a virus contaminant.

DISCUSSION

From the results of experiments in which virus was shaken in different diluents, it would appear that the diluents containing 10 per cent normal monkey serum or normal horse serum from carefully selected sera hold the titers of yellow fever and influenza viruses, respectively, slightly better than do diluents containing 0.2 per cent bovine albumin. In comparative titrations, when the suspensions were left for 2 hours at room and water bath (37 C) temperatures, 0.2 per cent bovine albumin was as effective as 10 per cent normal serum. Bovine albumin has the advantage in that, as far as is known, it contains no specific nor nonspecific virus inactivating substances, is of standard composition, and forms readily a clear solution.

Two per cent NHS contains 0.14 per cent protein (based on the usual content of mammalian sera of 7 per cent protein); 0.2 per cent bovine albumin solution was estimated to contain 0.19 per cent protein. If, as Adams (15) suggests, the virus particles are protected in shaking experiments by saturation of the gas-liquid interface by the protein of the diluent, it seems rational to expect that by increasing the protein content of the diluent, the stabilizing action of the diluent would be increased. This is confirmed by the fact that 0.2 per cent bovine-albumin diluent (protein, 0.19 per cent) proved to be more effective than 2 per cent NHS (protein, 0.14 per cent) but not as adequate as 10 per cent normal sera (protein, 0.7 per cent) if the virus dilutions were shaken for 24 hours.

The bovine-albumin diluent was employed as a 0.2 per cent solution primarily for comparison with 2 per cent NHS, and also because of the present high cost of bovine albumin. In spite of its cost, it seems to meet the demands of an effective and satisfactory diluent for viruses and merits further investigations with other viruses. Several subsequent experiments not here recorded indicate that the cheaper bovine albumin powder fraction V is as effective as the more expensive crystallized product.

SUMMARY

Two-tenths per cent bovine albumin in buffered saline solution has proved to be a highly satisfactory diluent for influenza and yellow fever viruses, having many advantages over serum. Chief among these are that it is easily prepared and, as far as is known, contains neither specific nor nonspecific virus-inactivating substances.

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