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PLAQUE FORMATION BY BLUETONGUE VIRUS

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The plaque assay of animal viruses in tissue culture has developed into a widely used technique since its first introduction by Dulbecco in 1952. In a comprehensive review, Cooper (1961) gives details of the variables which influence the sensitivity of the system and the special uses to which it has been put.

The property of an animal virus to produce plaques in a monolayer of susceptible cells is dependent on the production of demonstrable cytopathic changes within the cells during multiplication. These discrete areas of viral multiplication, localized by means of an agar overlay, can then be demonstrated and enumerated by selective staining methods.

The cytopathic changes produced by bluetongue virus were first observed by Haig, McKercher & Alexander (1956) in monolayer roller tube cultures of primary lamb kidney cells. Subsequently, Fernandes (1959) described the growth and cytopathic effects of a virulent as well as a homologous egg-adapted strain at the thirty-sixth passage level, in cultures of human amnion (Fernandes), Chang liver, clonal Hela HB_2, McCoy synovial, Henles intestine, bovine kidney, Chang conjunctiva and NCTC A2414 clonal human skin cells. By means of the fluorescent antibody staining technique, Livingstone & Moore (1962) concluded that multiplication of virus occurred within the cytoplasm of infected cells.

The purpose of this paper is to report plaque formation by bluetongue virus in a line of mouse fibroblast cells and to give details of some of the experimental variables and properties of the virus which might affect the further application of this technique.

Materials and Methods

Virus strains

Various strains of bluetongue virus were used during the course of this investigation.

(*a*) Sixteen strains of virus, referred to as " stock type strains ", stored in sealed ampoules at — 20°C in the freeze-dried form, were selected as representative of different serological types. These strains of virus had been previously isolated from naturally infected sheep or cattle, by the inoculation into six to seven-day embryonated fertile hens' eggs of blood, collected during the early febrile phase of the disease. After two to four serial passages in this host, the virus strains were adapted to primary monolayer cultures of lamb kidney cells and taken through six passages at limiting dilution, before freeze-drying.

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(*b*) Fourteen egg adapted strains of virus, homologous with the corresponding " stock type strains ", were also used. These strains were obtained by a continuation of the serial transfer of the virus in fertile hens' eggs to between the 60th and 80th passage levels. Suspensions of infective chick embryo tissue were freeze-dried and stored in sealed ampoules at — 20°C. For plaque assay, suitable dilutions of the freeze-dried virus strains were used either without further passage, or alternatively after two or three passages in monolayers of primary lamb kidney cells, in order to obtain a preparation of high infective titre.

Cells and monolayer cultures

(*a*) A derivative of Earle's strain mouse fibroblasts (NCTC clone 929 of Strain L), obtained from Prof. W. Schäfer, Tübingen, was maintained by subculture at three to four day intervals in Roux flasks. A modified Eagle's medium (Macpherson & Stoker, 1962), containing 10 per cent tryptose phosphate broth (Difco) and 10 per cent bovine serum, was used.

Confluent monolayer cultures were washed twice in pre-warmed Ca^{++} and Mg^{++} free phosphate buffered saline pH 7·4 and the cells removed from the surface of the glass by the addition of 20 ml of prewarmed buffer solution, containing 0·02 per cent sodium versenate and 0·25 per cent trypsin. After decanting, the cell suspension was centrifuged lightly, the versene-trypsin solution discarded and the cells suspended in nutrient medium and counted. Monolayer cultures for plaque assay were prepared in 45 or 65 mm petri plates, seeded with 5 or 10 ml respectively of a cell suspension containing 4 to 5 $\times$ 10^5 cells per ml.

Plates were incubated at 37°C in a humidified chamber which was gassed continuously with an atmosphere of 5 per cent CO_2 in air. Confluent monolayers were usually obtained from 24 to 48 hours after seeding. Before use, cultures were washed twice in prewarmed phosphate buffered saline (PBS) at pH 7·4 to equilibrate the pH and remove inhibitory substances, which might have been present in the nutrient medium.

Roller tube cultures were prepared by seeding 15 mm test tubes with 1·5 ml of the same cell suspension. The tubes were kept stationary until cell attachment was complete and were then rolled continuously in an incubator at a temperature of 37°C.

(*b*) Roller tube cultures of primary lamb kidney cells were produced by standard techniques using the trypsin dispersion method of Youngner (1954). Nutrient medium for these cultures was prepared by the addition of 10 per cent bovine serum to Hanks balanced salt solution containing 0·5 per cent lactalbumin hydrolysate.

All media contained antibiotics in the following concentrations: streptomycin 100 μg; penicillin 100 units and amphotericin B (Squibb) 2·5 μg per ml.

Virus inoculation and adsorption

Washed petri dish cultures were inoculated with 1·0 ml of the appropriate virus dilution prepared in chilled PBS containing 0·5 per cent bovine albumin. Unless otherwise stated, adsorption was allowed to proceed for one hour at 37°C. In order to reduce counting errors, dilutions were selected which gave no more than an anticipated 40 to 60 plaques per 65 mm plate or half this count with the smaller plates. Three to six plates were used for each dilution.

Overlay

The overlay was prepared by autoclaving a 1·0 per cent suspension of agarose* in Earle's saline at 15 lb/sq in. for 20 minutes. When this preparation had cooled to 44°C an equal volume of prewarmed Eagle's medium was mixed with the agarose. Finally the required amount of stock 3 per cent sodium bicarbonate solution was added, which unless otherwise stated, gave a final concentration of 1·1 gm per litre.

After absorption and without removal of the original inoculum, 8·0 ml of this overlay, held at 44°C, was added to each 65 mm plate and half this volume to the smaller sized plates. Plates were returned to the incubator and inverted on the second day.

Plaque identification

After incubation for the periods specified in the test, plates were flooded with a 1: 20,000 dilution of neutral red in PBS prepared from a stock solution stored in a non-actinic glass bottle. Plaques were counted after two to four hours further incubation at 37°C.

Diluents

(*a*) Phosphate buffered saline (PBS). This solution was prepared according to the standard formula of Dulbecco & Vogt (1954).

(*b*) Tris buffered saline (TBS). Three stock solutions were prepared. For the first 8·0 gm sodium chloride, 0·38 gm potassium chloride and 3·0 gm Tris-(hydroxy-methyl-aminomethane) were dissolved in 300 ml water. The second stock solution was prepared by dissolving 0·1 gm magnesium chloride in 100 ml water. The third stock solution contained 0·1 gm calcium in 100 ml water. The solutions were autoclaved separately, cooled, combined and brought up to 1 litre with sterile distilled water. The final solution was stored at 4°C.

(*c*) Buffered lactose peptone (BLP). A $\frac{M}{15}$ phosphate buffer of pH 7·2 containing 0·2 per cent neutral peptone (Difco) and 10 per cent lactose was sterilized by Seitz filtration and stored at 4°C.

Experimental Results

(*a*) *Growth cycle of bluetongue virus in Earle's strain of mouse fibroblasts*

A strain of Type 10 bluetongue virus, an antigenic group which has been most extensively studied in view of its worldwide distribution, was selected for a preliminary determination of the growth cycle of the virus in this cell line. A monolayer culture of L strain fibroblasts in a Roux flask was infected with an input multiplicity of 5 PFU/cell. After incubation at 37°C for one hour to facilitate adsorption of the virus, the medium was decanted, the monolayer washed twice with PBS and then incubated at 37°C in a medium composed of Eagle's nutrients plus 2 per cent foetal calf serum. Samples of the medium were removed at half-hourly intervals. These samples were centrifuged at 2000 rpm to remove cellular material and then assayed for infective virus by the plaque technique. The result of this experiment is shown graphically in Fig. 1. Under the conditions of this experiment there was a lag phase

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of approximately four hours, after which the virus multplied exponentially to reach a maximum extracellular concentration at 12 hours. Virus release continued for a further 16 hours after which a decrease in the infectivity titre was observed, presumably as a result of thermal inactivation. Cytopathic changes were first observed after 24 hours incubation, and continued to involve an ever-increasing number of cells until complete cell destruction was apparent after 72 to 96 hours incubation. There thus appeared to be a pronounced delay between virus production and cytopathic effect.

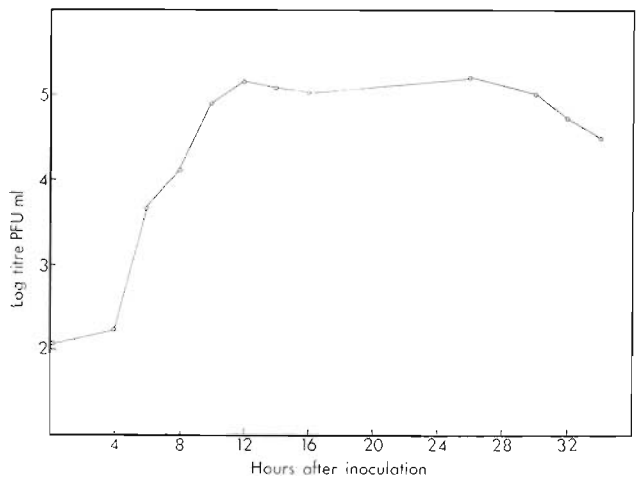

FIG. 1.—Multiplication of Type 10 bluetongue virus in MLF cells

(b) Production of plaques by immunologically different strains of bluetongue virus

After the susceptibility of Earle's L strain of mouse fibroblast to a strain of tissue culture adapted bluetongue virus had been established, the various selected " type strains ", as well as those strains which had been serially passaged in eggs, were examined by plaque assay. In this experiment the plates were stained on the 5th day after infection with serial tenfold dilutions of the freeze-dried virus strains. The observations and measurements made on these plates are summarized in Tables 1 and 2. It is noteworthy that plaques were formed by each of the strains included in the experiment, irrespective of whether the virus had been previously passaged in tissue culture or exclusively in fertile hens' eggs. Distinct morphological differences in plaque size and shape and of the perimeter, were found to be associated with different strains of virus. These differences not only existed between different immunological types, but also between the virulent and egg attenuated strains of the same virus type. Without exception each strain showed a wide variation in plaque size on individual plates and the percentage size distribution showed a marked lack of uniformity between the strains. An illustration of the variations in morphology and size encountered among the different strains is recorded in Plate 1.

TABLE 1.—*Size and plaque morphology of freeze-dried tissue culture type strains*

Strain	Percentage size distribution after 5 days incubation in 5 per cent CO_2 at 37° C				Morphology		
	Size in mm				Boundary	Shape	Centre
	<1·0	1·0–1·9	2·0–2·9	3·0 or>			
1.....	4	81	15	0	Sharp	Round	Clear
2.....	0	30	70	0	Sharp	Irregular	Clear
3.....	1	95	4	0	Sharp	Irregular	Clear
4.....	3	83	14	0	Sharp	Irregular	Clear
5.....	34	66	0	0	Sharp	Irregular	Clear
6.....	65	35	0	0	Sharp	Round	Clear
7.....	0	88	12	0	Sharp	Irregular	Clear
8.....	8	80	12	0	Sharp	Irregular	Clear
9.....	10	90	0	0	Sharp	Very irregular	Clear
10.....	0	19	53	28	Sharp	Round	Clear
11.....	0	45	50	5	Sharp	Irregular	Clear
12.....	3	32	62	3	Sharp	Irregular	Clear
13.....	1	42	51	6	Sharp	Irregular	Clear
14.....	0	56	43	1	Sharp	Round	Clear
15.....	69	31	0	0	Sharp	Round	Clear
16.....	12	86	2	0	Sharp	Irregular	Clear

TABLE 2.—*Size and plaque morphology of high passage egg adapted strains*

Strain	Percentage size distribution after 5 days incubation in 5 per cent CO_2 at 37° C				Morphology		
	Size in mm				Boundary	Shape	Centre
	<1·0	1·0–1·9	2·0–2·9	3·0 or >			
1.....	5	29	49	17	Diffuse	Round	Clear
2.....	52	48	0	0	Sharp	Round	Clear
3.....	3	37	41	19	Sharp	Round	Clear
4.....	12	47	41	0	Sharp	Irregular	Clear
5.....	95	5	0	0	Sharp	Irregular	Clear
6.....	9	64	26	1	Sharp	Round	Clear
7.....	2	42	53	3	Diffuse	Round	Clear
8.....	4	68	20	8	Sharp	Irregular	Clear
9.....	9	77	14	0	Sharp	Irregular	Clear
10.....	16	70	14	0	Sharp	Irregualr	Clear
11.....	4	56	40	0	Sharp	Round	Clear
12.....	14	65	21	0	Sharp	Irregular	Clear
13.....	4	29	47	20	Diffuse	Irregular	Clear
14.....	4	25	35	36	Diffuse	Round	Clear

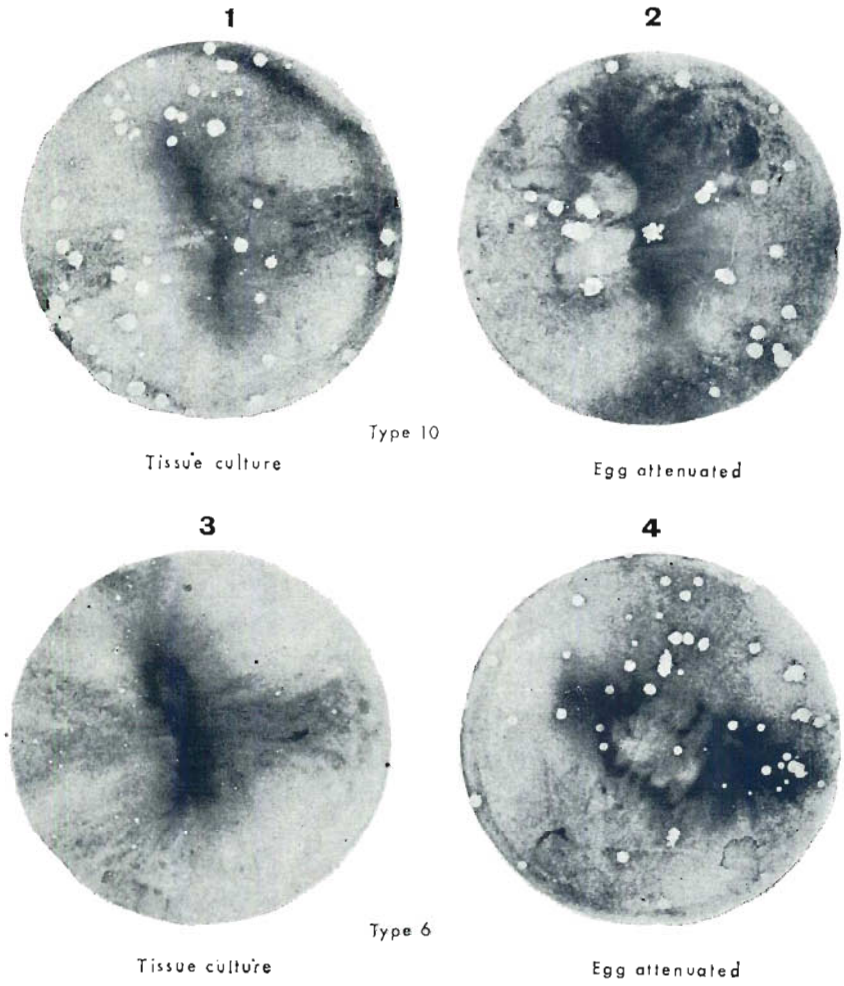

PLATE 1 —Variations in the morphology of plaques produced by bluetongue virus

(*c*) *Rate of plaque formation under agarose*

Before proceeding further with these experiments it was necessary to determine the optimal time at which final plaque counts should be made. This period of incubation would be influenced by such factors as the viability of the cells under agar, the appearance of the maximum number of plaques and the period of time before larger plaques obscured one another by overlapping.

For this purpose, a group of strains found to reflect the variations encountered in plaque size was selected and seeded in suitable dilutions, onto confluent monolayers. In this experiment neutral red at a final concentration of 1: 40,000 was

incorporated into the overlay. From the 3rd day onwards, when minute plaques first became visible, counts were made at daily intervals and were continued until the contrast between individual plaques and the monolayer became indistinct. Considerable difficulty was experienced in distinguishing between small primary and secondary plaques, thus only plates with widely dispersed plaques were used for the daily count.

The results of this experiment are recorded in Table 3 and represent the average plaque count of not less than four plates for each strain. It was concluded that the assay of the tissue culture type strains could be undertaken on the 5th day with the exception of Type 15 where the counts only became constant from the 6th day onwards. Plaques produced by the high egg passage strains appeared to develop earlier, but for the sake of uniformity were subsequently also recorded on the 5th day.

TABLE 3.—*Development of plaques under agarose*

Virus		Average plaque count						
		Days after seeding						
Origin	Immunological group	1	2	3	4	5	6	7
Freeze-dried tissue culture type strains	1	0	0	17	41	42	42	—
	2	0	0	6	17	21	21	—
	3	0	0	6	10	11	12	—
	4	0	0	1	9	13	13	—
	6	0	0	1	9	18	18	—
	15	0	0	6	20	36	45	45
High passage egg adapted.....	1	0	0	0	17	18	17	—
	2	0	0	9	38	44	45	—
	3	0	0	6	10	11	12	—
	4	0	0	2	12	12	13	—
	6	0	0	0	8	8	8	—

(*d*) *Adsorption of the virus*

In this experiment a Type 4 strain of virus was used. This selection was purely arbitrary and was made on the assumption that the experimental results would serve as a pattern for more detailed comparative studies on a future occasion.

Confluent monolayers were washed and inoculated with a virus dilution giving an anticipated count of 50 plaques per plate. At set intervals of time, three plates were removed from the incubator and unadsorbed virus neutralized by the addition of 1 ml of a 1: 40 dilution of homologous type specific antiserum. Each set of plates was then incubated for one hour at 37°C, after which the antiserum was removed and the monolayers once again washed before adding the overlay.

The result of this experiment is recorded graphically in Fig. 2. Under the conditions of this experiment with a small volume of inoculum at pH 7·4 the adsorption rate at 37°C was rapid, and approximately exponential during the first 15 minutes.

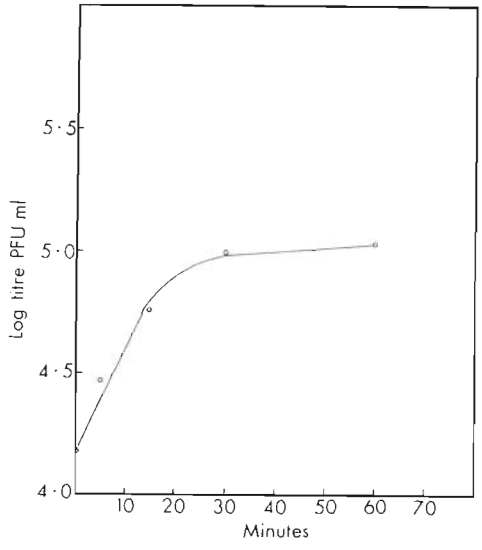

FIG. 2.—Adsorption of Type 4 bluetongue virus onto MLF cells at 37° C

(*e*) *Linearity*

Threefold dilutions of Type 4 virus were made in PBS plus 0·5 per cent bovine albumin and seeded in equal volumes onto confluent monolayers. The graphical representation of the results of this experiment (Fig. 3) gives the average count of quadruplicate determinations.

From this data it is apparent that there is a good linear relationship between plaque count and relative virus concentration, thus providing experimental evidence for the validity of this method of assay as a single particle count.

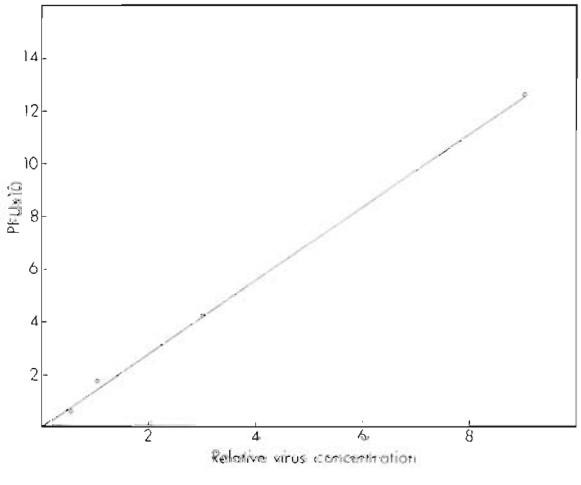

FIG. 3.—Relationship between plaque-forming units and dilution of virus

(f) Composition of the overlay

(i) *The influence of agarose concentration on plaque formation*

The more efficient gelling properties of agarose suggested that relatively lower concentrations than those employed with ordinary agar might be used in these experiments. It was believed that this would facilitate more rapid diffusion of released virus and the ultimate formation of larger and more distinct plaques. An experiment was, therefore, undertaken in which four separate overlays were prepared with agarose concentrations of 0·25, 0·5, 0·75 and 1·0 per cent respectively. A set of 20 plates was seeded with a suitable dilution of Type 1 virus and after adsorption each overlay was added to a group of five plates. Plaques were counted on the 5th day and size determinations made. Subsequently this experiment was repeated with a Type 4 strain of tissue culture virus. The results are given in Table 4. It is apparent from the results of this experiment that, within the range of 0·25 to 0·75 per cent agarose concentration, there is no significant effect by the overlay on either the sensitivity of the system or on the size of the plaques formed after five days incubation. However, a dramatic two-fold reduction in the number of plaques was encountered with the higher concentration of agarose. There was furthermore a distinct decrease in the size of the plaques, particularly on the 4th day of incubation which was more apparent than the analysis would appear to indicate.

TABLE 4.—*Influence of agarose concentration on plaque formation*

Strain of virus	Percentage agarose concentration	PFU	Percentage size distribution (mm)			
			<1·0	1·0–1·9	2·0–2·9	3·0 or >
1	0·25	72	0	89	11	0
	0·50	75	6	91	3	0
	0·75	71	2	92	6	0
	1·00	32	0	100	0	0
4	0·25	122	1	73	26	0
	0·50	124	3	94	3	0
	0·75	123	7	84	9	0
	1·00	61	10	90	0	0

(ii) *The effect of the depth of overlay*

It was anticipated that the depth of the overlay might affect plaque formation as a result of variations in the relative concentrations of gases and the products of cell metabolism in the zone immediately adjacent to the monolayer.

Groups of 20 plates were seeded with a suitable dilution of Types 1 and 4 virus. After adsorption, increasing volumes of standard overlay were added to give final depths of 1, 2, 4 and 6 mm in the 65 mm petri dishes.

The number of plaques and their size distribution were determined on the 5th day. These results are recorded in Table 5.

The influence of the overlay on plaque formation was found to be pronounced in this experiment, where with increasing depth of overlay a decrease in both the size and number of plaques was observed. Where an overlay of less than 2 mm depth was used, the cells deteriorated within 48 hours of adding the overlay and were therefore discarded.

TABLE 5.—*Effect of the depth of overlay on plaque formation*

Strain of virus	Depth of overlay (mm)	PFU	Percentage size distribution (mm)			
			<1·0	1·0–1·9	2·0–2·9	3·0 or >
1	2	41	1	33	62	4
	4	21	6	93	1	0
	6	23	12	88	0	0
4	2	57	9	88	3	0
	4	39	16	84	0	0
	6	12	26	74	0	0

(iii) *The action of neutral red incorporated in the overlay on plating efficiency*

The cytotoxic effect of neutral red on various cell types and the lowering of the plating efficiency by incorporating the dye in the overlay has been reported by various workers (Cooper, 1961). The effect of this dye on the system under investigation was determined. A 1: 200 stock solution of neutral red (Coleman & Bell) was prepared in distilled water, sterilized by autoclaving at 15 lb pressure for 20 minutes and then stored in non-actinic glass bottles.

Three overlays were prepared with neutral red added to give final concentrations of 1: 20,000, 1: 40,000 and 1: 80,000. Plates were seeded with a suitable dilution of virus and after adsorption the appropriate overlay was added. A duplicate set of control plates were overlayed with agarose without the addition of neutral red. In order to improve contrast in those plates with low neutral red concentration and in the unstained controls, all plates were restained on the 5th day and the number of plaques determined.

It was found with the particular batch of stain used in these experiments that a concentration of 1: 80,000 produced insufficient contrast to permit its use in routine assays. On the other hand a concentration of 1: 20,000 exhibited a definite inhibitory effect on strains producing smaller plaques, but apparently had little influence on plaque formation by other strains of virus (Table 6). For routine assay purposes, therefore, neutral red was incorporated into the overlay when required at a final concentration of 1: 40,000.

TABLE 6.—*Effect of neutral red on plating efficiency*

Type of plaque	Strain of virus	PFU			
		Final concentration of neutral red in overlay			
		1 : 20,000	1 : 40,000	1 : 80,000	Control
Large	1	54*	76	90	110
	4	33	32	41	53
Small	6	35	76	79	75
	15	45	62	61	53

*Average PFU counted on 5 plates

(iv) *Effect of sodium bicarbonate concentration on plaque formation*

Petri dishes used in these experiments were incubated throughout in an atmosphere of 5 per cent CO_2 in air, but for various reasons slight variations in the pH of the overlay at any given bicarbonate concentration were inevitable. For this experiment four type strains were selected together with their homologous egg attenuated counterparts. Plates were seeded with suitable dilutions of virus and allowed to adsorb. Thereafter groups of five plates were overlaid with nutrient agar containing either 0·55, 1·10 and 2·20 gm of sodium bicarbonate per litre respectively. The plaque counts made on the 5th day after infection are given in Table 7.

TABLE 7.—*Influence of sodium bicarbonate concentration on plaque formation*

Origin of Virus	Strain of Virus	PFU		
		Sodium bicarbonate concentration (gm/l)		
		0·55	1·10	2·20
Stock type strain	1	39	43	41
	2	82	82	85
	3	53	55	55
	4	94	92	95
Egg adapted	1	19	34	26
	2	64	60	61
	3	28	29	26
	4	59	60	54

It is apparent from these results that the pH of the overlay, within the range of sodium bicarbonate concentrations used, had no significant influence on plaque formation by either low passage or egg adapted strains of virus. Overlays containing higher concentrations of sodium bicarbonate were found to be cytotoxic to this particular type of cell.

(*g*) *Thermal stability of bluetongue virus*

Previous experience and preliminary experiments had indicated that bluetongue virus was unstable in various serum-free tissue culture media.

With the development of a more sensitive and reproducible assay system it was considered desirable to reinvestigate the stability of the virus with particular reference to the conditions which might be encountered during plaque assay or the storage of the virus. Furthermore it was imperative before continuing these studies, to obtain a diluent which would stabilize infectivity and at the same time have no adverse effect on virus adsorption and cell viability during the assay procedure. To this end, the stability of a Type 4 virus was determined, after storage for various periods, in different diluents over a temperature range of − 70°C to 37°C.

A freshly harvested virus preparation, grown in lamb-kidney monolayers in Eagle's medium without serum was divided into aliquots From stock solutions of the selected stabilizers sufficient was added to each to give the following respective concentrations: bovine albumin 1 per cent, glycerine 20 per cent, buffered-lactose-peptone (BLP) 50 per cent, and a combination of 16 per cent glycerine plus 0·8 per cent bovine albumin. The preparation in Tris-buffered saline (TBS) was prepared by first removing cellular debris by light centrifugation at 800 g and finally sedimenting the virus by ultracentrifugation at 35,000 g for 1½ hours. The pellet was then resuspended in the stock TBS solution. For comparative purposes samples of the virus suspended in BLP and 1 per cent bovine albumin were concurrently freeze-dried in a Speedivac Model 30Pl Centrifugal Freeze Drying Unit In the recorded results these preparations are indicated by the abbreviation F.D. in their respective graphs

The results of the titrations of virus infectivity of the various samples at given intervals are indicated in Fig. 4.

The virus was extremely unstable in TBS especially at 37°C, −20°C and −70°C. At 4°C it was relatively stable with intermediate values at room temperature (25–30°C). In the basic nutrient medium without serum, stability of the virus was only slightly improved, except at −70°C where infectivity was lost very slowly. This apparent stability at −70°C is in all probability due to the tryptose phosphate broth, added as an additional nutrient to the medium.

The presence of 1 per cent bovine albumin, 20 per cent glycerine or a combination of both appeared to improve stability at lower temperatures, but 50 per cent BLP was the most efficient stabilizer employed. Its wide use as a stabilizer for the freeze-drying of viruses at this Institute is thus substantiated, but unfortunately its cytotoxic effect, particularly at the concentration used in the present series of experiments, precluded its use as a diluent for direct inoculation of some types of cell culture. At this stage the best substitute, which does not induce cytotoxic effects, would therefore appear to be 1 per cent bovine albumin in PBS.

The most striking stabilizing effect of BLP is observed at −20°C, which focuses attention on the mechanism of virus inactivation consistently observed at this temperature with the other diluents. The observation that storage at −70°C is much less injurious to infectivity than at −20°C, provided some macromolecular substance is present, suggests that the rate of freezing might be an important factor. Subsequent experiments showed that samples of virus snap-frozen at − 70°C and then stored at −20°C retained 90 per cent more infectivity than a control sample frozen and stored at − 20°C.

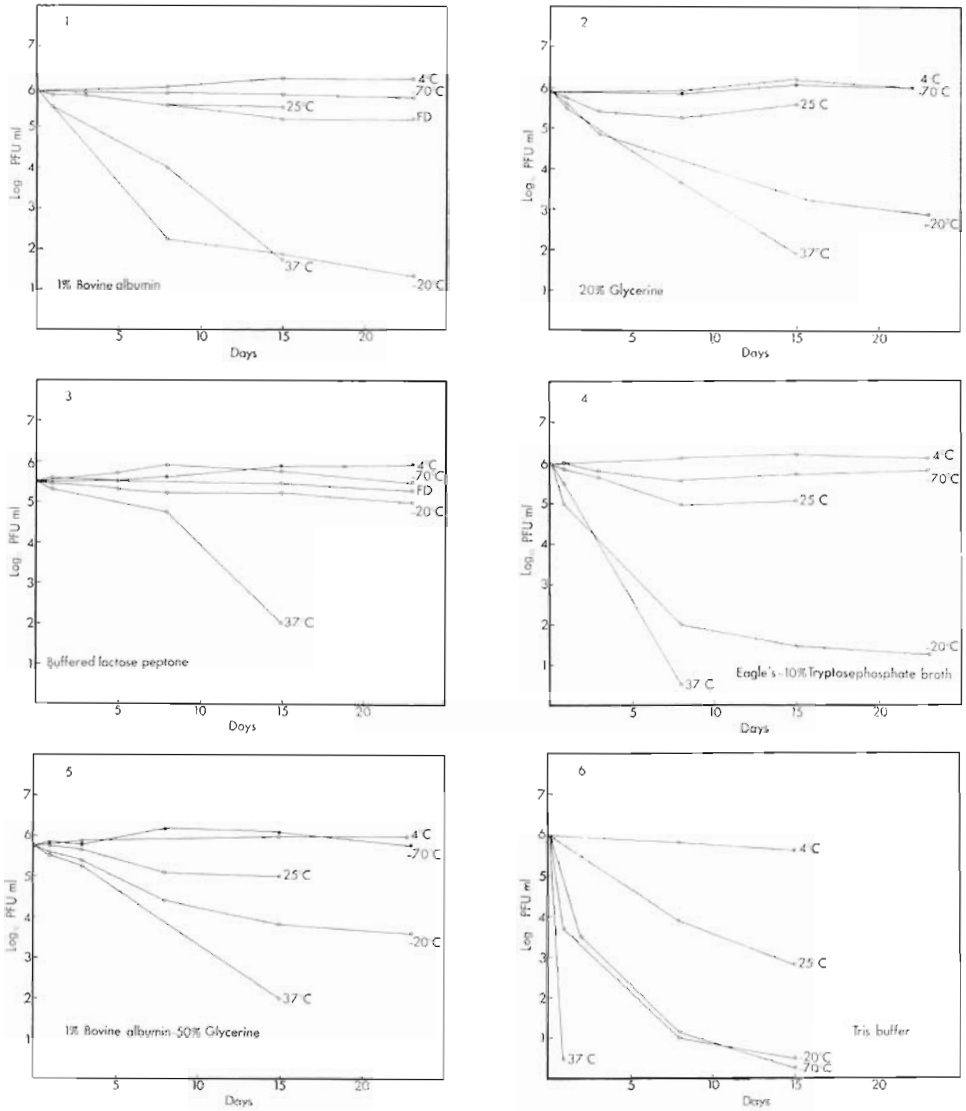

FIG. 4.—Assay of Type 4 bluetongue virus in various stabilizers at given temperatures

The minimal loss of infectivity during the process of freeze-drying is consistent with previous experience of large scale vaccine production. Unfortunately the limited period of the experiment during which titrations were conducted gives no indication of the end points in the persistence of virus infectivity, however; the loss of infectivity of stock strains stored in the freeze-dried form at −20°C has been negligible over the past eight years.

Discussion

In the past the assay of bluetongue virus has been confined to a limited range of laboratory host systems, of which fertile hens' eggs and primary cultures of lamb or calf kidney cells have been most frequently used. First attempts to demonstrate plaque formation on monolayer cultures of primary calf and lamb kidney and testes cells were not satisfactory, due either to the formation of minute indistinct plaques or alternatively to poor survival of the primary cells under an agarose overlay. Of various cell lines investigated, the most promising results were obtained with BHK/21 (clone 13) and the strain of mouse fibroblast described in this paper. Clear 2 to 4 mm plaques have been obtained in BHK cells suspended in agarose, according to the technique of Cooper (1955). However, in spite of the technical advantages associated with this method of plaque production, the system using BHK cells was found to be of no greater susceptibility than the monolayer technique described herein. Further attempts are being made to identify other cell types of greater susceptibility, particularly in regard to the assay of virulent unmodified strains of virus, for which an urgent need still exists.

In this preliminary study of strains of bluetongue virus, a highly heterogeneous group of antigens has been used. Fortunately, this factor would not appear to have prevented plaque formation, although a great variation was observed in plaque morphology and behaviour of the strains under agarose. These observations serve to emphasize the diverse characteristics which the immunologically distinct strains of a virus might be expected to exhibit on more critical investigation. Before further detailed studies can, however, be conducted, the preparation of genetically stable plaque purified strains of virus derived from a common source, would appear to be desirable.

Due to the present uncertainty which exists in regard to the classification of bluetongue virus, it is premature at this stage to attempt to evaluate the formation of plaques by this virus in relation to plaque formation by more well defined and systematically described viruses. On the other hand the introduction of the plaque technique will provide an invaluable aid in the determination of the physico-chemical properties of the virus and its final classification. At the same time, the numerous and diverse uses to which plaque formation has been applied in the fields of antibody assay, genetic studies and diagnosis, will materially assist in the study of the epizootiology and biological properties of the virus.

It is apparent from these preliminary observations that further experiments are necessary to determine the significance of the growth cycle and particularly the release of virus in relation to plaque formation by any particular strain. In this regard it has been shown by Schwöbel (1965) that the variation in plaque size encountered with a cloned strain of foot-and-mouth disease virus was dependent upon the variability of the time at which virus was released from primary infected cells and the variability of the time at which plaque growth commenced.

The variation in the morphology and size of the plaques produced by the selected strains of virus are significant in so far as they developed under a particular set of environmental conditions. Further experiments, however, have shown that although the overall size of plaques produced by a particular strain of bluetongue virus was a constant feature, the percentage size distribution of plaques produced by strains of uncloned virus were not highly reproducible. Serial passage of the virus in fertile hens' eggs to the levels used in this study, while modifying the size and morphology of the plaques of some of the strains, did not eliminate the variations in plaque size encountered within the type. Apart from the inherent variation in

plaque size between strains, other factors would also appear to influence the development of plaques. The metabolism of the cell and the conditions operating in the immediate environment as determined by increasing depth and concentration of agarose in the overlay, have been shown to decrease plaque size. These results would appear to be in accordance with the findings of Baron, Porterfield & Isaacs (1961) who showed that lowered oxygen concentration, obtained by varying the depth of overlay, inhibited the multiplication of six Group A arboviruses. Similarly the effect of the reduction of the oxygen tension and the resulting increase in the inhibitory effect of interferon (Isaacs, Porterfield & Baron, 1961) might account for the more uniform morphology of the plaques and the distinct absence of secondary plaques under these conditions. Variations in pH, which might be encountered under the conditions in which cultures were incubated, did not appear to have any significant effect on the sensitivity of this particular system.

The accuracy of the plaque assay technique is amongst other factors dependent upon a linear relationship between the plaque count and the relative concentration of virus inoculated. This basic requirement has been demonstrated experimentally in this paper, showing that the technique can be used as a particle counting method as well as for the cloning of strains of virus for various purposes.

The results of the experiments reported in this paper, where the stability of a Type 4 tissue culture adapted virus was determined under conditions which might be encountered during assay or in culture, showed that the virus was labile, unless suitable biological stabilizers were added to the medium. An extension of the experiments to include possible conditions of storage confirmed the marked drop in titre associated by earlier workers with slow freezing (Alexander, 1947). However it was found that this phenomenon could be reduced by the addition of suitable biological stabilizers. These results appear to be at variance with the conclusions of Svehag (1963) in whose experiments an egg adapted strain of virus was found to be markedly thermostable at pH 7 and to exhibit only negligible loss of infectivity during slow freezing. This author did not, however, use a defined diluent. The presence of embryo tissue material in the virus preparation, which itself differed in antigenicity (Type 10) and was considerably modified by egg propagation, precludes direct comparison of these conflicting results.

On the basis of the observations presented in this paper, the plaque assay of bluetongue virus does not at this stage appear to deviate from established techniques.

Summary

The formation of plaques in a line of mouse fibroblast cells under agarose, by 16 tissue culture and 14 egg adapted strains of bluetongue virus is described. Optimal conditions for routine plaque assay were determined and certain basic requirements substantiated. The technique was used to determine the thermal stability of the virus under various conditions and in selected biological stabilizers.

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