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INVESTIGATIONS INTO THE pH STABILITY OF BLUETONGUE VIRUS AND ITS SURVIVAL IN MUTTON AND BEEF

N. C. OWEN, Veterinary Research Institute, Onderstepoort

INTRODUCTION

The pH stability range has been determined for several animal viruses and Hamparian, Hilleman & Ketler (1963) have used their lability at pH 3 as a means of classification.

The influence of pH changes on the survival of acid-sensitive viruses in meat is well known. Henderson & Brooksby (1948) investigated the survival of foot-and-mouth disease virus in carcass meat and offal and found it to be dependent upon the post-mortal pH changes. Swine fever virus is known to persist for up to 95 days in frozen carcasses and can exist at pH values varying from pH 4 to pH 11 (I.B.A.H.—O.I.E., 1960).

The changes in the pH of meat after slaughter have been well studied by Schönberg (1950), who found that the pH of meat from healthy, well nourished and rested animals attains a value of 5·6 to 6·0 within 24 hours. However, the meat of diseased, exhausted or otherwise abnormal carcasses does not acidify appreciably, immaterial of the storage time. In abattoir practice carcass meat with an ultimate pH of 6·5 or more is generally considered to be of lower keeping quality.

Although there was no experimental evidence available Haig (1960) suggested that bluetongue virus may persist in carcass meat and offal for long periods, on the grounds that this virus is highly resistant.

This report records experiments to determine the pH stability of bluetongue virus *in vitro* as well as it's survival in meat of animals infected experimentally.

I.—THE pH STABILITY OF BLUETONGUE VIRUS *in Vitro*

As the pH stability of bluetongue virus is of considerable practical importance and has not yet been investigated, experiments were designed to examine this particular characteristic. The purpose of this work was not to determine the entire stability range, but to investigate the influence of those hydrogen ion concentrations which are normally encountered in the meat of slaughtered animals.

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Materials and Methods

Virus strains

The prototypes of Groups I and III bluetongue virus strains (Howell 1960), adapted to suckling mice by the method of van der Ende, Linder & Kaschula (1954) were used. These strains are stored in the lyophilized state at —20°C, at the 10th passage level. They are known as mouse brain adapted (M.B.) type 1 and M.B. type 3 respectively.

Buffer

Sorenson's phosphate buffer consisting of $\frac{m}{15}$ KH_2PO_4 and $\frac{m}{15}$ Na_2HPO_4 was mixed in varying amounts to give a pH range of 5·3 to 8. The entire range of buffer solutions was then sterilized by autoclaving and the pH of each solution finally checked with a battery operated meter equipped with glass electrodes and thermal control.

Mice

Four-day old suckling mice, grouped in families of seven, were obtained from the Institute's in-bred colony of Swiss Albino mice.

Procedure

The virus strains were reconstituted with distilled water and inoculated intracerebrally into six families of suckling mice. On the third day, when the mice were *in extremis*, the brains were harvested and a 10 per cent (w/v) suspension prepared. in physiological saline. The brain suspensions were clarified by centrifugation at 2,000 rpm for 10 minutes. One ml of the supernatant fluid was added to 9 ml of each of the buffer solutions within the pH range, giving a dilution of 1: 10.

After 24 hours at the desired temperature the pH of the virus-buffer solutions was checked and serial decimal dilutions made in phosphate buffered lactose peptone* (B.L.P.) with a pH of 7·2. One family of suckling mice was injected intracerebrally (0·03 ml) with each of the dilutions. Fifty per cent end points were calculated by the method of Reed & Muench (1938).

Three experiments were conducted according to this procedure. In the first, using mouse brain type III virus, the virus-buffer mixtures were held at 4°C for 24 hours and titrated immediately thereafter. In the next two experiments mouse brain type I virus was examined at 4°C and 37°C respectively. In these instances, the pH of the solutions was restored to neutrality prior to titration by dializing each solution against a large volume of phosphate buffer $\left(\frac{m}{15}\right)$ at 4°C.

Experimental Results

The results obtained from the above-mentioned experiments have been presented graphically with the virus titre of each solution plotted against the pH recording. Virus titres have been expressed as the negative logarithm of the 50 per cent dilution end point.

* $\frac{m}{15}$ phosphate buffer pH 7·2, 1 per cent difco peptone and 5 per cent lactose

Experiment I.—The pH stability of type III bluetongue virus at 4°C

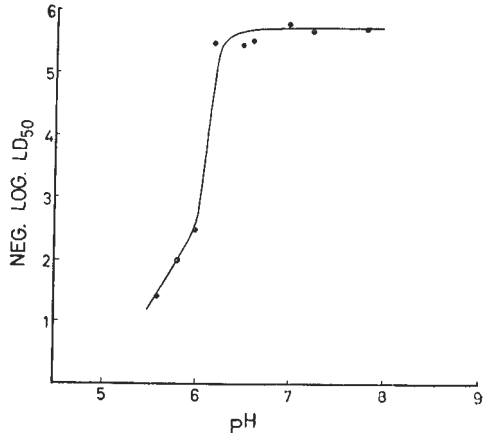

FIG. 1.—The influence of various hydrogen ion concentrations on the infectivity of mouse brain adapted type III bluetongue virus held for 24 hours at 4°C

The results presented in Fig. 1 clearly demonstrate a marked loss of infectivity between pH 6·0 and pH 6·2, with a stable region from pH 6·2 to pH 8·0.

Experiment II.—The pH stability of type I bluetongue virus at 4°C

FIG. 2.—The influence of various hydrogen ion concentrations on the infectivity of mouse brain adapted type I bluetongue virus held for 24 hours at 4°C

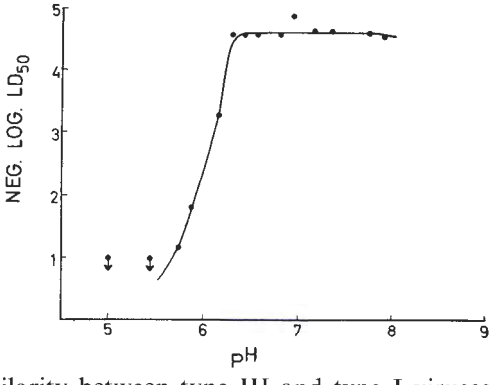

Figure 2 demonstrates the close similarity between type III and type I viruses. The latter shows a stable region between pH 6·3 and pH 8·0 while its infectivity is almost completely lost between pH 6·0 and 6·3.

Experiment III.—The pH stability of type I bluetongue virus at 37°C

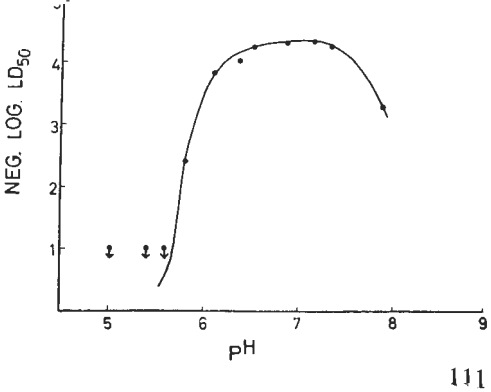

FIG. 3.—The influence of various hydrogen ion concentrations on the infectivity of mouse brain adapted type I bluetongue virus held for 24 hours at 37°C

Although the drop in infectivity below pH 6·2 is rapid, it appears to be more gradual at 37°C than was found at 4°C. In this instance the virus infectivity remained stable between pH 6·2 and pH 7·5, but dropped significantly at pH 8·0. It therefore appears that the pH stability is influenced by the storage temperature.

Although the initial virus titre and the rate of inactivation have not been taken into account in these experiments, all the results obtained demonstrate a dramatic loss of infectivity between pH 6·0 and pH 6·3 within 24 hours. These preliminary results stress the importance of working under controlled pH conditions in any experimental investigations with bluetongue virus.

II.—The Survival of Bluetongue Virus in Carcass Meat

The following experiments were conducted to investigate the survival of a strain of bluetongue virus in the carcass meat of experimentally infected animals.

Materials and Methods

Virus strain

Infective blood in oxalate-carbol-glycerine solution* (O.C.G.), representing the original virulent Cyprus strain of bluetongue virus (Neitz, 1948) was used. This virus has since been classified as Group III by Howell (1960).

Sheep

Bluetongue susceptible Merino sheep kept under insect-free conditions throughout, were used.

pH determination

The Schönberg Nitrazine Yellow colorimetric method as described by van den Heever (1959), was used to determine the pH of the meat specimens. All recordings were controlled with a battery operated pH meter.

Procedure

Two sheep (No. 12543 and 12592) were injected intravenously with 2 ml of virus each, while a bovine (crossbred Afrikaner, No. 624), reared under insect-free conditions received 5 ml of virus. The animals were carefully observed and their rectal temperatures recorded daily. All three animals were slaughtered, at a predetermined stage after infection, under routine abattoir conditions and after the onset of rigor mortis the carcasses were stored at 4°C. At suitably spaced intervals meat samples of approximately one square inch were taken from the M. gluteus and the M. longissimus dorsi after surface sterilization with a heated spatula. Particular care was taken to ensure that all specimens were collected and prepared separately. The pH of each specimen was recorded immediately after excision.

* 500 ml glycerine; 5 ml carbolic acid; 5 gm potassium oxalate and 500 ml water

It was then ground up with sterile alundum in 10 ml of B.L.P. and clarified by light centrifugation. The supernatant fluid from each specimen was injected intravenously into a susceptible sheep, the dose being 2 ml. The recipient sheep were observed daily and the clinical symptoms and rectal temperatures recorded. Reacting sheep were bled into O.C.G. during the febrile phase of the disease. Virus isolations were made in embryonated hen's eggs and thereafter propagated in lamb kidney cell cultures (Alexander, 1947; Haig, McKercher & Alexander, 1956). All sheep were bled for serum prior to infection and all surviving sheep were bled 35 days thereafter for convalescent phase serum. The sera were stored at 4°C until they were subjected to serum-virus neutralization tests to determine whether antibodies had developed to the strain of virus originally injected.

Neutralization tests

All virus isolations from reacting sheep were identified by using a screen neutralization test as described by Howell (1960). The identity of these virus isolations was based on complete neutralization in those cultures containing type specific antiserum at the time when the tubes containing the negative control serum showed complete cell destruction.

The specificity of the febrile reactions of the surviving sheep was confirmed by determining the neutralizing indices of the paired serum samples. Undiluted serum was mixed with falling 10-fold dilutions of tissue culture adapted type III virus. After contact for one hour at 4°C, two lamb kidney cell roller tubes were seeded with 0·2 ml of each serum virus mixture. The tubes were observed daily for specific cytopathic changes and the difference of the 50 per cent end points experssed as the neutralizing index.

Experimental Results

The Stability of Bluetongue Virus in Mutton

Sheep No. 12543: Sheep 12543 underwent a very severe bluetongue reaction. After an incubation period of three days, the body temperature rose to 105·6°F and remained above 104°F for four consecutive days. The sheep was slaughtered on the eighth day when hypothermia had set in and the animal was moribund. Ante-mortal examination revealed mild coronitis, buccal erosions, petechial haemorrhages in the mucous membranes of the mouth and tongue, lingual cyanosis, respiratory distress and a mucoid nasal discharge. On post-mortal examination there was a slight tumour hepatis, hyperaemia and oedema of the lungs with a pinkish discoloration of the fat and connective tissues due to engorgement of the capillaries. These pathological findings were of such a slight nature that a casual inspection, without ante-mortal examination, could have allowed the carcass to pass as fit for consumption.

The results of the examination of the meat specimens taken from this carcass are recorded in Table 1.

TABLE 1.—*Particulars of meat specimens taken from the carcass of sheep* 12543

Number of days after slaughter	Recipient sheep number	Specimen	pH of specimen	Reactions in recipient sheep				Virus isolation
				Incubation period in days	Duration of febrile reaction in days	Coronitis	Outcome	
0	12587	L	6·9	5	2	+	Died	+
	12556	G		4	3	—	Died	+
3	12594	L	6·4	3	5	—	Died	+
	12590	G		3	5	—	Died	+
6	12616	L	6·4	3	4	+	Died	—
	12584	G		2	4	+	Surv.	+
9	12608	L	6·4	4	6	+	Died	+
	12544	G		2	7	+	Died	+
12	12562	L	6·3	3	5	+	Died*	—
	12674	G		5	3	—	Died	+
16	12651	L	6·4	2	3	—	Died	+
	12613	G		7	3	—	Died	+
19	12567	L	6·5	3	5	+	Died	—
	12546	G		3	3	+	Died	+
30	12684	L	6·5	4	8	+	Surv.	+
	12574	G		7	2	+	Surv.	—

NOTE:—
* Besides the recorded symptoms, this sheep had a marked torticollis before it died.
L Represents specimens taken from the M. longissimus dorsi.
G Represents specimens taken from the M. gluteus.

The reactions occurring in all the test sheep were typical of bluetongue. In those instances where no coronitis has been recorded, the sheep died before any foot lesions could develop. In most cases it has been possible to isolate the virus and confirm the identity of the strain by neutralization with type III (Cyprus) antiserum. The technique of virus isolation is, however, not entirely satisfactory and in the few cases where no virus could be isolated, a positive diagnosis was based on the typical symptoms of bluetongue encountered.

The results in Table 1 show that in this sheep, slaughtered at a late stage of the disease, the pH failed to drop below 6·3 and the presence of infective bluetongue virus could be demonstrated for 30 days after slaughter. Although the virus could not be determined quantitatively, sufficient infective virus was still present on the 30th day to cause severe clinical bluetongue in the recipient sheep. The pH changes in the meat of the carcass were typical of a " fevered " carcass where insufficient lactic acid is released due to depletion of the glycogen reserves.

Sheep No. 12592: Sheep No. 12592 had an incubation period of six days. On the morning of the sixth day the rectal temperature was 104·4°F, rising to 106·4°F during the afternoon, at which stage the animal was slaughtered. This sheep was therefore killed during the early stages of the disease, before any additional symptoms had appeared. The post-mortal examination revealed general hyperaemia especially of the lungs, but otherwise the carcass appeared normal.

Meat specimens were collected at the time of slaughter and again at suitable intervals thereafter. The results obtained by injection of these specimens into bluetongue susceptible sheep, together with the pH recordings are presented in Table 2.

TABLE 2.—*Particulars of meat specimens taken from the carcass of sheep* 12592

Number of days after slaughter	Recipient sheep number	Specimen	pH of specimen	Reactions in recipient sheep				Neutralizing Index
				Incubation period in days	Duration of febrile reaction in days	Coronitis	Outcome	
0	12563	L	6·9	—	—	—	—	0
	13557	G		5	5	+	Surv.	4
3	12598	L	5·5	—	—	—	—	0
	12577	G		—	—	—	—	0
6	12604	L	5·4	—	—	—	—	0
	12615	G		—	—	—	—	0
9	12526	L	5·4	—	—	—	—	0
	12636	G		—	—	—	—	0

For note see Table 1

In this instance the neutralizing indices of the sera from the recovered sheep have been used to confirm the specificity of the clinical reactions. The pH changes recorded in the meat of this sheep were similar to those encountered in normal carcasses, dropping to an ultimate level of 5·4.

Bovine No. 624: Bovine No. 624 failed to react clinically to the injection of virulent type III (Cyprus) bluetongue virus. The animal was slaughtered on the tenth day with a normal temperature of 101·4°F. On ante-mortal examination no outward clinical symptoms of disease could be detected. The carcass also appeared completely normal on post-mortal examination and was passed as fit for consumption. Specimens were collected at the time of slaughter and again after six days and examined as described. The results obtained are recorded in Table 3.

TABLE 3.—*Particulars of meat specimens taken from the carcass of bovine* 624

Number of days after slaughter	Recipient sheep number	Specimen	pH of specimen	Reactions in recipient sheep				Virus isolation
				Incubation period in days	Duration of febrile reaction in days	Coronitis	Outcome	
0	12688	L	6·4	—	—	—	—	—
	12667	G		5	9	+	Died	+
6	12614	L	5·5	—	—	—	—	—
	12603	G		—	—	—	—	—

For note see Table 1

The M. gluteus specimen with a pH of 6·5 again proved positive, which indicated that this muscle contained infective virus at the time of slaughter. The M. longissimus dorsi specimen, taken together with the above specimen did, however, not contain infective virus. Meat specimens with a pH of 5·4 taken on the sixth day thereafter, also proved to be negative. The positive reaction in Table 3 was confirmed by virus isolation and neutralization with homologous immune serum.

Discussion

Laboratory investigation with a particular virus requires a sound knowledge of both its pH and thermal stability. As an example may be mentioned that the stability of the poliomyelitis virus (Loring & Schwerdt, 1944) over a wide range of pH allows for the use of techniques requiring acid conditions. On the other hand relatively low pH values can be used for the inactivation of acid-sensitive viruses.

The pH stability trials reported in this paper, although by no means comprehensive, indicate that a marked loss of infectivity can be expected in the region of pH 6·1 to pH 6·3. There appears to be no significant difference between types I and III at 4°C, but the results indicate that bluetongue virus has a wider stability range at this temperature than at 37°C.

A similar narrowing of the stability range at 37°C has been observed with mouse brain preparations of Columbia SK virus where Saunders & Jungeblut (1942) recorded a loss of infectivity on either side of neutrality. In these investigations type I bluetongue virus appeared to be less stable only under the more alkaline conditions.

The results presented in the latter part of this paper, dealing with the survival of bluetongue virus in carcass meat, show that the virus can occur in both mutton and beef. In mutton, the longevity of the virus depends upon the stage of disease in which the animal is slaughtered. It is unlikely that advanced cases of bluetongue will be slaughtered for commercial purposes. However, the importance of a thorough ante-mortal examination is stressed as the pathological lesions encountered in the dressed carcass may not be sufficiently apparent to preclude the passing of such a carcass as fit for consumption.

The meat of sheep slaughtered in the initial stages of the disease, before the clinical symptoms are apparent, will contain sufficient muscle glycogen to lower the pH beyond the stability range of the virus.

Du Toit (1962) has reported the high incidence of cattle harbouring bluetongue virus in their circulation under natural conditions during the summer months. At no stage during his investigations did any of these cattle exhibit any clinical signs of disease.

As the majorty of slaughter cattle in South Africa are sent directly from ranches to the abattoirs, it is likely that during the latter part of the summer season a large number of carcasses will contain infective bluetongue virus. However, the results presented show that the meat of bovines containing infective bluetongue virus will acidify sufficiently to lower the pH to a point at which this virus will no longer persist, provided such animals are otherwise healthy at the time of slaughter.

The point at which bluetongue virus is destroyed in meat appears to lie between pH 5·6 and pH 6·3. The *in vitro* stability trials reported in this paper substantiate this finding and indicate that point to be in the vicinity of pH 6·0. The failure in two instances (sheep 12592 and bovine 624) to demonstrate infective virus in the M. longissimus dorsi immediately after slaughter cannot be explained by the hydrogen ion concentration. It can thus only be suggested that this particular site probably becomes involved in the more advanced stages of the disease.

Tissues, such as the fat, lymph nodes, bone marrow and connective tissue, which do not acidify to any extent after slaughter, have not been taken into account in this investigation.

Summary

Experiments are reported in which the *in vitro* pH stability of bluetongue virus as well as the survival of this virus in muscle of mutton and beef carcasses, have been investigated.

(*a*) A marked loss of infectivity occurred between pH 6·1 and pH 6·3 under laboratory conditions when bluetongue virus was subjected to those hydrogen ion concentrations normally encountered in carcass meat.

(*b*) The survival of bluetongue virus in carcass meat appears to be dependent upon the post-mortal pH changes. The virus was shown to persist for a period of 30 days at 4°C in an ovine carcass, where the pH failed to drop below pH 6·3. On the other hand, meat with a pH in the region of 5·4, did not contain infective virus.

(*c*) A bovine carcass was shown to contain infective bluetongue virus on the tenth day after artificial infection, although no clinical signs of disease were encountered.

(*d*) There appeared to be a greater tendency for bluetongue virus to be present in the M. gluteus than in the M. longissimus dorsi.

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