

pH Stability and Purification of Lumpy Skin Disease Virus

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SUMMARY: Lumpy skin disease virus in allantoic fluid was found to be stable when subjected to wide variation of hydrogen-ion concentration under differing conditions of time and temperature. The virus could be concentrated by adsorption on the precipitate formed when allantoic fluid was dialysed at pH 4.5; adsorption on calcium phosphate was also demonstrated. Preliminary purification of the virus could be effected by these methods.

The isolation of a filtrable agent from a calf dead of lumpy skin disease (LSD) was reported by van den Ende, Don & Kipps (1949). In a later paper van den Ende & Turner (1950) described a method of titrating the virus and applied it in neutralization tests and in the determination of certain physical properties. The data reported in this communication are from continued observations on the properties and behaviour of the virus.

MATERIAL AND METHODS

Virus suspensions. These consisted of allantoic fluids from Leghorn chicken eggs inoculated on the 9th day of incubation and harvested after 3-4 days of further incubation at 37°. The eggs were chilled before harvesting to obtain fluids free from blood. The pooled fluids were either used immediately or stored at 0-4° until they were required. Material stored over long periods was kept at -70°.

Buffer solutions. For the pH range 2.0-8.0 McIlvaine's standard 0.1M-citric acid + 0.2M-disodium phosphate mixtures were used. For pH 10.0, 0.1M-glycine or 0.1M-disodium phosphate adjusted with 0.1M-sodium hydroxide was used. For pH 12.0, 0.1M-disodium phosphate was similarly adjusted with 0.1M-sodium hydroxide. pH 1.0 'buffer' consisted of 0.1M-hydrochloric acid.

Diluent. Dilutions for virus titrations were made in nutrient broth of pH 7.0.

Virus titrations. Tenfold serial dilutions were made in broth and 0.2 ml. amounts of the dilutions were inoculated sub-allantoically into 9-day Leghorn chick embryos. At least five eggs were used for each dilution. The eggs were 'candled' daily for 10 days and times of death recorded. Deaths which occurred on the 1st day after inoculation were regarded as being non-specific. LD₅₀ end-points were calculated by the method of Reed & Muench (1938). All inocula were tested for bacterial sterility on blood agar slopes. All solutions were prepared in distilled water and sterilized by filtration through Seitz pads where necessary. The pH values of buffer solutions were checked by glass

electrode measurements. Penicillin and streptomycin were added in manipulations where a high probability of bacterial contamination was considered likely.

RESULTS

Stability at different pH values. Volumes (10 ml.) of buffer solutions of pH values 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 were tubed in duplicate. To each tube was added 1.0 ml. of virus-containing allantoic fluid. The tubes were then held at 37° in a water-bath or in the refrigerator at 0–4° for various times. When the time periods had elapsed one series of tubes was used for a determination of pH value and the individual tubes of the other series were immediately adjusted to pH 7.0. Disodium phosphate or citric acid were used for

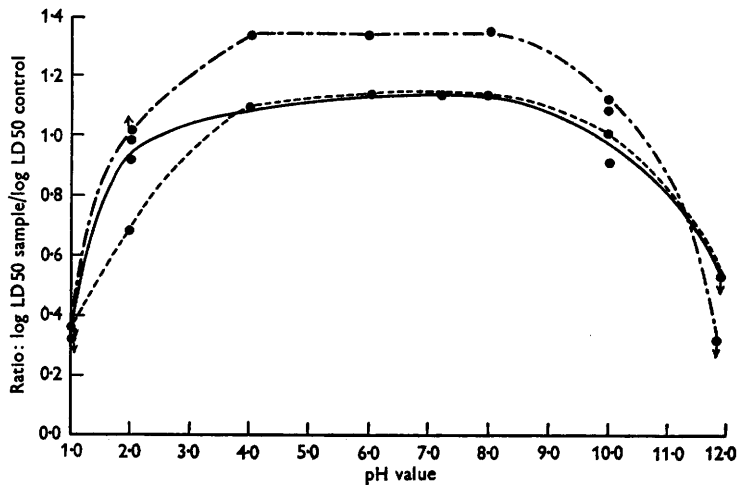


Fig. 1. Graph showing the stability of lumpy skin disease virus exposed to buffers of various pH values. — — —, 14 days at 0–4°; - · - · -, 4 hr. at 37°; —, 1 hr. at 37°.

neutralization of the pH 2.0–8.0 series and 0.1M-sodium hydroxide or 0.1M-hydrochloric acid for the remainder. The volume of each mixture was brought to 100 ml. with pH 7.0 buffer, thus giving a dilution of 1/100 with respect to the initial amount of virus added. Further tenfold dilutions were made from each of these samples and titrated in eggs. A control titration of untreated starting material was included with each experiment. The pH value of this initial allantoic fluid was 7.6. The pH values of the mixtures showed little variation during the time of the exposure except that the pH values of the samples at the extremes of the range had altered. The sample originally at pH 10 had fallen to pH 8.9 and that at pH 12.0 to 11.8; the samples at pH 1.0 and 2.0 originally, increased to 1.2 and 2.3 respectively.

The results have been recorded graphically in Fig. 1 and are expressed as ratios of \log_{10} LD₅₀ titre of the exposed sample to \log_{10} LD₅₀ titre of the untreated control. It will be seen that the virus infectivity remained relatively stable when it was subjected to wide variations in pH value of the suspending

fluid. For 1 hr. at 37° and 14 days at 0–4° the virus was stable at values from pH 2.0 to 10.0. Diminution of activity was apparent at pH 2.0 after 4 hr. exposure at 37°, and there was rapid inactivation at pH's 1.0 and 11.8 under all the tested conditions of time and temperature. It is of interest that over the range at which the virus remains stable the samples held diluted in buffer had higher titres than the control material.

Precipitation of the virus with a protein fraction of allantoic fluid

It was noted during the pH experiments that a precipitate occurred when allantoic fluid was exposed to a hydrogen-ion concentration of pH 4.0. Both infected and normal allantoic fluid behaved similarly in this respect, although the precipitate appeared to be heavier in the infected allantoic fluid. In preliminary experiments allantoic fluid was adjusted to *c.* pH 4.8 by the direct addition of 0.1 N-HCl. The bright yellow precipitate was redissolved in its original volume of 0.07 M-Na₂HPO₄ buffer at pH 8.2 and had a higher virus content than its supernatant fluid. The nature of the precipitate was examined electrophoretically. Samples of infected allantoic fluid (50 ml.) and of normal allantoic fluid (50 ml.) harvested from eggs of the same age were dialysed in viscose sausage casing bags against 2 l. citric phosphate buffer (pH 4.0) at 0–4° for 48 hr. This provided a mild means of changing the pH value of the fluids; the precipitates obtained were possibly heavier. The precipitates were recovered by centrifugation and each was redissolved in 7.5 ml. of pH 8.2 0.07 M-phosphate buffer. The solutions were dialysed overnight against 0.013 M-phosphate buffer (pH 8.2) containing 0.044 M-NaCl. After centrifugation at 10,000 r.p.m. for 15 min. the solutions remained slightly opalescent. They were then examined electrophoretically using a double cell (Polson, 1952) under a potential gradient of 5 V./cm. The material from the infected allantoic fluid showed no evidence of inhomogeneity and migrated as one boundary. It contained approximately 10 times as much protein as the material from normal fluid. Tryptic digestion followed by ultracentrifugation in both cases yielded surface layers of lipid, indicating that the precipitates were lipo-protein in nature. Later experiments, however, showed that lipid was neither a constant nor necessary factor in 'virus precipitation'.

The preliminary experiment quoted above indicated that protein precipitates might carry with them a large proportion of the virus content of infected allantoic fluid and could be utilized in the concentration and purification of the virus.

The pH value for optimum virus precipitation

Experiments were set up to determine the pH value at which maximal precipitation of virus activity occurred. Volumes (20 ml.) of infected allantoic fluid in viscose sausage casing were dialysed for 2 days at 0–4° against 1 l. volumes of McIlvaine's citric phosphate buffer at pH values 3.2, 3.6, 4.0 and 4.8. The precipitates obtained were recovered by centrifuging at 3000 r.p.m. for 30 min., and dissolved in 20 ml. (the original volume) of 0.07 M-phosphate buffer pH 8.2. These solutions, together with their respective supernatant

fluids, were then titrated in eggs. Controls to these titrations consisted of the original untreated allantoic fluid. It will be seen from Fig. 2 that over the pH range 3.2–4.4 there was approximately 100 times more virus in the precipitates than in their respective supernatants and an optimum is indicated between pH 4.0 and 4.4. At pH 4.8 only scanty precipitation occurred and, as the figure shows, almost identical titres were obtained for resuspended precipitate and supernatant fluid. The titres of precipitates obtained at pH 3.2 were still high, but in this region and below, pH values were approached at which the virus becomes unstable.

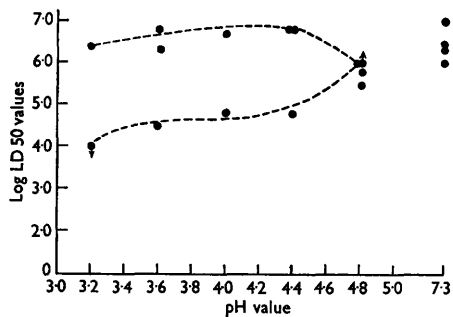


Fig. 2

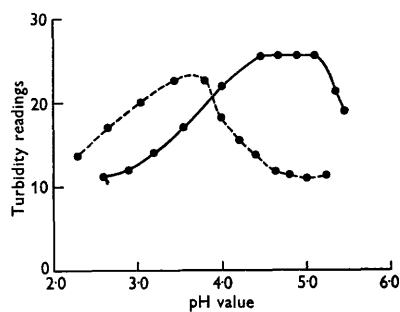


Fig. 3

Fig. 2. Titres (expressed in neg. log. LD 50) of the precipitates and supernatant fluid obtained from allantoic fluid dialysed against buffers of various pH values. Top curve = virus titres in precipitates. Bottom curve = virus titres in corresponding supernatant fluids. The values at pH 7.3 are those of the original untreated allantoic fluids.

Fig. 3. Turbidity readings for normal allantoic fluid diluted in McIlvaine's buffer at different pH values. Broken curve with standard buffer concentration; solid curve with buffer diluted 1/5 in distilled water.

Decrease of the salt concentration of the buffers used in the precipitation of the virus resulted in the production of heavier precipitates. In a preliminary experiment McIlvaine's buffer mixtures in 10 ml. volumes were prepared to cover a range from approximately pH 2.0 to 5.5, one series having the normal salt concentration and the other one-fifth of this amount. Samples (0.25 ml.) of infected allantoic fluid were added to each tube and the turbidities compared in a Klett colorimeter before coagulation had occurred. Water was used as a standard. Absolute values of optical density or light transmission were not calculated but colorimeter readings were directly plotted against pH values. Fig. 3 shows that with more dilute buffer a heavier precipitate was obtained and that the pH range at which optimal precipitation occurred was both extended and shifted to a slightly more alkaline level. Subsequent experiments were done with precipitates obtained by dialysis of allantoic fluid against McIlvaine's citric phosphate mixtures diluted 1/5 and having a pH value of 4.5.

It had been hoped that some purification could be effected by preferentially precipitating contaminating protein at one salt concentration and virus at another. Virus activity accompanied the heaviest precipitate and it seemed

probable that the precise nature of the protein precipitated was not a decisive factor in the 'precipitation' of the virus, the bulky protein precipitates behaving as adsorbents and carrying the virus with them. Confirmation of this was obtained in an experiment where the virus from 300 ml. infected allantoic fluid was concentrated by precipitation and partially purified by ether extraction and ultracentrifugation. When a protein solution, obtained from normal allantoic fluid, was added to this material it yielded on dialysis at pH 4.5 a precipitate which again carried with it the bulk of the virus. Some evidence was obtained that calcium phosphate could act as an adsorbent for LSD virus. In a typical experiment calcium phosphate was precipitated in the presence of allantoic fluid after the method described by Herbert & Todd (1941). Samples (4.0 ml.) of infected allantoic fluid were each mixed with 4.5 ml. 0.066 M-disodium hydrogen phosphate followed by 0.36 ml. of 1.0 M-calcium acetate. Precipitation was allowed to occur at room temperature for 20 min. The precipitates were collected by centrifuging and either immediately resuspended in broth to the original volume (4.0 ml.) or washed twice in 5.0 ml. saline and then resuspended. The washed and unwashed precipitates after resuspension together with the supernatant fluid were titrated in eggs. These results are recorded in Table 1.

Table 1. *Titration showing adsorption of lumpy skin disease virus on calcium phosphate precipitate*

Sample	Dilution of sample					LD 50
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
	Eggs dead/eggs inoculated					
Initial allantoic fluid	—	—	—	8/8	—	> 10 ^{5.0}
Supernatant after precipitation	7/12	—	1/10	—	1/11	10 ^{2.5}
Resuspended precipitate	11/12	—	10/12	—	4/12	10 ^{5.2}
Resuspended precipitate twice washed	5/5	—	5/6	—	1/6	10 ^{5.0}
Control calcium phosphate precipitate	2/8	—	—	—	—	< 10 ^{2.0}

Subsequent removal of the protein from redissolved virus-protein precipitates was attempted in various ways.

Tryptic digestion. Preliminary experiments indicated that the virus was stable when treated with crystalline trypsin in a concentration of 0.033% for periods up to 150 min. In a typical experiment 700 ml. infected allantoic fluid was dialysed against two changes of 10 l. of pH 4.5 McIlvaine buffer diluted 1/5 with distilled water. After 48 hr. the very heavy precipitate within the cellophan was collected by centrifuging at 2000 r.p.m. for 1 hr. This precipitate was dissolved in 35 ml. 0.2 M-phosphate buffer (pH 8.2). This solution was extracted with an equal volume of ether at 0° and centrifuged for 1 hr. at 2000 r.p.m. in a refrigerated centrifuge. No lipo-protein precipitate was present at the ether/water interface after centrifuging but the ether layer was yellow in colour. The ether layer was removed. A deposit of some crystalline material below the aqueous layer was extracted with 5 ml. saline and added to the aqueous layer. The ether extraction was repeated and residual dissolved

ether removed from the aqueous phase by evaporation at a water pump. The aqueous phase was then centrifuged at 20,000 r.p.m. for 15 min. in a Spinco Model L preparative centrifuge and the deposit discarded. The supernatant aqueous solution was then again centrifuged, this time at 30,000 r.p.m. for 2 hr. The pellet so obtained was dispersed in 20 ml. 0.07M-phosphate buffer and treated with 0.002 g. of crystalline trypsin for 1 hr. at 37°. The digested material was centrifuged at 30,000 r.p.m. for 2 hr. and yielded a pellet of volume 6–7 mm³. which was redispersed in 1 ml. 0.07M-buffer at pH 8.2. Samples of the original allantoic fluid and the final concentrate were titrated in eggs. The titres (LD 50) of the original and concentrated material were 5.6 and 8.2 respectively, indicating a concentration of approximately 400 times, i.e. 40% of the total infective virus was recovered.

Chloroform extraction of the virus-protein precipitate. The precipitate obtained from 100 ml. infected allantoic fluid dialysed at pH 4.5 as described previously was redissolved in 15 ml. of 0.07M-phosphate buffer (pH 8.2). The solution was mechanically shaken for 7 min. in an ice bath with an equal volume of chloroform containing 5.0% (w/v) amyl alcohol. The mixture was centrifuged for 15 min. at 3000 r.p.m. in a refrigerated centrifuge and the chloroform together with the heavy precipitate at the chloroform/aqueous interface discarded. The chloroform extraction was repeated, giving a total time of contact of 30 min. A second layer of precipitate at the liquid/liquid interface was discarded after centrifuging in the same way.

The aqueous layer was freed from chloroform under reduced pressure. Some odour of amyl alcohol was still apparent in the water-clear fluid, which gave no precipitate when tested with trichloroacetic acid. The fluid made up to its original volume (100 ml.), as well as a sample of the original allantoic fluid, was titrated in eggs. The log. LD 50 titres of original and chloroform-extracted material were 6.3 and 5.7 respectively. While there was therefore a decrease in titre the removal of large amounts of protein justified the use of chloroform in further experiments.

In another experiment 550 ml. virus-infected allantoic fluid were dialysed at pH 4.5 as before. The precipitate obtained was redissolved in 25 ml. 0.07M-phosphate buffer (pH 8.2) and centrifuged at 30,000 r.p.m. for 2 hr. The pellets were dispersed in 10 ml. pH 8.2 buffer and subjected to six successive extractions with chloroform + amyl alcohol mixture. The extractions were done at 0° and occupied a total time of 90 min. A water-clear protein-free fluid was obtained. A sample of this material was diluted to be comparable with the original allantoic fluid and titrated in eggs. The log. LD 50 titres of original and chloroform treated material were respectively 6.4 and <4.0. From these results it would appear that while chloroform was an admirable means of removing protein the drastic extractions described has a lethal effect on the virus or the virus remained adsorbed on the chloroform protein precipitate and was lost. Although a large part of the infectivity of the material was lost, on examination in the spectrophotometer it showed absorption in the range usually associated with nucleoprotein. No such absorption was observed when similarly processed material from normal allantoic fluid was examined.

DISCUSSION

The finding that lumpy skin disease virus is stable over a wide range of hydrogen-ion concentration provides information of use for further purification studies. Similar behaviour is exhibited by a number of other viruses. Under certain conditions of time and temperature, vaccinia (Beard, Finkelstein & Wyckoff, 1938), influenza (Stock & Francis, 1940), mumps (Weil, Beard, Sharp & Beard, 1948) and Lansing poliomyelitis virus (Bachrach & Schwerdt, 1952) all retained their infectivity when exposed to wide ranges of hydrogen-ion concentration. On the other hand, Alexander (1935) showed the extreme sensitivity of horsesickness virus which, while stable to a range of values above pH 5.96, underwent immediate inactivation when exposed to pH values even slightly less than this value.

Protein precipitation in the concentration of virus suspensions is not a novel process. Theiler & Gard (1940) with mouse encephalomyelitis showed that 90% of virus activity was found in the floccules which formed when mouse brain suspensions were adjusted to pH 4.5. The iso-electric precipitation of proteins from brain suspensions of virus has been utilized in concentrating strains of human poliomyelitis virus (Bourdillon & Moore, 1942; Herrarte & Francis, 1943; Bachrach & Schwerdt, 1952), the pH value for precipitation in each case being 4.6. It is significant that the proteins in normal allantoic fluid were precipitated at almost identical pH values and could be similarly utilized to concentrate the LSD virus.

Salk (1941) and Stanley (1945) used calcium phosphate adsorption techniques in the concentration of influenza virus. Similar procedures have been used here, and no claim is made for the selectivity of calcium phosphate as a specific adsorbent for LSD virus. Both precipitation procedures show that LSD virus shares a property of 'adsorbability' with other viruses, which can be utilized in effecting initial concentration.

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